Evaluation of the Probiotic Potential of \textit{Bacillus polyfermenticus} CJ6 Isolated from \textit{Meju}, a Korean Soybean Fermentation Starter

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To evaluate the probiotic potential of \textit{Bacillus polyfermenticus} CJ6 isolated from \textit{meju}, a Korean traditional soybean fermentation starter, its functionality and safety were investigated. \textit{B. polyfermenticus} CJ6 was sensitive to all antibiotics listed by the European Food Safety Authority. The strain was also non-hemolytic, carried no emetic toxin or enterotoxin genes, and produced no enterotoxins. The resistance of \textit{B. polyfermenticus} CJ6 vegetative cells and spores to simulated gastrointestinal conditions was high (60–100\% survival rate). \textit{B. polyfermenticus} CJ6 produced high amounts (0.36 g as a purified lyophilized form) of \(\gamma\)-polyglutamic acid (PGA). We speculate that the improved cell viability and the production of \(\gamma\)-PGA have a significant correlation. Adhesion of the strain to Caco-2 and HT-29 cells was weaker than that of the reference strain (\textit{Lb. rhamnosus} GG), but it was comparable to or stronger than those of reported \textit{Bacillus} spp. When \textit{B. polyfermenticus} CJ6 spores were given orally to mice, the number of cells excreted in the feces was 4-fold higher than the original inocula. This suggests the inoculated spores propagated within the intestinal tract of the mice. This idea was confirmed by field emission scanning electron microscopy, which revealed directly that \textit{B. polyfermenticus} CJ6 cells germinated and adhered within the gastrointestinal tract of mice. Taken together, these findings suggest that \textit{B. polyfermenticus} CJ6 has probiotic potential for both human consumption and use in animal feeds.

Keywords: Probiotic, \textit{Bacillus polyfermenticus}, safety, functional properties

The most commonly used probiotics for human consumption are lactobacilli and bifidobacteria [27]. Although less well known, \textit{Bacillus} species can also be used as probiotics [7, 19, 34]. For example, \textit{Bacillus}-based probiotics have already been introduced to the international market and are being used for bacteriotherapy and for bacterioprophylaxis of gastrointestinal (GI) disorders in humans [16, 18, 28]. These preparations consist of bacterial spores, which have a number of advantages over non-spore formers such as \textit{Lactobacillus} spp., including the abilities to survive the adverse conditions within the GI tract and to be stored at room temperature with no deleterious effects on viability [3, 35]. Foods fermented with \textit{Bacillus} spp. have a long history in Korea, Japan, and some African countries [1]. The species traditionally included in the \textit{B. subtilis} group (\textit{B. subtilis}, \textit{B. licheniformis}, \textit{B. amyloliquefaciens}, and \textit{B. pumilus}) have been given a “Qualified Presumption of Safety” (QPS) by the European Food Safety Authority (EFSA) [10]; nonetheless, the use of \textit{Bacillus} probiotics raises the question of safety, because several strains have been shown to be multidrug-resistant and to harbor toxin genes [8, 16]. Moreover, the number of reports showing the presence of toxins in several \textit{B. subtilis} strains is increasing [10, 34]. It would therefore seem appropriate that the safety of potential probiotic strains from this group be analyzed individually on a strain-by-strain basis [10].

To be used as a probiotic, a strain must fulfill various criteria related to safety (no pathogenicity or antibiotic resistance), functionality (survival, adherence, colonization, antimicrobial activity, immunostimulation, antigenotoxic activity, and prevention of pathogens) and technical issues (sensory properties, stability, phage resistance, and viability in process) [30]. \textit{Bacillus polyfermenticus} strains share the same morphological and biochemical properties with \textit{B. subtilis} strains. However, \textit{B. polyfermenticus} strains are different from \textit{B. subtilis} strains in terms of lactose utilization. \textit{B. polyfermenticus} produces large amounts of acetic acid and lactic acid from glucose and lactose, respectively [26]. We previously isolated \textit{B. polyfermenticus} CJ6 having antifungal [20] and antibacterial activities [21].

