Inhibition of *Bacillus cereus* Growth and Toxin Production by *Bacillus amyloliquefaciens* RD7-7 in Fermented Soybean Products

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*Bacillus cereus* is a gram-positive, rod-shaped, spore-forming bacterium that has been isolated from contaminated fermented soybean food products and from the environment. *B. cereus* produces diarrheal and emetic toxins and has caused many outbreaks of foodborne diseases. In this study, we investigated whether *B. amyloliquefaciens* RD7-7, isolated from rice doenjang (Korean fermented soybean paste), a traditional Korean fermented soybean food, shows antimicrobial activity against *B. cereus* and regulates its toxin gene expression. *B. amyloliquefaciens* RD7-7 exhibited strong antibacterial activity against *B. cereus* and inhibited the expression of *B. cereus* toxin-related genes (*groEL, nheA, nheC, and entFM*). We also found that addition of water extracts of soybean and buckwheat soksungjang (Korean fermented soybean paste made in a short time) fermented with *B. amyloliquefaciens* RD7-7 significantly reduced the growth and toxin expression of *B. cereus*. These results indicate that *B. amyloliquefaciens* RD7-7 could be used to control *B. cereus* growth and toxin production in the fermented soybean food industry. Our findings also provide a basis for the development of candidate biological control agents against *B. cereus* to improve the safety of fermented soybean food products.

**Keywords:** *Bacillus cereus*, *Bacillus amyloliquefaciens* RD7-7, *B. cereus* toxin-related genes, fermented soybean food products

**Introduction**

A wide range of traditional or indigenous fermented foods and beverages, representing approximately 5-40% of the total daily food intake, are produced and consumed globally. Fermented foods can play an important role in strengthening the livelihoods and improving the nutrition and social well-being of millions of people worldwide, and can provide food security for vulnerable and marginalized populations, in particular. For example, doenjang (fermented soy paste), a traditional Korean fermented food, is an excellent source of nutrition that is used as a flavoring ingredient in Korea, similar to miso (Japanese fermented soybean paste) in Japan and tempeh (Indonesian fermented soybean food) in Indonesia [14]. Doenjang has received considerable attention owing to its beneficial health-promoting properties, such as its anticancer, antioxidant, and fibrinolytic activities [23]. However, concerns remain about the safety of consuming fermented soybean products such as doenjang because these products are often contaminated with various foodborne pathogens, including *Bacillus cereus* [18].

*B. cereus* is a spore-forming, gram-positive bacterium that is responsible for diarrheal (heat-labile) and emetic (heat-stable) food poisoning, which are caused by enterotoxins and emetic toxins, respectively [17, 28]. Its ability to cause diarrhea is attributed to various enterotoxins and virulence factors such as non-hemolytic enterotoxin (Nhe), hemolysin BL, cytotoxin-K, and enterotoxin FM (EntFM), a group of heat-labile proteins that cause abdominal cramps, nausea, and, rarely, vomiting and watery diarrhea [7, 8]. The Nhe complex is composed of the NheA, NheB, and NheC proteins, which are encoded by *nheA, nheB*, and *nheC* [33]. The heat shock protein GroEL, which provides protection against physiological and heat stresses and is required for survival of *B. cereus*, has been used to reveal phylogenetic
relationships between bacteria [12, 26]. Emetic foodborne illness is induced by the small cyclic heat-stable toxin cereulide, which causes vomiting and nausea; the cereulide synthetase gene (ces) is only found in emetic toxin-producing \( B. \) cereus [11, 17].

\( B. \) cereus is commonly found in contaminated foods containing fermented soybeans, such as doenjang, and the South Korean food authority has reported that ingestion of more than \( 10^4 \) CFU of \( B. \) cereus per gram of fermented soybean products may cause food poisoning [27, 30]. Because spores of \( B. \) cereus are highly resistant to various stresses (heat, cold, radiation, desiccation, and disinfectants) and show excellent adhesion to food surfaces, \( B. \) cereus contamination is very difficult to control in the fermented soybean food industry [10]. However, some strains of bacteria produce antibacterial substances (bacteriocin-like peptides and antimicrobial lipopeptides) that have few or no undesirable effects, such as inhibition of the growth of fermenting bacteria or decrement of food quality. These strains have been investigated as beneficial additions to starter cultures in the industrial-scale production of fermented soybean foods [5, 29]. For example, \( B. \) amyloliquefaciens strain RD7-7, isolated from traditional fermented soybean foods, exhibits high enzymatic and antibacterial activities against foodborne pathogens.

