Understanding the ontogeny and succession of *Bacillus velezensis* and *B. subtilis* subsp. *subtilis* by focusing on kimchi fermentation

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*Bacillus subtilis* and *B. velezensis* are frequently isolated from various niches, including fermented foods, water, and soil. Within the *Bacillus subtilis* group, *B. velezensis* and *B. subtilis* subsp. *subtilis* have received significant attention as biological resources for biotechnology-associated industries. Nevertheless, radical solutions are urgently needed to identify microbes during their ecological succession to accurately confirm their action at the species or subspecies level in diverse environments, such as fermented materials. Thus, in this study, previously published genome data of the *B. subtilis* group were compared to exploit species- or subspecies-specific genes for use as improved qPCR targets to detect *B. velezensis* and *B. subtilis* subsp. *subtilis* in kimchi samples. *In silico* analyses of the selected genes and designed primer sequences, in conjunction with SYBR Green real-time PCR, confirmed the robustness of this newly developed assay. Consequently, this study will allow for new insights into the ontogeny and succession of *B. velezensis* and *B. subtilis* subsp. *subtilis* in various niches. Interestingly, in white kimchi without red pepper powder, neither *B. subtilis* subsp. *subtilis* nor *B. velezensis* was detected.

*Bacillus* species are ubiquitous, endospore-forming, gram-positive bacteria that are of high economic importance due to their specific characteristics, such as their ability to colonize plants; to produce spores, biofilms and antibiotics; and to induce the synthesis of plant hormones. Among *Bacillus* species, *B. subtilis* and *B. velezensis* have received considerable attention as biological resources in the food industry because they are accepted as safe, and their antagonistic activity may reflect competitive pressure in natural environments, leading to microbiota selection. Moreover, for *B. subtilis*, each subspecies presents different biological properties due to differences in metabolite synthesis. For example, *B. subtilis* subsp. *subtilis* strains were reported to synthesize either the lipopeptide surfactin, which has anti-Listeria activity, or surfactin and a novel bacteriocin, which have antibacterial properties. *B. velezensis* strains are potential protective starter cultures for the production of alkaline fermented foods, such as bikalgol. *B. velezensis* (its heterotypic synonyms include *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum*, and *B. oryzicola*) and its sister species *B. subtilis* compose an evolutionarily small but physiologically relevant group of bacteria that includes strains isolated from diverse habitats.

In general, the detection, quantitation, and identification of bacteria, including those in the genus *Bacillus*, are performed using various approaches, such as phenotypic, biochemical and immunological assays and molecular methods. However, when distinguishing between closely related species, these techniques have poor resolution and often result in the misclassification and misidentification of strains. In particular, while the relative abundance of the genus *Bacillus* has been described for several types of kimchi using pyrosequencing, few data at the species level have been reported.

For many years, 16S rRNA genes have been used to describe the microbiota composition of various environments. However, this analysis primarily identifies the relative abundances and diversity of bacteria and archaea in a sample. For the genus *Bacillus*, molecular analyses based on sequencing the 16S rRNA gene have been utilized...
as the primary tool for taxonomic assignment and phylogenetic tree construction of Bacillus species, including B. subtilis, B. licheniformis, and B. velezensis.4,13

However, species identification based on ribosomal RNA (rRNA) operon sequences, specifically the 16S rRNA genes, fails to distinguish one species from another, as their sequences have no significant differences. Thus, the use of these assays has significant drawbacks in the identification and detection of Bacillus groups, because they also detect other Bacillus species or subspecies. These technical limitations have become a significant obstacle that has prevented the elucidation of the microbial communities of various assayed samples, such as food or soil samples, even though pyrosequencing methods can provide insight into understanding the overall microbial composition of a particular niche13,16-18.

In the food industry, research has primarily focused on crucial bacteria, such as lactic acid bacteria (LAB) and bacilli, which play prominent roles in food fermentation processes and are thus of both scientific and industrial interest in food microbiology19,20. However, determining detection specificity, which can be influenced both by the uniqueness of DNA sequences within targeted bacterial genomes of interest and by the accurate annealing of primers or probes to their targets, is both critical for any PCR detection approach17,21 and urgently required.

The number of sequenced genomes has continued to increase with rapid advances in next-generation sequencing technology, allowing genes to be identified in the genome sequences of Bacillus strains that are unique to a taxon or a group of taxa. This achievement, accompanied with the availability of various bioinformatics tools, has led to the advent of higher-fidelity, faster, and more cost-effective techniques for identifying Bacillus species in diverse environments. However, notwithstanding the present-day scientific advancements made using Bacillus strains in microbial industries, currently available solutions for identifying, detecting and quantifying a targeted Bacillus species or subspecies remain mostly limited, as is the case for other bacteria.5

Consequently, in this study, the bacterial genome sequences available in the NCBI GenBank ftp site (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/bacteria/) were downloaded and compared to identify genes unique to B. subtilis subsp. subtilis or B. velezensis using a combination of bioinformatic tools.3,24 In silico analyses of the selected genes and designed primer sequences, in conjunction with SYBR Green real-time PCR, confirmed that this newly improved PCR assay is able to precisely identify and quantify the two most prominent Bacillus species (B. subtilis subsp. subtilis and B. velezensis) at the species or subspecies level during kimchi fermentation, which is necessary for their use in food biotechnology.