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The antibacterial activity was stable over a pH range of 3.0–9.0, and was partially inactivated by proteinase K, protease, and α-chymotrypsin [21]. The antibacterial activity appeared effective against a variety of pathogens, including Salmonella Typhi, Listeria monocytogenes, Escherichia coli O157:H7, and Staphylococcus aureus. Indeed, when equal numbers of cells were cocultured, B. polyfermenticus CJ6 completely suppressed these pathogens within 3–6 h [21].

In the present study, we assessed the probiotic potential of B. polyfermenticus CJ6 isolated from mejü [20] by evaluating its functionality (acid and bile tolerance, adherence to epithelial surfaces) and safety (antibiotic resistance, virulence traits). This study also included in vivo experiments, which confirmed the germination and colonization of B. polyfermenticus CJ6 within the mouse GI tract. The properties of B. polyfermenticus CJ6 described here suggest that this microorganism may have probiotic potential for both human consumption and use in animal feeds.

**Materials and Methods**

**Bacterial Strains and Media**

*B. polyfermenticus* CJ6 (GenBank Accession No. of the 16S rDNA gene: FJ436014) and *B. cereus* ATCC 14579 were cultured separately in tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37°C with shaking (200 rpm; Hanbaek, Seoul, Korea). *Lactobacillus rhamnosus* GG (ATCC 53103) was cultured in de Man, Rogosa, and Sharpe broth (MRS; Difco) at 30°C. *B. polyfermenticus* CJ6 was aerobically propagated in TSB agar and Mueller–Hinton (MH; Difco) media. ATCC strains were purchased from the American Type Culture Collection (Manassas, VA, USA).

**Preparation of Bacterial Cells**

**Vegetative Cells.** *B. polyfermenticus* CJ6 was cultivated in 50 ml of TSB medium with 1% (v/v) inoculum for 12 h at 37°C, and then centrifuged (9,500 × g, 3 min, 4°C), washed with 20 ml of phosphate buffered saline (PBS; pH 7.4, Hyclone, Logan, UT, USA), and resuspended in 10 ml of PBS.

**Spores.** Spores were prepared as described by Koransky et al. [24] with modification. *B. polyfermenticus* CJ6 was cultivated in 50 ml of TSB for 96 h at 37°C. The culture was then mixed with 50% ethanol to a 1:1 ratio and rotated in screw-capped tubes at 33 rpm on a circular rotator (Curtis Nuclear, Los Angeles, CA, USA) for 1 h at room temperature [4, 24]. The mixture was then centrifuged (9,500 × g, 15 min, 4°C), washed twice with 20 ml of PBS (pH 7.4), and resuspended in 10 ml of PBS. The resultant spore suspension was serially diluted and spread on TSB agar for cell counting.

**Analysis of Enterotoxins and Virulence Traits**

Hemolysis was detected by streaking bacterial cells on blood agar base containing 7% horse blood (Oxoid, Basingstoke, Hampshire, England). The plate was then incubated for 24 h at 37°C, after which it was screened for clear zones around colonies.

Polymerase chain reaction (PCR) was carried out using a Palmcycler (Corbett Research, Mortlake, Australia) to detect the putative *B. cereus* emetic toxin [23] and enterotoxin [15, 23] genes. Bacterial chromosomal DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Eleven sets of primers previously described by Guinebretière et al. [15] and Kim et al. [23] were used in this experiment.

The Hbl and Nhe enterotoxins were detected and measured using a *B. cereus* enterotoxin reversed passive latex agglutination (BCET-RPLA) kit (Oxoid) and a *Bacillus* diarrhoeal enterotoxin visual immunoassay (BDE-VIA) kit (Teca, New South Wales, Australia), respectively. These commercial kits were used according to the manufacturer's instructions.

**Antibiotic Susceptibility**

*B. polyfermenticus* CJ6 was evaluated for its susceptibility to antibiotics using the technical guidelines of the European Food Safety Authority [11]. The minimal inhibitory concentrations (MICs) of ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol (Sigma, St. Louis, MO, USA) were all determined. Cells cultured overnight (12 h) in TSB were centrifuged (9,500 × g, 3 min, 4°C) and resuspended in MH broth at a concentration of approximately 5.5 log CFU/ml (McFarland standard 0.5). Each antibiotic was added to the MH cell suspension and incubated for 24 h at 37°C. Growth of the cell suspension was observed visually and confirmed by measuring its turbidity at 600 nm (Ultrspec 2100 Pro; Amersham, Uppsala, Sweden). MIC values were determined using serial antibiotic dilution in MH broth. Culture in MH broth for 24 h at 37°C without an antibiotic served as a control.