Previous studies have characterized the prevalence of \( B. \) cereus in fermented soybean products, but little research has been conducted on the detection or characterization of the expression of genes encoding \( B. \) cereus toxins in these products [19, 25]. Therefore, we investigated the toxin gene profiles and toxin expression levels of \( B. \) cereus in fermented soybean samples co-cultured with \( B. \) amyloliquefaciens RD7-7. Strain RD7-7 exhibited strong antibacterial activity against \( B. \) cereus and reduced its toxin expression. Thus, \( B. \) amyloliquefaciens RD7-7 shows potential for use as a starter strain to prevent the growth of \( B. \) cereus during the industrial production of fermented soybean foods and has numerous potential applications for food preservation.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

\( B. \) amyloliquefaciens RD7-7 KACC 92071P, \( B. \) amyloliquefaciens KACC 15866 (used as a reference strain), and \( B. \) cereus KACC 10004 (indicator strain) were grown in Luria-Bertani (LB) broth (Difco, Becton Dickinson, Sparks, MD, USA) or on LB agar medium at 30°C. The strains were subcultured at 30°C for 24 h in LB broth and streaked on nutrient agar (NA; Difco, Becton Dickinson) plates, and then incubated at 30°C for 24 h before use.

**Transmitting Electron Microscopy (TEM)**

Bacterial cultures were centrifuged at 930 xg for 20 min and then washed twice with distilled water (DW). A carbon Formvar-coated 200-mesh copper grid was rendered hydrophilic by high-voltage glow discharge (JFC-1100E Ion Sputter, Jeol Co., Tokyo, Japan). Bacteria on the grid were negatively stained with 2% uranyl acetate for 315 sec and then rinsed three times with DW. The sample was examined under a Tecnai 12 transmission electron microscope (Philips, Eindhoven, The Netherlands) at an acceleration voltage of 120 kV.

**Scanning Electron Microscopy (SEM)**

Bacterial cells grown on LB for 24 h were fixed with Karnovsky’s fixative solution at 4°C for 24 h and then washed three times for 10 min each with 0.05 M cacodylate buffer (pH 7.2). The fixed specimens were post-fixed with 1% osmic acid for 2 h at 4°C, and then washed three times with DW for 10 min each. Specimens were dehydrated using a graded ethanol series (50%, 75%, 90%, and 95%) for 30 min each, with two final 30 min treatments with 100% ethanol. The specimen was transited with 100% amyl acetate at room temperature (RT) two times for 30 min each. After critical-point drying and gold coating, the sample was observed using the scanning electron microscope (Hitachi S-2460N; Hitachi, Ltd., Tokyo, Japan) at an acceleration voltage of 20 kV.

**Activity Against Pathogenic Bacteria**

Antimicrobial activity against several pathogenic bacteria was measured using the agar well diffusion method [13, 31]. Bacterial cultures grown for 24 h in LB broth were inoculated (3% (v/v)) to soft nutrient agar containing 0.7% agar, which was melted and then cooled to approximately 45-50°C. After a very vigorous homogenization, the inoculated agar was poured into standard plastic Petri dishes, and 3-mm diameter wells were bored into the agar plates. The plates were incubated for 24 h at 37°C and then the diameters of the inhibition halos were measured in centimeters.

**Co-Inoculation of \( B. \) cereus and \( B. \) amyloliquefaciens**

For co-culture experiments, bacterial strains were cultured at 30°C for 24 h in LB broth. When the optical density at 600 nm (\( \text{OD}_{600} \)) reached approximately 0.4, which is indicative of a bacterial density of \( 10^7 \) CFU/ml, \( B. \) cereus \( 5 \times 10^5 \) CFU/ml (0.5%) was inoculated with different concentrations of \( B. \) amyloliquefaciens RD7-7 (1.25 x 10^7 to 1.0 x 10^9 CFU/ml; 0.125%, 0.25%, 0.5%, and 1%) or \( B. \) amyloliquefaciens KACC 15866 (1.25 x 10^7 to 1.0 x 10^8 CFU/ml; 0.125%, 0.25%, 0.5%, and 1%) in LB broth at 30°C for 24 h.

**Isolation and Enumeration of \( B. \) cereus Cells by Selective Cultivation and Real-Time qPCR**

Following 10-fold serial dilution in 0.85% sterile saline, 100 µl aliquots of each diluted sample were spread on \( B. \) cereus chromogenic medium, CHROMagar \( B. \) cereus (CHROMagar Microbiology, Paris, France), and incubated at 30°C for 24 h for isolation and
identification of *B. cereus*. The co-cultured cells were harvested by centrifugation at 8,000 xg at 4°C for 10 min and the cell pellets were washed with 1 ml of sterile DW and centrifuged. After centrifugation, total genomic DNA was extracted using a QiAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified DNA was eluted in 100 µl of sterile DW. DNA isolated from 1 ml of each culture in LB broth at different concentrations (10^5, 10^6, and 10^7 CFU/ml) as determined by microbial counts on Plate Count Agar (Difco). DNA was isolated from 1 ml of each dilution. Cycle threshold (Ct) values were plotted against the colony forming units (CFUs) [12].