Our findings demonstrated that this genome-based approach is advantageous for the accurate identification, detection and quantification of each targeted species in various environments. Interestingly, B. subtilis subsp. subtilis and B. velezensis were not detected in white kimchi without red pepper powder. However, the density of B. subtilis subsp. subtilis and B. velezensis was not particularly high in red pepper kimchi.

Results

In silico assay evaluation and PCR confirmation. The oligonucleotide primers and genes (Table 1) selected from two Bacillus species, i.e., B. subtilis subsp. subtilis or B. velezensis, were evaluated and confirmed via a combination of bioinformatic tools.

In B. velezensis, the BLASTn searches revealed no substantial matches to the recognized reference sequences of other Bacillus species. The results of the BLASTx searches, which used the predicted protein sequence of our putative hydrolase gene, showed that the most similar protein was a Bacillus nakamuraí protein [identity = 61%, score = 564 bits (1,454), and expected = 0.0].

For B. subtilis subsp. subtilis, the BLASTn searches yielded no solid match to any of the other identified Bacillus reference sequences. The results of the BLASTx searches, which used the predicted protein sequence of our LyS-R family transcriptional regulator, showed that the most similar protein was from Brevibacterium halotolerans [identity = 94%, score = 413 bits (1,061), and expected = 2e-144].

Specificity tests were performed using conventional PCR techniques for each species or subspecies primer set against DNA samples from various Bacillus strains, including the type strain of each targeted species or subspecies (Tables 2 and 3). Each expected specific PCR product was confirmed for each PCR assay, all of which were performed using genomic DNA from B. velezensis and B. subtilis subsp. subtilis strains (Fig. 1). Incidentally, the B. subtilis strains 10113, 10114, and 11994 from the Korean Agricultural Culture Collection (KACC) (Tables 2 and 3) were identified as B. velezensis, as shown in Fig. 1. Taken together, the results of the in silico evaluation and PCR confirmation showed that the PCR assay reliably identified B. velezensis and B. subtilis subsp. subtilis.

Real-time PCR assay efficiency, LOD and LOQ. Threshold cycle values (Ct) were considered the primary outcome variables for this study (Tables 4 and 5). The standard curves for the type strain of each targeted species, including B. velezensis and B. subtilis subsp. subtilis, were generated by charting the mean threshold cycle

| Primer | Oligonucleotide sequence (5′-3′) | Annealing (°C) | Amplicon (bp) | Target gene (GenBank Accession No.) | Reference |
|--------|---------------------------------|----------------|---------------|-------------------------------------|-----------|
| Bam249F | GTCCGGGGCGATTGCGTGAG | 67 | 249 | putative hydrolase (NC020410.1) | This study |
| Bam249R | CCCCTGACATAGCAGGACTGA | | | | |
| BS110F | GGCCTATTGAACACCCTGATTTA | 67 | 310 | LysR family transcriptional regulator (NC020832.1) | This study |
| BS110R | CGGATGCGGCCTTCTTTTC | | | | |

Table 1. Primer sequences, their targets, and the annealing temperatures used in Bacillus velezensis and B. subtilis subsp. subtilis PCR screens.
Table 2. Bacterial strains used in the PCR specificity test for *Bacillus velezensis*. “Superscript “T” indicates type strain. aConventional or real-time assay; + and − indicate species detected or not detected, respectively. bN.D., Not determine.