**Acid and Bile Tolerance**

The ability of *B. polyfermenticus* CJ6 vegetative cells and spores to tolerate acid and bile salt was determined as described previously [6, 8] with modification. Approximately 8.1 log CFU/ml of vegetative cells or spores were resuspended in 0.2 M HCl buffer (pH 2.0) or simulated gastric juice (SGJ; 1 mg pepsin dissolved in 1 ml of 0.5% saline buffer, pH 2.0) and/or bile salt (0.3% or 0.5% oxgall dissolved in PBS, pH 7.4). The cells were incubated for 1 h at 37°C in 0.2 M HCl buffer, for 1 h or 2 h in SGJ, and for 3 h in bile salt. After incubation, the cells were harvested (9,500 × g, 15 min, 4°C) and resuspended in TSB. Numbers of viable cells were then counted on TSB agar after incubation for 24 h at 37°C. Run in parallel were control samples in which the vegetative cells or spores were suspended in TSB media without acids and bile salt.

**Adhesion to Caco-2 and HT-29 Cells**

The adherence of *B. polyfermenticus* CJ6 to Caco-2 and HT-29 cells was evaluated as described by Rowan et al. [29] with modification. Caco-2 and HT-29 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) and Rosewell Park Memorial Institute (RPMI; Hyclone) medium, respectively. Both media were supplemented with 10% (v/v) fetal bovine serum (FBS), 1% non-essential amino acids, 1% streptomycin/penicillin (10,000 IU/ml), 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1 mM sodium pyruvate, and 1 mM 1-glutamine. For adhesion assays, Caco-2 and HT-29 monolayers were prepared in 24-well tissue plates (Corning Costar, Cambridge, MA, USA). Prior to the assays,
the cell monolayers were washed twice with sterile PBS, and then each well was inoculated with approximately 7.3 log CFU of bacterial cells in 1 ml of DMEM or RPMI without antibiotics or PBS and incubated for 1 h at 37°C under a 5% CO₂ atmosphere (AsteC Sci 16SD; AsteC, Tokyo, Japan). After incubation, the monolayers were washed three times with sterile PBS to remove the unattached bacteria. The total number of adherent bacteria in each well was counted by lysing cells; 1 ml of 0.05% (v/v) Triton X-100 was added to each well, after which the plate was shaken (Green SSeiker, Vision, Gyeonggi-Do, Korea) for 10 min at 160 rpm at room temperature. The viable bacterial counts were plated onto TSB agar and incubated for 24 h at 37°C. *Lb. rhamnosus* GG, which is known for its adhesion properties [25], served as a positive control. In the *Lb. rhamnosus* GG experiment, all procedures were the same as with *B. polyfermenticus* CJ6, except MRS served as the counting medium.

**In vivo Adherence Assay**

Five-week-old mice (BALB/c AnNHsd) were purchased from Koatech (Gyeonggi-Do, Korea) and maintained at the animal holding unit of the Laboratory Animal Center in Chosun University, School of Medicine. Mice were provided free access to a standard diet and water for 1 week before the experiments to minimize the effects of changes in feeding schedules and diurnal rhythms on the data. This experimental design was approved by the Committee for Care and Use of Laboratory Animals at Chosun University. Mice were divided into three groups for use in a fecal cell count experiment (5 mice), a colonization analysis with mucosal samples (12 mice), and a control experiment (standard diet without spores supplement; 3 mice). Spores (9.18 ± 0.01 log CFU/0.2 ml of PBS) were administered by intragastric gavage to each mouse using a sterile stainless feeding needle once a day for 6 days. Thereafter, the mice had free access to the standard diet without spore administration until the end of the experiment (day 17).

**Counts of *B. polyfermenticus* CJ6 in Fecal Counts**

Fecal *B. polyfermenticus* CJ6 counts were carried out using the method of Hoa et al. [17] with modification. Fecal samples were collected once a day for 17 days. The collected samples were weighed and homogenized in PBS using a Cute mixer (CM-1000; Eyela, Tokyo, Japan). Aliquots were then serially diluted, plated on TSB agar, and incubated for 24 h at 37°C. *B. polyfermenticus* CJ6 was readily distinguished from other organisms based on its distinctive colony shape and color on TSB agar. The identity of *B. polyfermenticus* CJ6 was confirmed based on its biochemical characteristics [20] using an API 50 CHB system (Bio-Mérieux, Marcy l’Etoile, France).

