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Probiotic characterization of *Bacillus subtilis* P223 isolated from kimchi

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Abstract Probiotic characteristics of *Bacillus subtilis* P223 isolated from kimchi were investigated in this study. Spore cells of *B. subtilis* P223 showed high tolerance to artificial gastric juice (pH 2.5, 0.3% pepsin, 3 h) and bile salts (0.3% oxgall, 24 h). Spore cells of *B. subtilis* P223 showed more adherence to intestinal cells (HT-29 cells) than vegetative cells. In addition, *B. subtilis* P223 showed high autoaggregation ability, similar to a commercial strain (*Bacillus clausii* ATCC 700160). Moreover, its coaggregation abilities with pathogens were strong. The adherence of three pathogens (*Salmonella enteritidis* ATCC 13076, *Listeria monocytogenes* ATCC 15313, and *Escherichia coli* ATCC 25922) to HT-29 cells was inhibited by *B. subtilis* P223. It was found that *B. subtilis* P223 could not produce β-glucuronidase, a carcinogenic enzyme. However, it had amylase and protease activities. Antibiotic susceptibility was measured using disk diffusion assay. It was revealed that *B. subtilis* P223 was only resistant to streptomycin among eight kinds of antibiotics. In addition, *B. subtilis* P223 showed no hemolysis activity. It did not have enterotoxin genes. Results of this study suggest that *B. subtilis* P223 isolated from kimchi has potential as a probiotic strain.

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Introduction

Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' [1]. Probiotics can improve intestinal health, enhance immune response, and reduce serum cholesterol levels [2]. They can also help overcome lactose intolerance, *Helicobacter pylori* infections, and inflammatory bowel disease [3]. However, probiotics should be confirmed to have specific characteristics such as resistance to gastric and bile acid with adhesion activity to human epithelial cells to exert their probiotic potential [4]. Safety assessment of probiotics such as antibiotics resistance and hemolysis should also be tested for probiotics [5]. The majority of probiotics commercialized are isolated from human gastrointestinal tract and dairy products [2].

Kimchi is a Korean traditional vegetable fermented food made of cabbage or radish with ground red pepper, garlic, and diverse seasoning ingredients [6]. Kimchi is fermented in low temperature, usually under 10 °C. Diverse bacteria increase during fermentation [7]. Kimchi is known to have many beneficial effects to human body, including anti-cancer [8], anti-mutagenic [9], anti-oxidative [10], and anti-aging [11]. Its beneficial effects are due to components in kimchi, including fibers, vitamins, phytochemicals, and minerals derived from ingredients and bacteria in kimchi [12]. Many studies have reported lactic acid bacteria as probiotics isolated from kimchi. However, few research studies have reported probiotic *Bacillus* strains isolated from kimchi [13–15]. Many *Bacillus* species have been isolated from fermented soybean food and applied in industrial products as probiotics for many years [5, 16].

Bacillus species known as probiotics include *B. subtilis*, *B. cereus*, *B. clausii*, *B. coagulans*, and *B. licheniformis* [16]. *Bacillus* spp. produce spores when strains are exposed to extreme conditions of environment [17]. These spores are heat-stable therefore they can be stored during long period. They are resistant to low pH environment such as gastric acid [16, 18]. Therefore, *Bacillus* spp. have advantages over non-spore forming probiotics such as *Lactobacillus*, *Bifidobacterium*, *Leuconostoc*, and *Pediococcus* [5]. Enterogermina® is a probiotic product that contains *B. clausii* spores in water. It is expected to improve the delivery of spores to gastrointestinal (GI) tract [16].

The aim of this study was to identify *B. subtilis* P223 isolated from kimchi and investigate its probiotic properties, including its resistance to acid and bile salts, enzymatic activity, adhesion ability, and auto/co-aggregation. In addition, antibiotic susceptibility, hemolysis, and toxic gene analysis were determined to assess its safety.