| No. | Bacterial strains (Subjective synonym) | Sourcea | Host | This studyb |
|-----|----------------------------------------|---------|------|-------------|
| 1   | *Bacillus amyloliquefaciens* (Bacillus velezensis) | LMG 12331 | N.D. | + |
| 2   | *Bacillus amyloliquefaciens* | KACC 10116T | Takamine bacterial amylase concentrate | + |
| 3   | *Bacillus amyloliquefaciens* | KACC 17029 | Soil | + |
| 4   | *Bacillus amyloliquefaciens* | KACC 17030 | Soil | + |
| 5   | *Bacillus amyloliquefaciens* | KACC 17031 | Soil | + |
| 6   | *Bacillus amyloliquefaciens* | KACC 17032 | Soil | + |
| 7   | *Bacillus amyloliquefaciens* subsp. amyloliquefaciens | LMG 12325 | N.D. | + |
| 8   | *Bacillus amyloliquefaciens* subsp. amyloliquefaciens | LMG 12326 | N.D. | + |
| 9   | *Bacillus amyloliquefaciens* subsp. plantarum | LMG 26770T | N.D. | + |
| 10  | *Bacillus amyloliquefaciens* subsp. plantarum | LMG 12384 | N.D. | + |
| 11  | *Bacillus amyloliquefaciens* subsp. plantarum | LMG 17599 | N.D. | + |
| 12  | *Bacillus stamiensis* | KACC 15859 | Gochujang | + |
| 13  | *Bacillus subtilis* | KACC 10113 | Soil | + |
| 14  | *Bacillus subtilis* | KACC 10114 | Adenine and phenylalanine-requiring mutant | + |
| 15  | *Bacillus subtilis* | KACC 11994 | Rhizosphere of Brassica napus | + |
| 16  | *Bacillus methylotrophicus* | LMG 27586T | N.D. | + |
| 17  | *Bacillus subtilis* subsp. *subtilis* | LMG 7135T | N.D. | − |
| 18  | *Bacillus subtilis* | KACC 10111 | N.D. | − |
| 19  | *Bacillus subtilis* subsp. *spirizenii* | LMG 19156T | N.D. | − |
| 20  | *Bacillus licheniformis* | LMG 12363T | N.D. | − |
| 21  | *Bacillus pumilus* | LMG 18928T | N.D. | − |
| 22  | *Bacillus sonorensis* | LMG 21636T | N.D. | − |
| 23  | *Bacillus Vallismortis* | LMG 18725T | N.D. | − |
| 24  | *Bacillus atrophaeus* | KACC 12090T | Soil | − |
| 25  | *Bacillus alveayuensis* | KACC 13323T | Deep sea sediment of the Ayu Trough (4000 m below sea level) in the western Pacific Ocean | − |
| 26  | *Bacillus pechoomensis* | KACC 14006T | Soil, ginseng field | − |
| 27  | *Bacillus kribbensis* | KACC 14005T | Soil | − |
| 28  | *Bacillus circulans* | KACC 14392T | Soil | − |
| 29  | *Bacillus firmus* | KACC 10897T | N.D. | − |
| 30  | *Bacillus jeotgali* | KACC 17399T | Jeotgal | − |
| 31  | *Bacillus niabensis* | KACC 11279T | N.D. | − |
| 32  | *Bacillus litoralis* | KACC 12148T | Sea water | − |

(Ct) (n = 3) based on logarithmic concentrations of genomic DNA (*B. velezensis*, from 5 to 5 × 10⁻³ ng/µl; and *B. subtilis* subsp. *subtilis*, from 5 to 5 × 10⁻³ ng/µl), cloned DNA (*B. velezensis*, from 1.42 × 10⁹ to 1.42 × 10³ copies/µl; and *B. subtilis* subsp. *subtilis*, from 1.39 × 10⁹ to 1.39 × 10⁷ copies/µl), and cell suspensions (*B. velezensis* and *B. subtilis* subsp. *subtilis* from 0.1 × 10⁶ to 0.1 × 10⁻³ OD₆₅₀ units of cells per reaction). Limit of quantitation (LOQ) assays yielded linear responses and high correlation coefficients (*B. velezensis*, R² = 0.999; and *B. subtilis* subsp. *subtilis*, R² = 0.999). Standard curve analyses of the linear portions of the slopes for *B. velezensis* and *B. subtilis* subsp. *subtilis* produced coefficients of −3.353 and −3.400, which yielded PCR efficiencies of 98.7% and 96.8%, with y-intercept values of 28.942 and 31.640, respectively (Fig. 2). Melting analysis (curve, temperature, and peaks) of the real-time PCR reactions performed with the above *Bacillus* species provided reproducible melting temperatures (*B. velezensis*, 86.0°C; and *B. subtilis* subsp. *subtilis*, 83.5°C) and specific peaks (Fig. 2). The genomic DNA (*Bacillus velezensis*, R² = 0.999, slope = −3.336; and *B. subtilis* subsp. *subtilis*, R² = 0.997, slope = −3.456) and cell suspension (*B. velezensis*, R² = 0.992, slope = −3.575; *B. subtilis* subsp. *subtilis*, R² = 0.991, slope = −3.128) standard curves for each of the *Bacillus* species were linearly correlated with their respective Ct values, and their real-time PCR limits of detection (LOD) were 50–500 fg/µl of reaction mix and 0.1 × 10⁻³ OD₆₅₀ units of cells per reaction (Tables 4 and 5). The SYBR Green real-time PCR assays for each *Bacillus* species presented excellent quantification and detection.