**Counts of *B. polyfermenticus* CJ6 in the Intestinal Tract**

Colonization by *B. polyfermenticus* CJ6 in the mouse intestinal tract was investigated using the method of Wang et al. [38] with modification. At selected times following gavage with *B. polyfermenticus* CJ6 (days 7, 10, and 17), four mice were sacrificed under anesthesia with diethyl ether. Prior to sacrifice, the mice were fasted for at least 12 h, but water remained available. The small and large intestines were removed aseptically (small intestine length: 39.7–43.5 cm; large intestine length: 10.6–14.2 cm) and then cut longitudinally, after which the feces were removed using a sterile scraper (SPL, Gyeonggi-Do, Korea). The intestines were then washed twice with sterile PBS buffer, diced, suspended in sterile PBS buffer, and vortex mixed to break bacterial clumps away from the tissue pieces. Aliquots of the resultant suspension were serially diluted with PBS buffer, plated on TSB agar, and incubated for 24 h at 37°C. *B. polyfermenticus* CJ6 was distinguished from the other strains and identified based on its colony shape and on its morphological and biochemical characteristics. The strain was ultimately confirmed as *B. polyfermenticus* CJ6 by 16S rRNA sequencing (1,520 bp).

**Viscosity Determination**

The viscosity of the *B. polyfermenticus* CJ6 culture supernatant was measured by a viscometer (Brookfield, Middleboro, MA, USA) equipped with a sample adapter using spindle No. 18. *B. subtilis* ATCC 6633 culture was used as a control.

**Preparation and Analysis of Poly-γ-Glutamic Acid (PGA)**

 Purification and analysis of PGA from *B. polyfermenticus* CJ6 was carried out as described previously [14] with modification. *B. polyfermenticus* CJ6 culture broth containing PGA was mixed with the same volume of distilled water and centrifuged (20,000 × g, 20 min, 4°C). Four volumes of cold ethanol were poured into the supernatant and left overnight, and the resultant precipitate was collected by centrifugation (25,000 × g, 30 min, 4°C). The precipitate was dissolved in distilled water and insoluble materials were removed by centrifugation. The aqueous solution was dialyzed against distilled water at 4°C for 24 h using dialysis tubing with a cut-off of MW<1,000 (Spectra/Por 6 membrane; Spectrum Labs, Rancho Dominguez, CA, USA) and the dialysis sample was then lyophilized to give pure PGA.

Analysis of PGA was done with high-performance liquid chromatography (HPLC; Agilent, Santa Clara, CA, USA) using a Inno C18 column (4.6 mm × 150 mm; Innopia, Gyeonggi-Do, Korea) and FL detector (Agilent). Sample was prepared by hydrolysis of the purified PGA using 6 N HCl at 130°C for 24 h. The running buffers used were buffer A and buffer B [acetonitrile:methanol:water = 45:45:10 (v/v/v)]. The column was eluted at a flow rate of 1.5 ml/min. The running conditions were 100% buffer A for 25 min, followed by 55% buffer A to 45% buffer B for 2 min, and 0% buffer A to 100% buffer B for 4 min. One nmol/µl of 17 amino acids (Agilent) including glutamic acid dissolved in 0.1 N HCl was used as the standard.
Table 1. Potential virulence characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>B. polyfermenticus CJ6</th>
<th>B. cereus ATCC 14579</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysisa</td>
<td>γ</td>
<td>β</td>
</tr>
<tr>
<td>Emetic toxin geneb</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ces</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hbl gene complexc</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>hblA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>hblB</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>hblC</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>hblD</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Nh gene complexd</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>nheA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nheB</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nheC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other enterotoxin genes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bceT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cytK</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>entFM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hbl enterotoxin</td>
<td>0</td>
<td>128</td>
</tr>
<tr>
<td>Nhe enterotoxin</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