**Quantitation of Expression of Toxin-Related Genes in *B. cereus* by Real-Time qPCR**

Total RNA was isolated from cells using the RNasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions. Isolated RNA was quantified on a Synergy Mx microplate reader (BioTek Instruments, Winooski, VT, USA). The first-strand cDNA was synthesized from 1 µg of isolated RNA template and amfiRivert Platinum cDNA synthesis Master Mix (GenDEPOT; Barker, TX, USA). A subset of genes was amplified with amfiEco Taq DNA polymerase (GenDEPOT) and the gene-specific primers listed in Table 1. The qPCR analyses were performed with the C1000 Thermal Cycler equipped with a CFX96 Real-Time System (Bio-Rad) in a total volume of 10 µl containing 5 µl of SYBR Green Supermix (Bio-Rad), 200 nM of each of the primers listed in Table 1, and 2 µl of cDNA. The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 10 sec, 55°C for 10 sec, and 72°C for 10 sec. 16s rRNA was used as an internal control for data normalization.

**Production of Fermented Soybean Products and Isolation of Bacteria**

The soybeans (500 g) were washed and soaked in potable water for 16 h at RT. After the soaking water was drained, the weight of the soaked soybeans had increased by approximately 2-fold. The drained soybeans were steamed for 3 h at 100°C and cooled below 40°C. *B. cereus* and *B. amyloliquefaciens* RD7-7 cultures were inoculated to a final cell density of approximately 10^7 CFU/ml (OD_600 = 0.4) after 24 h of incubation in LB broth at 30°C. After cooling, the surfaces of the cooked soybeans were inoculated with 1% (v/w) (10^7 CFU/g) inoculum (mixed culture of *B. amyloliquefaciens* RD7-7 and *B. cereus* at a ratio of 10:0 (sample 2), 0:10 (sample 3), 9:1 (sample 4), or 5:5 (sample 5)). The sample 1 group was treated with sterile saline solution (0.85% NaCl), as a negative control. The mixtures were fermented at 35°C for 36 h. To extract bacterial DNA, RNA, and protein, 50 g of fermented soybeans was mixed with 450 ml of sterile saline and shaken for 15 min and then filtered through No. 2 Whatman filter paper. The extracts were centrifuged at 8,000 xg for 10 min and the residue was collected in

### Table 1. Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>groEL-L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TGGCAACTGTATTAGCCAAGCT-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>groEL-R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TTATCATCCTTCTGCTG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>nheA-L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-ACGAATGTAATTTGAGTCG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>nheA-R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TGGATTCCAAGATGTAACG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>nheC-L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-AGAGTTTGATCCTGGCTCAG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>nheC-R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-GGTGACACATTATCATATAAGGTG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>entFM-L&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-AAAGAATAATTAGGACCAABTTC-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>entFM-R&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-CAACTGTATTAGCCAAGCT-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>ces-L&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-GGTGACACATTATCATATAAAGGTG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>ces-R&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-CAACTGTATTAGCCAAGCT-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>16s-rRNA-L</td>
<td>5'-AGAGTTTGATCCTGCTG-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
<tr>
<td>16s-rRNA-R</td>
<td>5'-GGCTACTCTTGTTACGACTT-3'</td>
<td>RT-PCR, qPCR</td>
</tr>
</tbody>
</table>

* Primer sequences have been described previously [12].
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Table 2. Detection of Bacillus cereus in co-culture with B. amyloliquefaciens RD7-7 and B. amyloliquefaciens KACC15866 in Luria-Bertani broth after 24 h of co-cultivation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ct (Mean ± SD)</th>
<th>y = -3.9756x + 49.693 (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (B. cereus&lt;sup&gt;1&lt;/sup&gt; 0.5%)</td>
<td>21.93 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.98</td>
</tr>
<tr>
<td>Ratio of B. cereus : B. amyloliquefaciens RD7-7</td>
<td>23.07 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>23.14 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.68</td>
</tr>
<tr>
<td></td>
<td>26.24 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.90</td>
</tr>
<tr>
<td></td>
<td>28.92 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.23</td>
</tr>
<tr>
<td>Ratio of B. cereus : B. amyloliquefaciens&lt;sup&gt;2&lt;/sup&gt;</td>
<td>20.31 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.39</td>
</tr>
<tr>
<td></td>
<td>20.04 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.46</td>
</tr>
<tr>
<td></td>
<td>20.61 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.32</td>
</tr>
<tr>
<td></td>
<td>19.77 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.53</td>
</tr>
</tbody>
</table>

Individual cycle threshold (Ct) values correspond to standard curves derived from the B. cereus PCR detection kit with serial 10-fold dilutions.

<sup>1</sup>B. cereus KACC 10004.

<sup>2</sup>Standard curve of B. cereus KACC 10004.

<sup>3</sup>B. amyloliquefaciens KACC 15866.

<sup>4</sup>Each value indicates the mean ± SD of three replicates. Within each row, means indicated with different superscript letters differ significantly p < 0.05 (one-way ANOVA, followed by Duncan’s multiple comparison test).