Quantitative analysis of *Bacillus velezensis* and *B. subtilis* subsp. *subtilis* in kimchi samples using SYBR Green real-time PCR. Each kimchi sample was incubated at three different temperatures (4°C, 15°C, and 25°C) and was subsequently tested for the presence and abundance of *B. velezensis* and *B. subtilis* subsp. *subtilis* using SYBR Green real-time PCR assays.
Bacillus velezensis was not detected in any of the white kimchi samples (including the 4 °C, 15 °C, and 25 °C samples), but the corresponding samples from whole kimchi produced opposite results (Fig. 3). Generally, the 4 °C whole kimchi sample exhibited more delayed-fluorescence signals for the Ct value than those stored at 15 °C and 25 °C.

Similar to the findings for Bacillus velezensis, all white kimchi samples (including the 4 °C, 15 °C, and 25 °C samples) tested negative for Bacillus subtilis subsp. subtilis. In whole kimchi, the proportional changes of Bacillus subtilis subsp. subtilis were similar to those observed for Bacillus velezensis at 4 °C, 15 °C and 25 °C (Fig. 3). The samples collected from whole kimchi stored at 15 °C and 25 °C had lower Ct values than those stored at 4 °C between days 0 and 1. However, no significant difference in bacterial density was observed between the whole kimchi samples stored at 15 °C and those stored at 25 °C.

Therefore, the ontogeny of Bacillus velezensis and Bacillus subtilis subsp. subtilis was observed only in red pepper powder kimchi, regardless of the fermentation period. Furthermore, neither Bacillus velezensis nor Bacillus subtilis subsp. subtilis was a dominant species during the kimchi fermentation process.

Discussion
Bacillus species continue to be leading bacterial workhorses in microbial industries. In particular, Bacillus velezensis, which produces a natural antibiotic protein, an alpha amylase, a protease, and a restriction enzyme, and Bacillus subtilis, one of the best understood prokaryotes, possess excellent genetic characteristics that have provided powerful tools and industrial resources to investigate targeted bacteria. However, although many approaches have been used to study the ecology and roles of various properties of Bacillus species, little is known about their ecological composition and succession in specific environments at the species or subspecies level.

| No. | Bacterial strains                  | Sourcea | Host                                      | This studyb |
|-----|-----------------------------------|---------|-------------------------------------------|-------------|
| 1   | Bacillus subtilis subsp. subtilis | LMG 7135T | N.D.†                                    | +           |
| 2   | Bacillus subtilis                 | KACC 10111 | N.D.                                    | +           |
| 3   | Bacillus subtilis                 | KACC 10113 | Soil                                     | -           |
| 4   | Bacillus subtilis                 | KACC 10114 | Adenine and phenylalanine-requiring mutant | -           |
| 5   | Bacillus subtilis                 | KACC 11994 | Rhizosphere of Brassica napus             | -           |
| 6   | Bacillus subtilis subsp. spizizenii | LMG 19156T | N.D.                                    | -           |
| 7   | Bacillus amylobioquefaciens       | KACC 10116T | Takamine bacterial amylase concentrate   | -           |
| 8   | Bacillus amylobioquefaciens       | KACC 17029 | Soil                                     | -           |
| 9   | Bacillus amylobioquefaciens       | KACC 17030 | Soil                                     | -           |
| 10  | Bacillus amylobioquefaciens       | KACC 17031 | Soil                                     | -           |
| 11  | Bacillus amylobioquefaciens       | KACC 17032 | Soil                                     | -           |
| 12  | Bacillus amylobioquefaciens       | LMG 12325 | N.D.                                    | -           |
| 13  | Bacillus amylobioquefaciens subsp. amylobioquefaciens | LMG 12326 | N.D.                                    | -           |
| 14  | Bacillus amylobioquefaciens subsp. amylobioquefaciens | LMG 26770T | N.D.                                    | -           |
| 15  | Bacillus amylobioquefaciens subsp. planatarum | LMG 12384 | N.D.                                    | -           |
| 16  | Bacillus amylobioquefaciens subsp. plantatarum | LMG 17599 | N.D.                                    | -           |
| 17  | Bacillus amylobioquefaciens subsp. plantatarum | KACC 15859 | Gochujang                              | -           |
| 18  | Bacillus siamensis                | KACC 12536 | N.D.                                    | -           |
| 19  | Bacillus methylotrophicus         | LMG 27586T | N.D.                                    | -           |
| 20  | Bacillus licheniformis            | LMG 27586T | N.D.                                    | -           |
| 21  | Bacillus pumilus                  | LMG 18928T | N.D.                                    | -           |
| 22  | Bacillus sonorenseis              | LMG 18928T | N.D.                                    | -           |
| 23  | Bacillus variisomertis            | LMG 18928T | N.D.                                    | -           |
| 24  | Bacillus atrophaeus               | KACC 12090T | Soil                                     | -           |
| 25  | Bacillus alvearensis              | KACC 13323T | Deep sea sediment of the Ayu Trough (4000 m below sea level) in the western Pacific Ocean | -           |
| 26  | Bacillus pocheomensis             | KACC 14005T | Soil, ginseng field                      | -           |
| 27  | Bacillus kribbensis               | KACC 14005T | Soil                                     | -           |
| 28  | Bacillus circulans                | KACC 14392T | Soil                                     | -           |
| 29  | Bacillus firmus                   | KACC 10897T | N.D.                                    | -           |
| 30  | Bacillus jeotgali                 | KACC 17399T | Jeotgal                                | -           |
| 31  | Bacillus niabensis                | KACC 11279T | N.D.                                    | -           |
| 32  | Bacillus litoralis                | KACC 12148T | Sea water                                | -           |