aHemolysis on horse blood agar; γ, no change; β, complete hemolysis with a clear zone around colonies.
bGene encoding components of the Hbl or Nhe enterotoxins or other B. cereus enterotoxins or emetic toxin were analyzed using PCR: +, a PCR product of the expected size was amplified; −, no PCR product was detected.
cProduction of the Hbl enterotoxin in growing cells was determined using a BCEF-RPLA toxin kit (Oxoid) and expressed as an index, where a value of 0 is negative. The sensitivity of the test is 2 ng/ml.
dProduction of Nhe enterotoxin in growing cells was measured using the Bacillus Diarrhoeal Enterotoxin Visual Immunoassay kit (Tecra Diagnostics). Strains with an index of <3 are considered negative. The sensitivity of the test is 1 ng/ml.

**RESULTS AND DISCUSSION**

**Presence of Potential Virulence Factors**

*B. polyfermenticus* CJ6 did not induce hemolysis when plated on horse blood agar. The strain can therefore be considered γ-hemolytic. By contrast, *B. cereus* ATCC 14579 produced a clear zone around its colony on horse blood agar (β-hemolysis).

To detect the presence of *Bacillus* emetic toxin and enterotoxin (diarrheal toxin) genes using PCR, one set of primers for the emetic toxin gene and 10 sets of primers for the enterotoxin genes were used to evaluate *B. polyfermenticus* CJ6. The genes investigated included the *nhe* genes (3 sets), encoding a nonhemolytic enterotoxin; *hbl* genes (4 sets), encoding hemolysin BL, which is the primary virulence factor in *B. cereus*-associated diarrhea; *bceT*, *cytK*, and *entFM* genes (one set each), encoding the single component toxin enterotoxin T, cytotoxin K, and enterotoxin FM, respectively [32]; and *ces* gene, encoding cereulide synthetase, which is responsible for the emetic type of GI disease caused by *B. cereus* [12]. *B. cereus* ATCC 14579, which is known to produce enterotoxins [23], served as a positive control. Table 1 shows that *B. polyfermenticus* CJ6 did not carry the *B. cereus* emetic toxin or enterotoxin genes. In vivo analysis was also carried out for the Hbl and Nhe *B. cereus* enterotoxins, and *B. polyfermenticus* CJ6 tested negative. By contrast, *B. cereus* ATCC 14579 tested positive in Nhe and Hbl enterotoxin production assays and was found to carry all of the tested *B. cereus* enterotoxin genes. *B. cereus* ATCC 14579 is known to be a cereulide-negative strain [12] and did not produce the *ces* gene fragment in PCR using a *ces* primer set (Table 1). When assessing the safety of a bacterium with probiotic potential, it is necessary to ensure that it produces no enterotoxins and emetic toxins. Many *Bacillus* strains are known to carry one or more enterotoxin and emetic toxin genes (*hbl, nhe, cytK, bceT, entFM, and ces*) in their chromosome [15, 23, 29]. On the other hand, *B. polyfermenticus* CJ6 did not carry the emetic toxin or enterotoxin genes, and produced neither. Moreover, *B. polyfermenticus* CJ6 did not induce hemolysis. Given the absence of *Bacillus* toxins and sensitivity to the antibiotics listed by EFSA, it seems reasonable to conclude that *B. polyfermenticus* CJ6 does not present a risk to human health. This strain thus appears to meet the currently recommended in vitro properties of *Bacillus* spp. with QPS status [11].

**Antibiotic Resistance**

*B. polyfermenticus* CJ6 was evaluated for its resistance to nine antibiotics highlighted by the EFSA [11]. According to the EFSA guidelines, strains with MICs lower than or equal to the EFSA breakpoint are considered susceptible [11]. *B. polyfermenticus* CJ6 can be categorized as...
susceptible to all of the antibiotics tested (Table 2), though it was somewhat more resistant to clindamycin than to the others. Other investigators have reported that many *Bacillus* strains show resistance to clindamycin and suggested that this may be an intrinsic characteristic of *Bacillus* species [19, 34]. Our findings were consistent with those earlier reports [19, 34]. Only the clindamycin MIC of *B. polyfermenticus* CJ6 reached the EFSA breakpoint (although it did not exceed it); the MICs for the other antibiotics tested were far below the EFSA breakpoint.