1 ml of sterile saline solution.

Production of Fermented Buckwheat Soksungjang Products and Isolation of Bacteria

To prepare buckwheat meju (Fermentation agent for Deonjang), 2.5 kg of soybeans was cleaned and soaked in drinking water for 24 h at RT and then drained. The drained soybeans were steamed at 100°C for 5 h and then crushed. Then, 1.4 kg each of crushed soybean and buckwheat (soybean : buckwheat = 7:3) was mixed in water to make a paste. The surfaces of the mixed samples were inoculated with 1% (v/w) (10<sup>7</sup> CFU/g) inoculum (mixed culture of B. amyloliquefaciens RD7-7 and B. cereus at a ratio of 0:10 (sample 2) or 9:1 (sample 3)). The sample 1 group (NT) was treated with sterile saline (0.85% NaCl), as a negative control. The paste was molded into 500 g discs with a consistent diameter of 12 cm and a thickness of 3 cm. After the discs were dried in the shade for 24 h, they were fermented for 7 days at 30°C with 80% relative humidity, and dried for 1 day. Then, the discs were crushed, and 1 kg of buckwheat meju, 220 g of sun-dried salt, and 1.2 L of DW were mixed and naturally fermented in a jar (Kalsantoki, Hongsung, Korea). Samples were collected at 7-day intervals for 8 weeks. To extract bacterial RNA and protein, 50 g samples of buckwheat soksungjang were mixed with 450 ml of sterile saline and shaken for 15 min, and then filtered by suction through a Whatman No. 2 filter paper. Samples were harvested by centrifugation at 8000 x g for 10 min and the residue was resuspended in 1 ml of saline solution.

Total Protein Extraction and Western Blot Analysis

To measure expression of the B. cereus atpB gene, co-cultures of B. cereus and B. amyloliquefaciens were incubated under the culture conditions described above. The cultured cells were collected by centrifugation at 10,000 × g for 5 min, and then the resulting pellet was resuspended in 30 µl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For extraction of cellular proteins, bacteria isolated from fermented soybean products and buckwheat soksungjang samples were centrifuged at 10,000 × g for 10 min and the collected pellets were resuspended in 30 µl of SDS-PAGE sample buffer, boiled for 10 min, and analyzed by western blotting. Equal amounts of protein from each sample were resolved on 12% SDS-PAGE gels and then subjected to western blot analysis. The separated proteins were transferred to a nitrocellulose membrane by electroblotting (120 V, 1 h) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The nitrocellulose membranes were blocked with 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h and then incubated for 1 h with a primary antibody, polyclonal anti-AtpB (the beta subunit of ATP synthase) (the beta subunit of ATP synthase) (1:3,000; Agrisera, Vannas, Sweden). After washing with TBS-T, the membranes were incubated with horseradish-peroxidase-conjugated goat IgG secondary antibodies (1:3,000; Bio-Rad) for 1 h. The blots were analyzed using an enhanced BM chemiluminescence blotting substrate (POD; Roche, Mannheim, Germany).

Statistical Analysis

All statistical analyses were performed in duplicate, using three replicates of each experiment. Using Statistical Package for the Social Sciences (SPSS), ver. 12.0, one-way analysis of variance (ANOVA) was applied to determine whether differences between treatments were significant. The means were compared using
Results and Discussion

Morphological Characteristics of *B. amyloliquefaciens* RD7-7 and Its Activity Against Pathogenic Bacteria

We previously demonstrated that *B. amyloliquefaciens* RD7-7, isolated from *rice doenjang*, a traditional fermented soybean paste, has the potential to improve the quality of fermented soybean food products. When this strain was added to the starter culture, the resulting food products showed high enzymatic activity and amino-type nitrogen contents [19]. To further characterize *B. amyloliquefaciens* RD7-7, we performed a morphological analysis using TEM and SEM techniques. As shown in Figs. 1A and 1B, *B. amyloliquefaciens* RD7-7 is a rod-shaped bacterium with flagella, similar to other *B. amyloliquefaciens* strains. We next evaluated the antibacterial activity of *B. amyloliquefaciens* RD7-7 against various pathogenic strains of bacteria, such as *B. cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes*. *B. amyloliquefaciens* RD7-7 had antagonistic activity towards all five pathogenic bacteria and produced inhibition zones >1.3 cm; in particular, it produced the largest zone of inhibition (1.51–1.80 cm) against *B. cereus* (Fig. 1C). This result indicates that *B. amyloliquefaciens* RD7-7 has broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria and the highest antimicrobial potential against *B. cereus*. Thus, *B. amyloliquefaciens* RD7-7 shows potential as a candidate novel antimicrobial agent to prevent or treat many important bacterial diseases.