Table 3. Bacterial strains used in the PCR specificity test for Bacillus subtilis subsp. subtilis. aSuperscript “T” indicates type strain. bConventional or real-time assay; + and – indicate species detected or not detected, respectively. cN.D., Not determine.

Bacillus velezensis was not detected in any of the white kimchi samples (including the 4 °C, 15 °C, and 25 °C samples), but the corresponding samples from whole kimchi produced opposite results (Fig. 3). Generally, the 4 °C whole kimchi sample exhibited more delayed-fluorescence signals for the Ct value than those stored at 15 °C and 25 °C.

Similar to the findings for Bacillus velezensis, all white kimchi samples (including the 4 °C, 15 °C, and 25 °C samples) tested negative for Bacillus subtilis subsp. subtilis. In whole kimchi, the proportional changes of Bacillus subtilis subsp. subtilis were similar to those observed for Bacillus velezensis at 4 °C, 15 °C and 25 °C (Fig. 3). The samples collected from whole kimchi stored at 15 °C and 25 °C had lower Ct values than those stored at 4 °C between days 0 and 1. However, no significant difference in bacterial density was observed between the whole kimchi samples stored at 15 °C and those stored at 25 °C.

Therefore, the ontogeny of Bacillus velezensis and Bacillus subtilis subsp. subtilis was observed only in red pepper powder kimchi, regardless of the fermentation period. Furthermore, neither Bacillus velezensis nor Bacillus subtilis subsp. subtilis was a dominant species during the kimchi fermentation process.

Discussion
Bacillus species continue to be leading bacterial workhorses in microbial industries. In particular, Bacillus velezensis, which produces a natural antibiotic protein, an alpha amylase, a protease, and a restriction enzyme, and Bacillus subtilis, one of the best understood prokaryotes, possess excellent genetic characteristics that have provided powerful tools and industrial resources to investigate targeted bacteria. However, although many approaches have been used to study the ecology and roles of various properties of Bacillus species, little is known about their ecological composition and succession in specific environments at the species or subspecies level.
subsp. trial scale by biotechnology companies. A role of a particular bacterium has been presented. Furthermore, B. methylotrophicus has been reported to have enormous potential to support crop production in agricultural applications. The various benefits of these species and their products have been demonstrated, no clear evidence describing the role of a particular bacterium has been presented. Furthermore, B. methylotrophicus has been reported to have enormous potential to support crop production in agricultural applications. 

In early studies of Bacillus subtilis group communities, conventional approaches that used morphological or phenotypic identification methods of colonies grown on agar-solidified media were used. However, because these methodologies were unsatisfactory, DNA-based approaches began to be included to distinguish between B. subtilis subsp. subtilis and subspecies via 16S rRNA amplification. However, because 16S rRNA sequences exhibit over 98% similarity within this group, this method does not reliably distinguish or differentiate Bacillus species or subspecies. Recently, whole genome shotgun sequencing was developed as an alternative approach to 16S rRNA-based methodologies that were unsatisfactory, DNA-based approaches began to be included to distinguish between B. subtilis subsp. subtilis and subspecies via 16S rRNA amplification. However, because 16S rRNA sequences exhibit over 98% similarity within this group, this method does not reliably distinguish or differentiate Bacillus species or subspecies. 

Recently, diverse Bacillus species have been identified in fermented foods, such as Korean cheonggukjang and kimchi, Chinese douchi, and Japanese natto. Of these species, the B. subtilis group contains the closely related taxa B. subtilis subsp. subtilis, B. licheniformis, B. velezensis, B. atrophaeus, B. mojavensis, B. vallismortis, and B. subtilis subsp. spizizenii. In particular, B. subtilis subsp. subtilis and B. velezensis are the most frequently isolated bacilli from fermented foods or soil. 

For example, bacilli spores can persist for many years, but little is known about the dynamics of the germination, proliferation, movement, and sporulation for the spores of a given species in different niches, even though these bacteria are of increasing interest for use as biocontrol and probiotic agents. 

In view of this finding, establishing reliable, efficient and specific molecular probes for the quantitative detection of a targeted bacterium in various niches is crucial, because such probes would enable the detection of individual species while providing an overall profile of the fluctuations in community structure in response to variations in time and temperature.