**PGA Production**

Several *Bacillus* species have been shown to produce γ-PGA on their exterior, and this viscous compound is freely secreted into the growth medium of *Bacillus* spp. as a product of fermentation [2, 33]. When we measured the viscosity of the *B. polyfermenticus* CJ6 supernatant, we found that it was four times more viscous than that of *B. subtilis* ATCC 6633 (4.69 ± 0.12 cP vs. 1.54 ±0.14 cP). γ-PGA production was confirmed by the detection of only glutamic acid in the hydrolysate of the purified compound using HPLC analysis (Fig. 1). When we purified PGA from *B. polyfermenticus* CJ6, 0.36 ± 0.08 g of purified PGA (lyophilized form) was obtained from 100 ml of culture broth.

**Acid and Bile Tolerance of Spores and Vegetative Cells**

We assessed the survival of spores and vegetative cells in 0.2 M HCl buffer (pH 2.0) or simulated gastric juice (SGJ; 1 mg/ml pepsin, pH 2.0) and/or bile salt (0.3% or 0.5%, pH 7.4). The acid and bile tolerance of *B. polyfermenticus* CJ6 (vegetative cells and spores) was quite high, so that the numbers of log CFU before and after treatment were similar (Table 3).

With *Bacillus* probiotics, it is particularly important that acid and bile tolerance tests are evaluated with spores and vegetative cells, as consumed *Bacillus* spores germinate in the GI tract [3]. The resistance of *B. polyfermenticus* CJ6 vegetative cells and spores to simulated GI stress was quite high, with 72–100% and 60–96% survival for spores and vegetative cells, respectively, after treatment. This is consistent with previously reported rates of survival among *Bacillus* spores (53–100%) after acid and bile treatments [8, 9]. However, whereas we found high viability (60–

### Table 3. Acid and bile tolerance of *B. polyfermenticus* CJ6.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vegetative cells</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>8.21 ± 0.30</td>
<td>8.08 ± 0.23</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 M HCl buffer, 1 h</td>
<td>8.20 ± 0.09</td>
<td>8.08 ± 0.10</td>
</tr>
<tr>
<td>SGJ*, 1 h</td>
<td>8.12 ± 0.11</td>
<td>8.07 ± 0.15</td>
</tr>
<tr>
<td>SGJ, 2 h</td>
<td>7.99 ± 0.08</td>
<td>8.00 ± 0.42</td>
</tr>
<tr>
<td>Bile salt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxgall (0.3%), 3 h</td>
<td>8.15 ± 0.04</td>
<td>8.06 ± 0.21</td>
</tr>
<tr>
<td>Oxgall (0.5%), 3 h</td>
<td>8.15 ± 0.10</td>
<td>8.02 ± 0.22</td>
</tr>
<tr>
<td>Acid and then bile salt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGJ (pH 2.0), 1 h and then oxgall (0.3%), 3 h</td>
<td>8.09 ± 0.08</td>
<td>8.00 ± 0.10</td>
</tr>
<tr>
<td>SGJ (pH 2.0), 1 h and then oxgall (0.5%), 3 h</td>
<td>8.00 ± 0.13</td>
<td>7.93 ± 0.10</td>
</tr>
</tbody>
</table>

*SGJ: simulated gastric juice (1 mg/ml pepsin in 0.5% saline buffer, pH 2.0).*

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**Fig. 1.** HPLC analysis of the hydrolysate of the purified γ-PGA. (A) Authentic glutamic acid; (B) hydrolysate of purified compound.
96%) among *B. polyfermenticus* CJ6 vegetative cells under simulated gastric conditions, others found rather poor cell viability (0.0002–0.0011%) when vegetative cells were exposed to acid and bile salt [9]. We speculate that the improved cell viability we observed could reflect the production of γ-PGA by *B. polyfermenticus* CJ6. Similar results were obtained with exopolysaccharide (EPS)-producing lactic acid bacteria (LAB) [39]. With LAB, there was a significant (P < 0.05) correlation between the quantity of EPS produced by strains and their acid resistance, and it was suggested that EPSs may be protective against low pH and bile salts [39]. We suggest that γ-PGA plays a similar role for *B. polyfermenticus* CJ6. Further investigation will be needed to test that idea, however. Additionally, the ability to produce γ-PGA may be considered as an additional functional benefit of *B. polyfermenticus* CJ6, since γ-PGA is not toxic to humans or the environment, and promotes the absorption of minerals, sequesters high metals, and facilitates the decreased accumulation of body fat [33, 37].