Materials and methods

Bacterial strains and growth conditions

B. subtilis P223 was isolated from kimchi. As a control, *B. clausii* ATCC 700160 used as commercial probiotic strain was obtained from Korean Collection for Type Cultures (KCTC, Daejeon, Korea). *B. subtilis* P223 and *B. clausii* ATCC 700160 were cultured in tryptic soy broth (TSB; Becton–Dickinson, Sparks, MD, USA) with shaking at 150 rpm. *B. subtilis* P223 and *B. clausii* ATCC 700160 were incubated at 37 and 30 °C, respectively. To obtain spore cells, *B. subtilis* P223 was incubated at 37 °C for 72 h while *B. clausii* ATCC 700160 was incubated at 30 °C for 7 days. After incubation, strains were heated at 80 °C for 30 min to kill vegetative cells. *Staphylococcus aureus* ATCC 6538, *S. enteritidis* ATCC 13076, *L. monocytogenes* ATCC 15313, and *E. coli* ATCC 25922 were incubated in TSB at 37 °C.

Identification of *Bacillus* strain

B. subtilis P223 was identified based on 16S rRNA sequencing analysis by Bionics Inc. (Seoul, Korea). Sequencing results were analyzed by searching database of National Center for Biotechnology Information Nucleotide using BLAST web site (http://blast.ncbi.nlm.nih.gov).

Resistance to artificial gastric juice and bile salts

Resistance of *Bacillus* strains to artificial gastric juice and bile salts was determined following protocol published by Jeon et al. [14]. Resistance of *Bacillus* to artificial gastric juice was assessed using TSB containing 0.3% (w/v) pepsin (Sigma-Aldrich, St. Louis, MO, USA) adjusted to pH 2.5 with 0.1 M HCl and incubated for 3 h with shaking (150 rpm). Resistance of *Bacillus* to bile salts was assessed by incubating in TSB containing 0.3% (w/v) oxgall (Becton–Dickinson, Sparks, MD, USA) for 24 h with shaking (150 rpm). Survival rate was determined by counting viable cells on TSA plates.

Adhesion capacity to HT-29 cells

Human colon adenocarcinoma cell (HT-29, KCLB 30038) was used to test the adhesion capacity of *Bacillus* strains. Briefly, 1×10^5 cells/well of HT-29 cells were seeded into 24-well cell culture plate and incubated at 37 °C for 24 h. After incubation, *Bacillus* strain was inoculated to HT-29 cells and incubated at 37 °C for 2 h. Non-adherent bacterial cells were removed by washing three times with phosphate-buffered saline (PBS; Gibco Life Technologies, Invitrogen, Carlsbad, CA, USA). Adherent cells were then detached using 1% (v/v) Triton X-100 (Sigma) solution. The number of adherent cells was counted by viable cell count method.

Autoaggregation and coaggregation

Autoaggregation and coaggregation abilities of bacteria were measured as described by Tareb et al. [19] with some modifications. Cultured bacteria were centrifuged to obtain bacterial cells. Cells were washed twice with PBS and resuspended in PBS to reach absorbance value of 0.3 ± 0.05 at 600 nm.

To determine autoaggregation percentage, bacterial suspension was incubated at 37 °C for 4 and 24 h. Absorbance at 600 nm was measured at 0, 4, and 24 h after incubation. The percentage of autoaggregation was expressed as follows:

$$\text{Autoaggregation } (\%) = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

where A_0 and A_t represented the absorbance at 0 h and at indicated incubation time (4 and 24 h), respectively.

For coaggregation, each *Bacillus* bacterial suspension (2 mL) was mixed with each pathogen (2 mL). The mixture (4 mL) was incubated at 37 °C for 4 and 24 h. The percentage of coaggregation was expressed as follows:

$$\text{Coaggregation } (\%) = \left(\frac{((A_P + A_B)/2) - A_{mix}}{(A_P + A_B)/2}\right) \times 100$$

where A_P and A_B represented the absorbance of the pathogen and the *Bacillus* strain at 0 h, respectively, and A_{mix} represented the absorbance of the mixed culture after indicated incubation time.