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Table 4. Mean cycle threshold (CT) end-point fluorescence of 10-fold serial dilutions of Bacillus velezensis KACC 10116 cloned DNA, genomic DNA and a cell suspension determined with real-time PCR assay. aSD, Three reactions standard deviation. bOD = 600 nm. cN.D., Not detected.

| Weight/µl reaction mix | Ct ± SDa | Weight/µl reaction mix | Ct ± SD | Cell densityb | Ct ± SD |
|------------------------|---------|------------------------|---------|--------------|---------|
| 5 pg (1.42 × 109 copies) | 8.93 ± 0.16 | 5 pg (1.42 × 109 copies) | 16.55 ± 0.15 | OD600 = 0.1 | 22.22 ± 0.11 |
| 50 pg (1.42 × 109 copies) | 12.22 ± 0.12 | 50 pg (1.42 × 109 copies) | 19.73 ± 0.07 | OD600 = 0.01 | 25.34 ± 0.15 |
| 500 pg (1.42 × 109 copies) | 15.51 ± 0.19 | 50 pg (1.42 × 109 copies) | 23.11 ± 0.05 | OD600 = 0.001 | 28.76 ± 0.41 |
| 5 pg (1.42 × 109 copies) | 18.83 ± 0.14 | 5 pg (1.42 × 109 copies) | 26.40 ± 0.05 | OD600 = 0.00001 | 33.00 ± 0.41 |
| 500 fg (1.42 × 103 copies) | 21.96 ± 0.28 | 500 fg (1.42 × 103 copies) | 29.91 ± 0.23 | OD600 = 0.000001 | N.D. |
| 50 fg (1.42 × 103 copies) | 25.53 ± 0.05 | 50 fg (1.42 × 103 copies) | 33.14 ± 0.31 | OD600 = 0.0000001 | N.D. |
| 5 fg (1.42 × 103 copies) | 29.21 ± 0.09 | 5 fg (1.42 × 103 copies) | N.D. | OD600 = 0.00000001 | N.D. |
rRNA amplicon sequencing and uses sequencing with random primers to sequence overlapping regions of a genome. However, this approach is more expensive and requires more extensive data analysis51.

Moreover, these techniques are not appropriate for determining the succession of a targeted bacillus at the species or subspecies level in various habitats.

Thus, the development of a reliable and effective procedure for quantitatively detecting specific bacilli used in commercial or scientific products is imperative. Fortunately, progress on the structural and functional genomics of a variety of B. subtilis group strains has provided insights into the microbial community dynamics of different environmental samples, revealing the parameters that influence variations in microbial communities. Determining the community structure of a microbiome and its key microbes has become easier due to the growing number of available microbial genome sequences. In particular, the availability of complete or draft Bacillus genome sequences provides an opportunity to improve their existing molecular detection and quantification tools by identifying new targets for more specific and sensitive detection.

To date, over 100 species of LAB and several yeast strains have been identified in kimchi, including Weissella, Leuconostoc, and Lactobacillus species. However, similar to other environments, there is little information regarding the succession or ontogeny of Bacillus species at the species level during kimchi fermentation, even though they are essential microorganisms in the food industry52.

In this study, we identified species or subspecies-specific genes using BLAST searches and designed primer sets to evaluate the population dynamics and ontogeny of B. velezensis and B. subtilis subsp. subtilis. The species-specific primer sets were designed using the whole genome sequences of B. amyloliquefaciens subsp. planatarum strain UCMB5036 (GenBank accession no. NC_020832.1) and B. subtilis subsp. subtilis strain BAB-1 (GenBank accession no. NC_020832.1) (Table 1). Selected primer sets, obtained through in silico analysis, in conjunction with SYBR Green real-time PCR, confirmed that this de novo qPCR assay is able to precisely identify and quantify the two most prominent Bacillus species, B. subtilis subsp. subtilis and B. velezensis, at the species or subspecies level55.

Recent reports on kimchi metabolites have indicated that the metabolite concentrations in red pepper powder kimchi are greater than those in kimchi without red pepper powder, likely because the addition of red pepper powder affects the metabolites in kimchi supernatants. In one case study of the food microbial community in kimchi, the percentage of Weissella was observed to be higher in red pepper powder kimchi than in kimchi without red pepper powder, whereas the abundances of Leuconostoc and Lactobacillus were lower in red pepper kimchi. In particular, the ontogeny of W. cibaria is significantly influenced by red pepper powder12,13.

Interestingly, as was observed for W. cibaria, the presence of B. velezensis and B. subtilis subsp. subtilis was only detected in whole kimchi with red pepper, regardless of the fermentation period. However, the abundances of these two microbes were not very high in red pepper kimchi during kimchi fermentation.