*B. cereus* Survival and Toxin Gene Expression in Co-Culture with *B. amyloliquefaciens* RD7-7

To test whether *B. amyloliquefaciens* RD7-7 could inhibit the growth and toxin production of *B. cereus*, we co-cultured the two strains in broth and quantified *B. cereus* survival and toxin gene expression. As shown in Fig. 2, the growth of *B. cereus* was inhibited when it was grown in co-culture with *B. amyloliquefaciens* RD7-7, but the survival of *B. cereus* was not affected by co-culture with *B. amyloliquefaciens* KACC 15866. The survival and growth of *B. cereus* decreased to approximately 7.01, 6.01, and 3.80 log CFU/ml in the presence of 0.25%, 0.5%, and 1% *B. amyloliquefaciens* RD7-7, respectively, compared with co-culture with *B. amyloliquefaciens* KACC 15866 (8.31, 8.37, and 8.42 log CFU/ml) and the control (8.52 log CFU/ml). The initial bacterial populations of *B. amyloliquefaciens* KACC 15866 and *B. amyloliquefaciens* RD7-7 did not differ significantly after co-cultivation (data not shown). Thus, *B. amyloliquefaciens* RD7-7 showed potent antibacterial activity against *B. cereus*.

Detection of *B. cereus* was performed using a *B. cereus* real-time PCR kit using the *groEL* probes, and a linear standard curve for real-time PCR amplification was achieved for purified DNA of the *B. cereus* KACC10004 strain at concentrations ranging from $2 \times 10^5$ to $2 \times 10^8$ CFU/ml. The Ct values for *B. cereus* *groEL* after co-culture with 0.25%, 0.5%, and 1% *B. amyloliquefaciens* RD7-7 at 6.68, 5.90, and 5.23 log CFU/ml were 23.14 ± 0.02, 26.24 ± 0.07, and 28.92 ± 0.01, respectively; thus, the Ct values increased as the template quantity decreased. The absolute value of the correlation coefficient of the calculated standard curve

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**Fig. 1.** Microscopy and antimicrobial activity analyses of *B. amyloliquefaciens* RD7-7. Transmission electron microscopy (A) and scanning electron microscopy (B) images of *B. amyloliquefaciens* RD7-7 grown on Luria-Bertani medium after 24 h at 30°C. Scale bars indicate 1 µm and 5 µm, respectively. Representative images of three independent experiments are shown. (C) The antimicrobial activity of *B. amyloliquefaciens* RD7-7 was detected using an agar spot test. The diameters of the inhibition zones of various pathogenic bacteria were measured. The data shown are representative of at least three independent experiments. Diameter of the inhibition zone: (+) weak (≤1.3 cm), (+++) intermediate (1.31–1.50 cm), (+++) strong (1.51–1.80 cm).

Duncan’s multiple comparison test, and $p < 0.05$ was considered to indicate statistical significance.

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generated from \textit{B. cereus} KACC10004 \textit{groEL} was 0.9641. In contrast, the Ct values for \textit{groEL} from \textit{B. cereus} co-cultures with \textit{B. amyloliquefaciens} KACC 15866 were similar to those of \textit{B. cereus} KACC10004 (control strain).

To examine the expression of \textit{B. cereus} toxin-related genes in co-culture with \textit{B. amyloliquefaciens} RD7-7, we performed in vitro transcription and translation reactions. Expression levels of transcripts of the \textit{B. cereus} toxin-related genes \textit{groEL}, \textit{nheA}, \textit{nheC}, and \textit{entFM} were down-regulated in \textit{B. cereus} co-cultured with \textit{B. amyloliquefaciens} RD7-7, whereas the \textit{B. cereus} KACC10004 control and co-culture with \textit{B. amyloliquefaciens} KACC 15866 did not differ significantly (Fig. 3A). The \textit{ces} gene encoding the emetic toxin was not detected by qPCR analysis in any of the bacterial strains (data not shown); thus, \textit{B. cereus} KACC10004 is a diarrheal toxin-producing strain. \textit{groEL} is common to all strains of \textit{B. cereus}, whereas \textit{ces} is detected only in emetic toxin-producing \textit{B. cereus}; therefore, simultaneous amplification of \textit{groEL} and \textit{ces} could facilitate detection and differentiation of non-emetic and emetic \textit{B. cereus} in food products [23]. The \textit{nheA} and \textit{nheC} genes of the Nhe complex was detected in most \textit{B. cereus} strains tested in this study, consistent with previous findings that most isolates from food products contained \textit{nhe}. The enterotoxin \textit{EntFM} contributes to the cytotoxic and hemolytic activities of \textit{B. cereus} and to its adhesion to host epithelial cell monolayers [9, 16].