Inhibition of adherence of pathogens to HT-29 cells

Inhibitory activity of bacterial adhesion was performed using the method of Lee et al. [4]. Briefly, HT-29 cells (1×10^5 cells/well) were seeded into 24-well cell culture plates and incubated for 24 h. About 10^6 cells/well of pathogen and mix culture (pathogens with *Bacillus* strain) was added onto the plate and incubated at 37 °C for 2 h. After the incubation, non-adherent bacterial cells were washed thrice with PBS. HT-29 cells were detached by adding 1 mL of 1% (v/v) Triton X-100 solution. The solution was diluted and spread onto XLD (Becton–Dickinson, Sparks, MD, USA) agar for *S. enteritidis*, Oxford agar (Becton–Dickinson) with supplement for *L. monocytogenes*, and EMB (Becton–Dickinson) agar for *E. coli* to count viable cell numbers.

Enzyme production

API ZYM kit (BioMerieux, Lyon, France) was used to determine enzymatic activity. *Bacillus* strains were centrifuged (14,240×*g*, 5 min, 4 °C) to obtain cell pellet. The pellet was washed and suspended with suspension medium (BioMerieux, Lyon, France). Then 10^6 CFU/mL of bacteria were inoculated into each cupule. After incubation at 37 °C for 4 h, ZYM A and ZYM B reagents were dropped to each cupule. Color change was detected to assess the level of enzyme activity after 5 min of reaction.

Amylase activity was measured using modified method of Bernfeld [20]. Briefly, 1 mL culture supernatant and 1 mL of 1% soluble starch solution were mixed at 25 °C for 3 min. DNS solution was added to the mixture followed by boiling for 15 min. After cooling on ice, 9 mL of distilled water was added to the mixture. The absorbance of the solution was then measured at 540 nm. One unit of amylase activity indicated the release of 1 mg of maltose from starch in 3 min.

Protease activity of *Bacillus* was determined using the method described by Lee et al. [21] with some modifications. Briefly, 1 mL of the culture supernatant was reacted with 1 mL of 0.6% casein solution (in 0.05 M potassium phosphate buffer, pH 7.5) at 37 °C for 10 min. Two milliliters of 0.4 M TCA solution was added to the solution to stop the reaction. The solution was filtered with 0.45 μm membrane filter. After filtering, 5 mL of 0.4 M sodium carbonate and 1 mL of 3×Folin and Ciocalteu's phenol reagent (Sigma Aldrich, St. Louis, MO, USA) were added to the filtered solution followed by incubation at room temperature for 20 min. Absorbance of the solution was measured at wavelength of 660 nm. One unit of protease activity indicated the released of 1 μM of tyrosine from casein per minute.

Antibiotic susceptibility

Antibiotic susceptibility of *Bacillus* was tested using disk diffusion method according to CLSI performance standards for antimicrobial susceptibility testing [22]. Eight kinds of antibiotics were used: ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), and doxycycline (30 μg). *Bacillus* strains (1×10^6 CFU/mL) were spread onto TSA plates. Antibiotics were loaded onto paper disks. The diameter of inhibition zone by each antibiotic was detected after incubation at 37 °C for 24 h.

Hemolysis

Columbia blood agar plate (Becton–Dickinson, Sparks, MD, USA) containing 5% (w/v) sheep blood was used to determine hemolysis. *Bacillus* strains were streaked onto blood agar plate and incubated 37 °C for 24 h. Plate with clear zone around the strain indicated β-hemolysis. Green-hued zone around the strain indicated α-hemolysis while no change around the strain indicated γ-hemolysis.

Detection of toxic genes

Bacillus strains were inoculated in 10 mL of TSB and incubated overnight at 37 °C. All culture medium was centrifuged (14,240×*g*, 4 °C, 10 min), washed with PBS, and the pellet was suspended in 200 μL of PBS. DNA was extracted from bacteria using *AccuPrep*® Genomic DNA Extraction Kit (Bioneer Corp., Daejeon, Korea). Purity and concentration of DNA were assessed using a Nanodrop spectrophotometer. Primers used in this work are listed in Table 1 [23]. All primers were synthesized by Bionics Inc. (Seoul, Korea). PCR mixture (Solgent Co., Ltd., Daejeon, Korea), 1 μg of DNA, and water were used for PCR reactions. A total of 20 μL of PCR reaction mixture was processed according to the annealing temperature of each primer. PCR products were subjected to 1.2% (w/v) agarose gel electrophoresis.