Accordingly, different types of kimchi and cheonggukjang were used to further determine whether red pepper powder affects the succession or density of B. velezensis and B. subtilis subsp. subtilis during kimchi fermentation. Both species were detected in cheongguk kimchi (with red pepper powder) and red pepper cheonggukjang, a fermented soybean paste. However, contrary to our expectations, while they were both detected and identified in cheonggukjang without red pepper powder, they were not detected in watery kimchi without red pepper powder (data not shown).

Consequently, microbial metabolites present in kimchi are predicted to be influenced by red pepper powder, thereby influencing the ontogeny of B. subtilis subsp. subtilis and B. velezensis in whole kimchi, even though these are not the dominant species in the fermentative process. Moreover, the abundances of B. subtilis subsp. subtilis and B. velezensis were not significantly affected by the kimchi fermentation temperature conditions (4 °C, 15 °C and 25 °C). Changes in the proportion of B. subtilis subsp. subtilis were similar to those of B. velezensis at 4 °C, 15 °C and 25 °C (Fig. 3).

In conclusion, our results revealed that the de novo real-time PCR assay developed in this study has helped to overcome limitations in detecting B. subtilis subsp. subtilis and B. velezensis that were previously observed due to a lack of specificity or reproducibility in conventional and real-time PCR protocols. In addition, the good

| Cloned DNA | Genomic DNA | Cell suspension |
|------------|-------------|----------------|
| Ct ± SD ($n = 3$) | Ct ± SD ($n = 3$) | Ct ± SD ($n = 3$) |
| 5 ng (1.39 × 10^7 copies) | 11.29 ± 0.18 | 5 ng (1.39 × 10^7 copies) | 17.86 ± 0.07 |
| 500 pg (1.39 × 10^7 copies) | 14.59 ± 0.06 | 500 pg | 21.15 ± 0.19 |
| 50 pg (1.39 × 10^7 copies) | 18.05 ± 0.12 | 50 pg | 24.83 ± 0.03 |
| 5 pg (1.39 × 10^6 copies) | 21.41 ± 0.14 | 5 pg | 28.33 ± 0.48 |
| 500 fg (1.39 × 10^7 copies) | 24.81 ± 0.19 | 500 fg | 31.55 ± 0.54 |
| 50 fg (1.39 × 10^6 copies) | 28.34 ± 0.24 | 50 fg | N.D. |
| 5 fg (1.39 × 10^5 copies) | 31.60 ± 0.45 | 5 fg | N.D. |

Table 5. Mean cycle threshold (CT) end-point fluorescence of 10-fold serial dilutions of Bacillus subtilis LMJ 7135 cloned DNA, genomic DNA and a cell suspension determined with real-time PCR assay. aSD, Three reactions standard deviation. bOD = 600 nm. cN.D., Not detected.
Figure 2. Specificity, melting peak and standard curve analysis of the SYBR Green qPCR assay with the Bam249F/R and BS310F/R primer sets. (A) B. velezensis. (a) Fluorescence intensity as a function of template concentration. For each assay, a series of 10-fold dilutions of cloned DNA (ranging from $1.42 \times 10^3$ to $1.42 \times 10^9$ copies/µl) was used as the template (1–7, sample dilutions). (b) Standard curve derived from the amplification plot. (c) Melting curve analysis (1–7, sample dilutions). (d) Melting peak analysis (1–7, sample dilutions). The amplified product derivatives of the relative fluorescence units $[-d(RFU)/dT]$ were plotted as a function of temperature (amplified product, 86.0°C). The large peak indicates the amplified product, while the small peak indicates the no-template control. (B) B. subtilis subsp. subtilis. (a) Fluorescence intensity as a function of template concentration. For each assay, a series of 10-fold dilutions of cloned DNA (ranging from $1.39 \times 10^3$ to $1.39 \times 10^9$ copies/µl) was used as the template (1–7, sample dilutions). (b) Standard curve derived from the amplification plot. (c) Melting curve analysis (1–7, sample dilutions). (d) Melting peak analysis (1–7, sample dilutions). The amplified product derivatives of the relative fluorescence units $[-d(RFU)/dT]$ were plotted as a function of temperature (amplified product, 83.5°C). The large peak indicates the amplified product, while the small peak indicates the no-template control.
performance of the assay was also confirmed by quantifying and identifying these Bacillus strains, even with DNA isolated from kimchi samples. Therefore, we believe that this approach can become the new gold standard method, as this evaluation will enable the rapid, culture-independent species- or subspecies-specific identification and quantification of these Bacillus strains in various industries, including fermented food.