We subsequently performed western blot analysis using an anti-AtpB antibody for the detection and quantification of \textit{B. cereus}. The beta subunit of ATP synthase, AtpB, which has been reported to control growth in \textit{B. cereus}, is the universal enzyme that synthesizes ATP from ADP and phosphate using the energy stored in a transmembrane ion gradient [1, 12]. As shown in Fig. 3B, AtpB expression levels were markedly lower in \textit{B. cereus} co-cultured with \textit{B. amyloliquefaciens} RD7-7 than in the control or in \textit{B. cereus} in co-culture with \textit{B. amyloliquefaciens} KACC 15866. These results demonstrate that inhibition of \textit{B. cereus} growth was mediated by suppression of AtpB expression, and that \textit{B. amyloliquefaciens} RD7-7 may inhibit the growth of \textit{B. cereus} by inhibiting expression of toxin-encoding genes.

Arguelles-Arias \textit{et al.} [3] investigated the effect of \textit{B. amyloliquefaciens} GA1, which produces antibiotic compounds, for the development of biocontrol agents for use as “green” pesticides. Thus, it seems likely that \textit{B. amyloliquefaciens} RD7-7 could produce an antibacterial substance that could be used as an agent for biocontrol of \textit{B. cereus}.

\textbf{Inhibition of \textit{B. cereus} Growth and Toxin Production by \textit{B. amyloliquefaciens} RD7-7 in Fermented Soybean Products}

We tested whether \textit{B. amyloliquefaciens} RD7-7, which showed significant antimicrobial activity against \textit{B. cereus} in broth, had the same antimicrobial effect in a fermented soybean product. Consistent with the results shown in Fig. 4, growth of \textit{B. cereus} was significantly inhibited in a soybean product fermented with \textit{B. amyloliquefaciens} RD7-7. The growth of \textit{B. cereus} decreased to approximately 4.17 log CFU/ml (a 1.9-fold reduction) and 4.33 log CFU/ml (a 2.1-fold reduction) at co-culture ratios of 5:5 and 1:9 \textit{B. cereus} KACC 10004 to \textit{B. amyloliquefaciens} RD7-7, respectively.
compared with control *B. cereus* (9.1 log CFU/ml) at 24 h (Fig. 4B). Thus, *B. amyloliquefaciens* RD7-7 effectively inhibited the growth of *B. cereus*. Fermented soybean foods that contain many beneficial microorganisms (bacteria and fungi) are commonly contaminated with *B. cereus* during fermentation [15]. Fig. 4A shows that the surface of a fermented soybean product inoculated with *B. amyloliquefaciens* RD7-7 was almost completely covered by a gelatinous, slippery, and viscous slime that was not present in the product inoculated with the control *B. cereus* KACC 10004 strain (sample 3), suggesting that slime production may play an important role in the bactericidal activity of *B. amyloliquefaciens* RD7-7.

Slime, which is also referred to as a self-produced matrix of extracellular polymeric substances, is a polymeric conglomeration generally composed of extracellular biopolymers in various structural forms [6]. Mucilage or slime on fermented soybean food products has been shown to inhibit the growth of bacteria by damaging the cellular membrane of pathogenic bacteria and some harmful yeast.
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The γ-polyglutamic acid (γ-PGA) produced by *B. amyloliquefaciens* C06 enhances colonization of *Bacillus* cells by improving biofilm formation, surface adhesion, and swarming motility; these findings suggest that γ-PGA producers might be used to improve biological control of *Bacillus* strains with enhanced surface colonization abilities [22]. In addition, a bacteriocin-like substance produced by a strain of *B. amyloliquefaciens* isolated from the Brazilian

![Fig. 4. Photographic images of fermented soybean products (A) and growth inhibition of *Bacillus cereus* KACC10004 (B) in soybean products fermented with *B. cereus* KACC10004 and *B. amyloliquefaciens* RD7-7.](image)

The error bars indicate the mean ± SD of three individual experiments, and within each row, means indicated with different superscript letters differ significantly (*p* < 0.05; one-way ANOVA, followed by Duncan's multiple comparison test). ND: not detected. Sample 1: Non-treatment (NT); Sample 2: *B. amyloliquefaciens* RD7-7; Sample 3: *B. cereus*; Sample 4: *B. amyloliquefaciens* RD7-7 and *B. cereus* (9:1); Sample 5: *B. amyloliquefaciens* RD7-7 and *B. cereus* (5:5).

![Table 3. Detection of *Bacillus cereus* in soybean products fermented with *B. cereus* KACC10004 and *B. amyloliquefaciens* RD7-7.](table)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strains</th>
<th>Ct (Mean ± SD)</th>
<th>y = -3.9756x + 49.693</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(log CFU/ml)</td>
</tr>
<tr>
<td>Sample 1</td>
<td>Non-treatment: NT</td>
<td>29.56 ± 0.03↑</td>
<td>5.06</td>
</tr>
<tr>
<td>Sample 2</td>
<td><em>B. amyloliquefaciens</em> RD7-7</td>
<td>37.80 ± 0.06↑</td>
<td>2.99</td>
</tr>
<tr>
<td>Sample 3</td>
<td><em>B. cereus</em> 5 do</td>
<td>20.26 ± 0.02↑</td>
<td>7.40</td>
</tr>
<tr>
<td>Sample 4</td>
<td><em>B. amyloliquefaciens</em> RD7-7: <em>B. cereus</em> (9:1)</td>
<td>36.80 ± 0.05↑</td>
<td>3.24</td>
</tr>
<tr>
<td>Sample 5</td>
<td><em>B. amyloliquefaciens</em> RD7-7: <em>B. cereus</em> (5:5)</td>
<td>35.33 ± 0.05↑</td>
<td>3.61</td>
</tr>
</tbody>
</table>

Individual cycle threshold (Ct) values correspond to standard curves derived from the *B. cereus* PCR detection kit with 10-fold serial dilutions of *B. cereus*.