Statistical analysis

Obtained data are expressed as mean ± standard deviation of triplicates. Differences between groups were analyzed using Student's *t* test and analysis of variance (ANOVA) was used to analyze differences in several groups. Duncan's post hoc tests were performed to evaluate significant difference ($p < 0.05$) using SPSS 24 software (SPSS Inc, Chicago, IL, USA).

Table 1 Oligonucleotide primer pair sequence

Primer	Sequence (5′-3′)	Size (bp)	Reference
hblA-F	AAGCAATGGAATACAATGGG	1154	[23]
hblA-R	AGAATCTAAATCATGCCACTGC		
hblC-F	GATACTCAATGTGGCAACTGC	740	
hblC-R	TTGAGACTGCTCGTCTAGTTG		
hblD-F	ACCGGTAACACTATTCATGC	829	
hblD-R	GAGTCCATATGCTTAGATGC		
nheA-F	GTTAGGATCACAATCACCGC	755	
nheA-R	ACGAATGTAATTTGAGTCGC		
nheB-F	TTTAGTAGTGGATCTGTAGC	743	
nheB-R	TTAATGTTCGTTAATCCTGC		
nheC-F	TGGATTCCAAGATGTAACG	683	
nheC-R	ATTACGACTTCTGCTTGTGC		

Results and discussion

Isolation and identification of *Bacillus* strain

Bacillus strain was isolated from kimchi by heating at 80 °C for 30 min and identified by 16S rRNA gene sequence analysis. By comparing sequencing results to nucleotide sequence database of National Center for Biotechnology Information Nucleotide using BLAST, P223 was identified as *B. subtilis*, sharing 99% sequence identities (similar to GenBank accession number, HQ202545.1) with 16S rRNA of known *B. subtilis* strains (data not shown). Our results indicated that *B. subtilis* P223 could be investigated for probiotic use because it is generally recognized as safe (GRAS) [16].

Resistance to artificial gastric juice and bile salts

Tolerance of *B. subtilis* P223 to artificial gastric juice (pH 2.5, 0.3% pepsin, 3 h) and bile salt (0.3% oxgall, 24 h) was measured to evaluate the potential of this *Bacillus* strain as probiotic. Results for the number of cells after treated with artificial gastric juice and bile salts are shown in Table 2. Vegetative cells of *B. subtilis* P223 were reduced by 2.73 log CFU/mL whereas *B. clausii* ATCC 700160 did not grow under acidic condition. On the other hand, the number of spore cells of *B. subtilis* P223 treated with artificial gastric acid showed no significant ($p > 0.05$) difference with the initial cell number while *B. clausii* ATCC 700160 was only reduced by 0.38 log CFU/mL. In the presence of artificial bile salts for 24 h, the number of *Bacillus* strains of both vegetative and spore cells was increased more than that in the control. Lee et al. [15] have reported that the number of vegetative cells of *B. polyfermenticus* KU3 is decreased by 3.18 log CFU/mL when it is treated with artificial gastric juice (pH 2.5, 0.1% pepsin, 2 h) and by 1.26 log CFU/mL when it is treated with artificial bile salts (0.3% oxgall, 24 h) while the number of spore cells of the strain is decreased only by 0.08 log CFU/mL in the presence of artificial gastric juice and by 0.16 log CFU/mL when treated with artificial bile salts. Our results are similar to results of a previous study showing that vegetative cells of *B. megaterium* JHT3 and *B. subtilis* DET6 had poor resistance to artificial gastric acid whereas their spore cells were resistant to gastric conditions [24].

Adhesion capacity to HT-29 cells

Adhesion ability of *Bacillus* to human epithelial cells was determined for its potential to colonize human intestine epithelial cells. Capacity for adhesion was tested for vegetative and spore cells (Table 2). The number of vegetative cells of *B. clausii* ATCC 700160 and *B. subtilis* P223 was reduced by 2.57 and 2.44 log CFU/well, respectively, while the number of their spore cells was reduced by 1.18 and 1.27 log CFU/well, respectively. Lee et al. [15] have reported that *B. polyfermenticus* SCD was inoculated at a concentration of 8.06 ± 0.02 log CFU/well to Caco-2 cells. After 2 h of incubation, 6.85 ± 0.08 log CFU/well of cells were found to be attached to intestinal cells [15]. Additionally, *L. rhamnosus* GG was inoculated to HT-29 cells at a concentration of 8.0 log CFU/well. After 2 h of incubation, 6.93 log CFU/well of bacterial cells were attached [7]. Therefore, the capacity for adhesion of *B. subtilis* P223 was sufficient to colonize intestinal epithelial cells.