Methods

**Bacterial strains, culture conditions and genomic DNA isolation.** A panel of 32 Bacillus reference strains was used for the specificity assay (Tables 2 and 3). Reference cultures were supplied by the Korean Agricultural Culture Collection (KACC) and the Belgian Coordinated Collections of Microorganisms (BCCM). The bacterial isolates were cultured on nutrient agar plates (BD Difco™, USA) for 48 h at 30 °C under aerophilic conditions and were subcultured at least twice before use. Total genomic DNA was extracted from bacterial cells scraped from plates as previously described and was spectrophotometrically quantified (NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies, USA)31. All extracted genomic DNA samples were stored at -80 °C until their use in further experiments.

**DNA extraction from kimchi samples.** Total DNA was extracted from kimchi samples using a Fast DNA Spin kit (MP bio, USA) according to the manufacturer’s instructions31. One microliter of each sample (5 ng/μl) was diluted individually for quantitative analysis.

In preparation of kimchi samples, two types of kimchi were obtained from a commercial factory in the Republic of Korea to investigate the ontogeny and succession of each targeted bacillus, B. velezensis and B. subtilis subsp. subtilis, during kimchi fermentation. Twenty-six batches of the two types of kimchi (13 batches each) were stored at 4 °C, while fifteen batches of each of the two types of kimchi stored at 15 °C and 25 °C were used, a total of 30 batches. The kimchi samples utilized in this experiment were obtained on the same day they were produced and were stored at different incubation temperatures (4 °C, 15 °C, and 25 °C) until sampling. As shown in Fig. 3, each sample was periodically filtered through sterilized coarse gauze (Daehan Co., Korea) during the fermentation period to collect the fluid portion. The filtrates were collected and then centrifuged (13,000 rpm for 10 min at 4 °C) to obtain the microorganisms in the kimchi.

**Candidate gene selection and oligonucleotide primer design for species- and subspecies-specific PCR assays.** The comparative genomic analysis method was used to integrate the computational steps, which improved the candidate gene selection pipeline described by Chen and Lang via computational clustering23,24. The whole genome sequences (FASTA format) from B. amyloliquefaciens subsp. plantarum str. UCMB5036, B. subtilis subsp. subtilis str. BAB-1 and the other Bacillus species strains were downloaded from ftp://ftp.ncbi.nlm.nih.gov/genomes/bacteria/4,5. From this gene selection pipeline, target candidate genes sharing no significant homology with other B. subtilis group strains were designated PCR targets. The specific primers used for B. velezensis and B. subtilis subsp. subtilis were generated using the DNASTAR Lasergene primerselect module (version 7.0) (Table 1). Primer synthesis was performed by the Bioneer Corporation (Daejeon, Korea). Each primer set
amplifies a specific DNA fragment from only the targeted species or subspecies. The nucleotide sequences of each primer set were assessed for their specificity via NCBI BLAST modules, including BLASTn and BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Species - and subspecies-specific PCR conditions.** All conventional PCR reactions were performed in a total volume of 25 μl (1 × buffer, 0.2 mM of each dNTP, 4.0 mM MgCl2) with 1.25 U of GoTaq® Flexi DNA polymerase (Promega, USA), 25 ng of template DNA and a 0.2 μM final concentration of each primer (Table 1). PCR was conducted using a PTC-225 thermocycler (MJ Research, Watertown, MA, USA) with the following steps: an initial denaturation period of 5 min at 95 °C, followed by 35 cycles of denaturation (1 min at 95 °C), annealing (30 s at 67 °C for both B. velezensis and B. subtilis subsp. subtilis), and extension (1 min at 72 °C), with a final extension period of 7 min at 72 °C. PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gels in 1 × TBE buffer solution at 60 V for 2 h. All PCR products were stained using LoadingStar (DYNEBIO, Korea). Gel images were captured and documented using a VersaDoc 1000 gel imaging system (Bio-Rad Laboratories, USA).

**Quantitative PCR assay.** Each qPCR reaction was performed in triplicate using the SYBR® Premix Ex Taq™ kit (Takara Bio, Japan). The total reaction mixture (20 μl) contained 10 μl of SYBR Green Mix, 1 μl of each primer (100 pM), 7 μl of RNase free water, and 1 μl of template. Samples were processed with a CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using the following thermal cycling program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. Melting curve analyses of PCR amplicons were initiated at 60 °C, with an incremental increase of 1 °C until a final temperature of 95 °C was reached. The standard curves of B. velezensis and B. subtilis subsp. subtilis were created by plotting the cycle threshold (CT) values of the qPCRs performed using a dilution series of genomic DNA, cloned DNA or a bacterial cell suspension. Absolute quantification and data analysis was performed automatically by the Bio-Rad CFX Manager™ Version 1.6 suite. The copy number of the cloned DNA was calculated based on a previously described formula.

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Author Contributions
D.S. Park conceived the study and wrote the manuscript; M.S. Cho, Y.J. Jin, B.K. Kang, Y.K. Park and C. Kim performed the data analysis and interpretation; and all authors reviewed the manuscript.

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