**In Vitro Adhesion Properties**

When we assessed the adhesion of *B. polyfermenticus* CJ6 to Caco-2 and HT-29 cells, we found that 5.15 ± 0.04 log CFU/ml adhered to Caco-2 cells, and 5.28 ± 0.02 log CFU/ml adhered to the HT-29 cells. By comparison, 6.04 ± 0.15 log CFU/ml and 6.00 ± 0.16 log CFU/ml of *Lb. rhamnosus* GG cells, the positive control, adhered to Caco-2 and HT-29 cells, respectively. Thus *Lb. rhamnosus* GG showed approximately 5- to 7-fold greater adherence than *B. polyfermenticus* CJ6. Although the adhesion rate of *B. polyfermenticus* CJ6 was clearly lower than that, the adhesion of *B. polyfermenticus* CJ6 was significantly higher (0.68% as Caco-2) than other *Bacillus* strains. For example, *B. indicus* HU36 [19], *B. subtilis* var. Natto, *B. subtilis* PY79 [19], *B. subtilis* BS3 [34] and *B. licheniformis* BL31 [34] all showed adhesion rates to Caco-2 cells that were less than 0.5%.

**Fate of Spores in the Mouse GI Tract (In Vivo Adhesion)**

*B. polyfermenticus* CJ6 colonies have a characteristic ruffled shape and dark ivory color on TSB agar. This enabled *B. polyfermenticus* CJ6 cells from fecal samples to be easily counted among the other isolates present on the TSB agar. By contrast, *B. polyfermenticus* CJ6 colonies were never obtained from control samples from mice fed standard diet without sporulation supplement. As shown in Fig. 2, cell counts from the feces were 9.80 ± 0.19 log CFU/mouse/day for the 6 days of spores administration; thereafter, the counts declined to 6.63 ± 0.24 log CFU/mouse/day and were maintained at that level until day 17, even though spore administration had been stopped. We found that the total number of *B. polyfermenticus* CJ6 cells excreted (10.58 ± 0.11 log CFU/mouse over 17 days) was larger than the number in the original inocula (9.95 ± 0.02 log CFU/mouse for 6 days).

To determine the fate of *B. polyfermenticus* CJ6 spores within the mouse GI tract, we prepared spores and dosed mice with 9.18 ± 0.01 log CFU spores/mouse/day. Oral inoculation of spores was carried out for 6 days (arrow indicates the final inoculation). The cumulative spore counts (open symbols) and daily spore counts (filled symbols) were obtained from the feces of mice collected individually at the indicated times.

**Fig. 2.** *B. polyfermenticus* CJ6 counts in feces from Balb/c AnNlHsd mice.
The dashed line shows the number of spores inoculated: 9.18 ± 0.01 log CFU spores/mouse/day. Oral inoculation of spores was carried out for 6 days (arrow indicates the final inoculation). The cumulative spore counts (open symbols) and daily spore counts (filled symbols) were obtained from the feces of mice collected individually at the indicated times.
(antifungal and antibacterial) activities [20, 21]. Although probiotics are now considered to have a health benefit, the complete occupation of the GI tract by probiotic bacteria is not recommended, as we know little about the function of the native bacteria. We suggest that the ability to co-exist with the native bacteria is one of the most important characteristics of probiotics in the normal healthy intestine. What makes Bacillus spp. attractive is the low cost of their production and their easy preparation, resistance to the stresses associated with processing, and long shelf life over a wide range of temperatures [3]. However, it is becoming increasingly clear that a more rigorous selection process is required to ensure the safety of Bacillus probiotic candidates, as the occasional presence of Bacillus cereus toxins in strains belonging to several Bacillus spp. has been reported [29, 31]. In addition, before a probiotic can be considered beneficial to human health, it must exhibit (i) acid tolerance and tolerance to human gastric juice, (ii) bile tolerance, (iii) adherence to the epithelial surface and persistence within the human GI-tract, and (iv) antagonist activity against pathogens [30]. From the results of the present study, B. polyfermenticus CJ6 appears to satisfy all of these criteria; thus, further in vivo study of its health benefits in humans and its application in the food industry would seem warranted.

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**References**