1) *B. cereus* KACC 10004
2) Standard curve of *B. cereus* KACC 10004
3) Each value indicates the mean ± SD of three replicate analyses. Within each row, means indicated with different superscript letters differ significantly *p* < 0.05 (one-way ANOVA, followed by Duncan’s multiple comparison test).
Atlantic forest was shown to inhibit pathogenic and food-spoilage bacteria, such as *L. monocytogenes*, *B. cereus*, and *Serratia marcescens* [21]. Thus, gluey, stringy, and mucilaginous substances, such as the bacteriocin-like substance produced by *B. amyloliquefaciens* RD7-7, may be good candidates in the search for natural antimicrobial agents.

Growth of *B. cereus* co-cultured with *B. amyloliquefaciens* RD7-7 in fermented soybeans was measured by quantitative real-time PCR analysis of *groEL*. The growth of *B. cereus* was reduced to approximately 3.24 log CFU/ml (36.80 Ct) and 3.61 log CFU/ml (35.33 Ct) at co-culture ratios of 1:9 and 5:5 *B. cereus* KACC 10004 to *B. amyloliquefaciens* RD7-7, respectively, compared with the control *B. cereus* (7.4 log CFU/ml, 20.26 Ct); thus, high Ct values represent low numbers of *B. cereus* (Table 3). Therefore, reduced expression of *groEL* in *B. amyloliquefaciens* RD7-7–treated fermented soybean products indicates inhibition of *B. cereus* growth.

For detection of expression of *B. cereus* toxin genes at the transcript level in fermented soybean products, we used qPCR for sensitive detection of *groEL*, *nheA*, *nheC*, and *entFM* transcripts in *B. cereus* co-cultured with *B. amyloliquefaciens* RD7-7. As shown in Fig. 5A, *groEL*, *nheA*, *nheC*, and *entFM* transcript levels were reduced in the 5:5 and 1:9 co-cultures of *B. cereus* KACC10004 and *B. amyloliquefaciens* RD7-7, compared with monoculture fermentation with *B. cereus* KACC10004. These results indicate that the inhibition of *B. cereus* growth in the presence of *B. amyloliquefaciens* RD7-7 may be regulated by *groEL*, *nheA*, *nheC*, and *entFM*.

We next performed western blot analysis using an anti-AtpB antibody for the detection and quantification of *B. cereus* in fermented soybean products. Fig. 5B shows that the fermented soybean product (5:5 and 9:1 ratio) inoculated with *B. amyloliquefaciens* RD7-7 showed low or undetectable levels of AtpB expression compared with the *B. cereus* KACC10004 control, suggesting a significantly lower expression level of the AtpB protein in the *B. amyloliquefaciens* RD7-7–treated fermented soybean samples, and indicating inhibition of the growth of *B. cereus* (Fig. 5B). Together, these results suggest that *B. amyloliquefaciens* RD7-7 may decrease the survival of *B. cereus* by reducing the expression of toxin-related genes in fermented soybean products.

**Growth and Toxin Expression by *B. cereus* Co-Cultured with *B. amyloliquefaciens* RD7-7 in a Buckwheat Soksungjang Product**

We measured the growth and toxin gene expression of *B. cereus* in a buckwheat soksungjang product inoculated with *B. amyloliquefaciens* RD7-7.

**Fig. 5.** Expression of *Bacillus cereus* spore-related genes and AtpB levels of fermented soybean products in the presence of *Bacillus amyloliquefaciens* strains. (A) Expression of the *Bacillus cereus* spore-related genes *groEL*, *nheA*, *nheC*, and *entFM* was determined by qPCR of fermented soybean product samples. All qPCR data were normalized to 16S rRNA and reported relative to the control culture. Graphs indicate the relative mRNA levels for *groEL*, *nheA*, *nheC*, and *entFM*. (B) The AtpB levels in whole cell extracts from fermented soybean products were determined by immunoblotting. Expression of AtpB was quantified by densitometry. The data shown are representative of three independent experiments. Error bars indicate the mean ± SD. Sample means were compared by one-way ANOVA followed by Duncan’s multiple comparison test. Means of different samples labeled with different letters are significantly different (*p* < 0.05). ND: not detected.