Autoaggregation and coaggregation activities

To investigate colonization of bacteria to intestinal cells and its inhibition on GI tract infection by pathogens, autoaggregation and coaggregation were performed as described previously [25]. After 4 h of incubation, *B. clausii* ATCC 700160 and *B. subtilis* P223 showed

Table 2 Probiotic characterization of *Bacillus* strains

Treatment	Viable cell number (Log CFU/mL)			
	Vegetative cell		Spore cell	
	B. clausii ATCC 700160	*B. subtilis* P223	*B. clausii* ATCC 700160	*B. subtilis* P223
Resistance to artificial gastric juice and bile salts				
Initial cell number	6.82 ± 0.35^{a1}	6.88 ± 0.18^{b}	5.69 ± 0.21^{b}	5.04 ± 0.57^{a}
0.3% (w/v) pepsin, pH 2.5, 3 h	ND[2]	4.15 ± 0.22^{a}	5.31 ± 0.16^{a}	5.38 ± 0.59^{a}
0.3% (w/v) oxgall, 24 h	7.50 ± 0.49^{a}	7.14 ± 0.32^{b}	7.86 ± 0.10^{c}	7.07 ± 0.36^{b}
Adhesion to HT-29 cell				
Initial cell number	7.57 ± 0.78^{a}	6.96 ± 0.09^{a}	5.57 ± 0.08^{a}	4.63 ± 0.05^{a}
Adhesion cell number	5.00 ± 0.05^{b}	4.52 ± 0.09^{b}	4.39 ± 0.08^{b}	3.36 ± 0.10^{b}
β-glucuronidase	–	–	NT[3]	NT
Hemolysis	–	–	NT	NT
Toxin genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB, nheC)*	–	–	NT	NT

[1a–c] Different superscript letters in the same row indicate statistical differences in each characteristic ($p < 0.05$). Values are expressed as mean ± SD ($n = 3$)

[2] Not detected

[3] Not tested

autoaggregation abilities of 85.10 ± 1.56% and 88.13 ± 3.55%, respectively (Table 3). After 24 h incubation, the value of *B. clausii* ATCC 700160 was increased while the value of *B. subtilis* P223 remained the same. Among different pathogens, *S. aureus* ATCC 6538 showed the highest activity after 4 h incubation while *S. aureus* ATCC 6538 and *L. monocytogenes* ATCC 15313 showed the highest activity after 24 h incubation. However,

Table 3 Autoaggregation and coaggregation activities of *Bacillus* strains and pathogens

Microorganisms	4 h	24 h
Autoaggregation activity (%)		
Bacillus		
B. clausii ATCC 700160	85.10 ± 1.56^{d1}	93.42 ± 0.86^{e}
B. subtilis P223	88.13 ± 3.55^{e}	86.03 ± 2.46^{d}
Pathogens		
S. aureus ATCC 6538	29.42 ± 2.14^{c}	66.35 ± 5.16^{c}
S. enteritidis ATCC 13076	8.88 ± 0.94^{a}	21.19 ± 4.69^{a}
L. monocytogenes ATCC 15313	12.26 ± 1.59^{b}	66.16 ± 3.67^{c}
E. coli ATCC 25922	13.26 ± 3.38^{b}	51.90 ± 3.41^{b}
Coaggregation activity (%)		
B. clausii ATCC 700160 with		
S. aureus ATCC 6538	62.37 ± 4.83^{e1}	72.52 ± 2.48^{c}
S. enteritidis ATCC 13076	50.32 ± 3.66^{ab}	63.08 ± 2.46^{a}
L. monocytogenes ATCC 15313	51.73 ± 3.32^{bc}	72.20 ± 3.25^{c}
E. coli ATCC 25922	51.81 ± 3.11^{bc}	70.16 ± 2.73^{bc}
B. subtilis P223 with		
S. aureus ATCC 6538	57.09 ± 3.48^{d}	68.96 ± 3.24^{b}
S. enteritidis ATCC 13076	46.86 ± 4.43^{a}	61.03 ± 4.98^{a}
L. monocytogenes ATCC 15313	54.07 ± 2.49^{cd}	79.19 ± 3.07^{d}
E. coli ATCC 25922	49.74 ± 2.45^{ab}	70.19 ± 1.46^{bc}