Buckwheat soksungjang is a *bealmijang* manufactured with buckwheat and soybeans. Buckwheat protein, which is mainly composed of lysine, arginine, albumin, globulin, and glutelin, shows various health benefits against several diseases, such as hypertension, hypercholesterolemia,
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Diabetes, and obesity; thus, buckwheat, used as a protein source complementary to grain (cereal) foods and vegetables, has gained an excellent reputation for its nutritious qualities [2, 20]. Therefore, we tested the efficacy of *B. amyloliquefaciens* RD7-7 in preventing *B. cereus* contamination of a soksungjang product made using buckwheat.

As shown in Fig. 6A, the growth of *B. cereus* was inhibited in the buckwheat soksungjang product fermented with *B. amyloliquefaciens* RD7-7, but did not differ significantly from the growth of control *B. cereus*. The survival and

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**Fig. 6.** Growth and toxin expression by *Bacillus cereus* co-cultured with *Bacillus amyloliquefaciens* RD7-7 in a buckwheat soksungjang product.

(A) Inhibition of *B. cereus* KACC10004 growth in buckwheat soksungjang products fermented with *B. amyloliquefaciens* RD7-7. Symbols: trapezoid: NT (non-treatment); squares: *B. cereus*; triangles: *B. amyloliquefaciens* RD7-7 : *B. cereus* (9:1). (B) Expression of *B. cereus* spore-related genes *groEL*, *nheA*, *nheC*, and *entFM* was determined by qPCR of fermented buckwheat soksungjang product samples. qPCR expression data were normalized to 16S rRNA and reported relative to the control culture. The bar graphs indicate the mean relative mRNA levels for *groEL*, *nheA*, *nheC*, and *entFM*. (C) The levels of AtpB in whole-cell extracts from fermented buckwheat soksungjang product were determined by western blot analysis. The expression of AtpB was quantified by densitometry. All experiments were performed independently three times and representative data are shown. The error bars correspond to the mean ± SD of three individual experiments, and within each row, means with different superscripts letters are statistically significant (*p* < 0.05; one-way ANOVA, followed by Duncan’s multiple comparison test). ND: not detected.
growth of *B. cereus* decreased to approximately 2.74 log CFU/ml (a 2.4-fold reduction) and 0.30 log CFU/ml (a 22.2-fold reduction) at co-culture ratios of 1:9 *B. cereus* KACC 10004 to *B. amyloliquefaciens* RD7-7, respectively, in comparison with control *B. cereus* (6.66 and 6.68 log CFU/ml) at 21 and 49 days.

Expression of transcripts of *B. cereus* toxin-related genes (*groEL*, *nheA*, *nheC*, and *entFM*) was downregulated in *B. cereus* co-cultured with *B. amyloliquefaciens* RD7-7 at 21 and 49 days, and did not differ at the three time points in the *B. cereus* control (Fig. 6B).

In addition, AtpB expression levels were markedly lower in *B. cereus* co-cultured with *B. amyloliquefaciens* RD7-7 compared with the control strain at 21 and 49 days. Together, these results suggest that *B. amyloliquefaciens* RD7-7 decreases the survival of *B. cereus* by reducing expression of toxin-related genes in a buckwheat soksungjang product. The use of antagonistic substances such as bacteriocins, bacteriocin-like substances, and antibacterial lipopeptides (surfactin, fengycin, and iturin) produced by *B. amyloliquefaciens* strains has been reported to prevent growth of *B. cereus* in cheese, milk, rice-based foods, cooked rice, beef gravy, and chilled dairy products [15, 22, 32]. Recent studies have shown that bacteriocin J4 produced by *B. amyloliquefaciens* J4, isolated from traditional fermented soybean paste, exhibits specific antagonistic activity against various foodborne pathogens such as *Micrococcus luteus*, *Vibrio parahaemolyticus*, *S. aureus*, and *L. monocytogenes* [22]. Arguelles-Arias et al. [4] observed that the *B. amyloliquefaciens* GA1 strain produces an antimicrobial peptide, named amylolysin (a novel antibiotic), active against an array of gram-positive bacteria, including methicillin-resistant *S. aureus*. Our results indicate that the novel antibacterial peptides produced by *B. amyloliquefaciens* RD7-7 will play important roles as bacterial biocontrol agents for the inhibition of *B. cereus* growth in food production.

In conclusion, *B. amyloliquefaciens* RD7-7 isolated from rice doenjang exhibited a significant antibacterial effect against *B. cereus* and reduced the expression of its enterotoxin genes. *B. amyloliquefaciens* RD7-7 shows potential for use as an efficient biological control agent in fermented soybean products to exclude pathogenic *B. cereus* during manufacturing, without decreasing food quality or inhibiting fermentation by *Bacillus* spp. It may also have other uses in the food, agricultural, biotechnology, and pharmaceutical industries. Hence, further studies are required to identify and characterize the novel antagonistic substances such as bacteriocins, bacteriocin-like substances, and antibacterial lipopeptides produced by *B. amyloliquefaciens* RD7-7.

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