[1a–e] Different superscript letters in the same row indicate statistical differences in each characteristic ($p < 0.05$). Values are expressed as mean ± SD ($n = 3$)

autoaggregation abilities of pathogens were much lower than those of *Bacillus* strains after 4 or 24 h incubation. Patel et al. [24] have reported that autoaggregation activity of *B. subtilis* DET6 is about 60% after 1 h incubation at 37 °C. Autoaggregation percentages of *L. rhamnosus* GG are reported to be 14.2 ± 4.4% and 48.2 ± 3.5% after 4 and 24 h of incubation at room temperature, respectively [19].

Coaggregation activity of *B. clausii* ATCC 700160 (62.37 ± 4.83%) and *B. subtilis* P223 (57.09 ± 3.48%) showed the highest properties with *S. aureus* ATCC 6538 after 4 h of incubation. After 24 h of incubation, *S. aureus* ATCC 6538 and *L. monocytogenes* ATCC 15313 showed higher coaggregation activities compared to other pathogens. In comparison with autoaggregation results, autoaggregation properties of pathogens affected coaggregation activities of *Bacillus*. Coaggregation percentages of *Bacillus subtilis* KATMIRA1933 in the presence of *S. aureus, S. enteritica, L. monocytogenes,* and *E. coli* were 34, 47.4, 48.2, and 50.3%, respectively [26].

Inhibition of *Bacillus* on adherence of pathogens to HT-29 cells

Adhesion to HT-29 cells was measured by counting viable cells (Fig. 1). Adherence of *S. enteritidis* ATCC 13076 to HT-29 cells was reduced by 0.29 and 0.49 CFU/mL when it was added with *B. clausii* ATCC 700160 and *B. subtilis* P223, respectively. When *L. monocytogenes* ATCC 15313 single culture was added to HT-29 cells, 5.90 log CFU/mL of *L. monocytogenes* ATCC 15313 were attached to HT-29 cells. However, when *B. clausii* ATCC 700160 and *B. subtilis* P223 was added into each well, the number of *L. monocytogenes* ATCC 15313 cells attached to HT-29 cells was decreased to 0.21 and 3.64 log CFU/mL, respectively. The adherence of *E. coli* ATCC 25922 to HT-29 cells was decreased more when it was incubated with *B. subtilis* P223 (0.42 log CFU/mL) compared to that when it was incubated with *B. clausii* ATCC 700160 (0.05 log CFU/mL). These results indicated that *B. subtilis* P223 could inhibit the adherence of pathogens (both Gram-positive and Gram-negative) to intestinal cells more than *B. clausii* ATCC 700160.

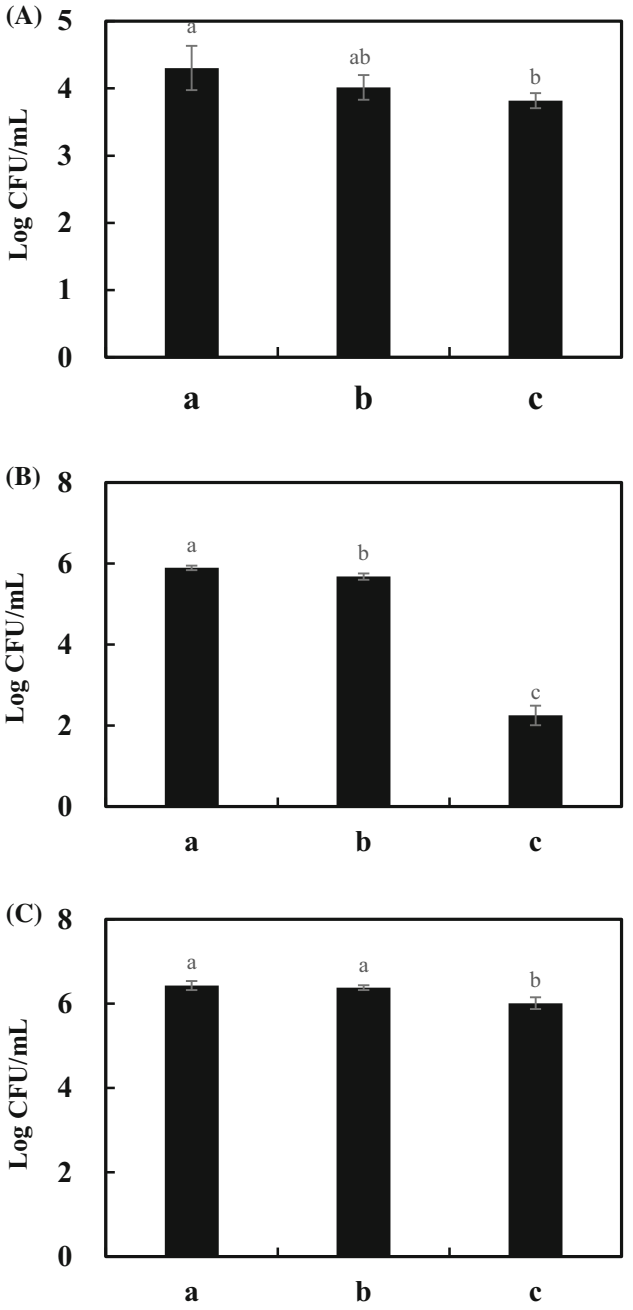

Fig. 1 Inhibition activity of *Bacillus* strains against adherence of pathogens to intestinal cells. (**A**) *S. enteritidis*; (**B**) *L. monocytogenes*; (**C**) *E. coli*. a, pathogen; b, pathogen with *B. clausii* ATCC 700160; c, pathogen with *B. subtilis* P223. *Different letters* on each *bar* represent significant difference between values ($p < 0.05$)

Production of enzymes

Bacillus strains were tested for production of enzyme using API zym kit. *B. clausii* ATCC 700160 and *B. subtilis* P223 did not produce β-glucuronidase, a carcinogenic enzyme (Table 2). Amylase activity of *B. clausii* ATCC 700160 (1.32 ± 0.11 U/mL) was higher than that of *B. subtilis* P223 (0.73 ± 0.10 U/mL). Protease activity of *B. subtilis* P223 (448.08 ± 12.42 U/mL) was similar to that of *B. clausii* ATCC 700160 (431.63 ± 13.53 U/mL). Amylase and protease activities are necessary for probiotics with digestive effect as starter of fermented foods because these enzymes can generate amino acids, sugars, organic acids, and diverse small compounds [5].

Antibiotic susceptibility of *Bacillus* strains

Antibiotic susceptibility of probiotics should be measured for safety purpose. Antibiotic resistance gene transmission can occur due to transposons, plasmids, and bacterial gene mutation, leading to new antibiotic resistant strains [27]. *B. clausii* ATCC 700160 and *B. subtilis* P223 were found to be only resistant to streptomycin among 8 kinds of antibiotics (data not shown). This is similar to *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895. They are also resistant to streptomycin and intermediately resistant to chloramphenicol [26].

Hemolysis and enterotoxin production

B. clausii ATCC 700160 and *B. subtilis* P223 did not show hemolysis on sheep blood agar (Table 2). Similarly, *B. polyfermenticus* CJ6 does not cause hemolysis on horse blood agar [28]. α-Hemolysis and no-hemolysis are considered to be safe whereas β-hemolysis is considered harmful [5].

For detection of enterotoxins, PCR and electrophoresis were performed. *B. cereus* KCCM 11341 carried enterotoxin genes (*hblA*, *hblC*, *hblD*, *nheA*, and *nheC*; data not shown). However, *B. clausii* ATCC 700160 and *B. subtilis* P223 did not carry any of these six genes (Table 2). *B. cereus* probiotics from products Subtyl and Biosubtyl^{DL} were found to carry enterotoxin genes, whereas *B. subtilis* PY79 did not carry such genes [29].

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