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Add the following:

▲ *Bacillus inaquosorum*

DEFINITION

Bacillus inaquosorum (formerly known as *Bacillus subtilis* subsp. *inaquosorum*) is a Gram-positive, spore-forming, rod-shaped bacterium that is aerobic. *Bacillus inaquosorum* is manufactured as a spore preparation of the specified strain. The formulated product may be blended with suitable diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of the specified strain of *Bacillus inaquosorum*.

- **Strain DE111:** *Bacillus inaquosorum* DE111 is a pure, specific strain of *Bacillus inaquosorum*.

IDENTIFICATION

• A. MICROSCOPIC IDENTIFICATION

Analysis: Proceed as directed in the Assay and perform microscopic examination.

Acceptance criteria: Rod-shaped bacterium with or without ellipsoidal endospores

• B. NUCLEIC ACID-BASED IDENTIFICATION

[NOTE—The nucleic acid-based identification method detailed in *Identification B* applies only to *Bacillus inaquosorum* DE111.]

Sample preparation reagent: Use a DNA extraction kit.¹

Sample: Dilute *Bacillus inaquosorum* DE111 powder in phosphate buffer² and then extract with a kit. Weigh out 1 g of *Bacillus inaquosorum* DE111 powder and add 99 mL of Butterfield's phosphate buffer. Dispense 200 µL into a 2.0-mL microcentrifuge tube and centrifuge at 13,000 rpm (16,200 × g) for 1 min, discarding the supernatant. Using the pellet, follow the manufacturer's instructions to extract the genomic DNA from the *Bacillus inaquosorum* DE111 powder.

Primer set: Use a primer set of forward primer sequence (5'–3') GAACTCCTAAAACTGCAAC and reverse primer sequence (5'–3')

AGCAACTTCACCCTCATAA.³ Dilute primers in [sterile water](#) to a stock concentration of 100 µM and store at –80°. Immediately before use, dilute an aliquot of each primer with [sterile water](#) (1:10, v/v). The annealing temperature for the primer set is 57°, but real-time polymerase chain reactions (PCRs) are run at an annealing temperature of 60°.

PCR sample preparations (for real-time and non-real-time PCR amplification): For each PCR amplification process, prepare a *PCR sample preparation* that contains 12.5 µL of a SYBR green⁴ mastermix, 1 µL of diluted primer forward (approximately 10 pmol), 1 µL of diluted primer reverse (approximately 10 pmol), 2 µL of *Sample* containing template DNA, and 8.5 µL of [sterile water](#).

PCR negative control: Prepare as directed for the *PCR sample preparations*, except replace the *Sample solution* with 1 mL of [sterile water](#).

PCR positive control: Prepare as directed for the *PCR sample preparations*, except replace the *Sample solution* with 2 µL of genomic DNA from the reference strain or replace the strain-specific primers with species-specific universal primers.

PCR amplification

For real-time PCR: Perform PCR on *PCR sample preparation*, *PCR negative control*, and *PCR positive control* using a real-time PCR system.⁵ Incubate the first cycle at 95° for 10 min, followed by 40 cycles at 95° for 15 s, with the annealing temperature at 60° for 30 s, extension at 72° for 30 s, and a melt curve cycle from 60° to 95°.

For non-real-time PCR: Perform PCR on *PCR sample preparation*, *PCR negative control*, and *PCR positive control* using an appropriate thermal cycler.⁶ Incubate at 95° for 10 min (step 1); 95° for 15 s (step 2); 57° for 30 s (step 3); and at 72° for 1 min (step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min, and hold at 4°.

Analysis

For real-time PCR amplification: Analyze the results of the *PCR amplification* using the software appropriate for the real-time PCR system.

Analysis of the *PCR positive control* should result in a cycle threshold (CT) value close (within 3.2 CT values) to that of the sample.

Analysis of *PCR negative control* should result in the absence of any amplification or a CT value close to 40 and at least 5 CT values higher than any sample. If the results are not as expected, the preparation of the *PCR sample preparations*, *PCR negative control*, and *PCR positive control* must be repeated, followed by real-time *PCR amplification* and *Analysis*.

For non-real-time PCR amplification: Analyze the results of the *PCR amplification* for each *PCR sample preparation*, *PCR negative control*, and *PCR positive control* using a precast agarose electrophoresis system.⁷ Follow the manufacturer's instructions for analysis.

Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 3% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris-hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with deionized water. [CAUTION—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.]

Use a DNA ladder standard⁸ suitable for determining the size of linear double-stranded DNA fragments amplified with the primers. The

ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons. Analysis of the *PCR positive control* must result in the amplification of specific base pairs as indicated in the *Acceptance criteria* if using genomic DNA of the strain or must result in the amplification of specific base pairs as found in the published literature if using species-specific universal primers. Analysis of the *PCR negative control* must result in the absence of any amplification products. If the results are not as expected, preparation of the *PCR sample preparation*, *PCR positive control*, and *PCR negative control* must be repeated, followed by non-real-time *PCR amplification and Analysis*.

Acceptance criteria

For real-time PCR amplification: The *PCR sample preparation* produces an amplification product of a low CT value and a unimodal melt curve with a melting temperature of around 74.6°.

For non-real-time PCR amplification: The *PCR sample preparation* produces an amplification of 173 base pairs.

ASSAY

• ENUMERATION

Peptone diluent: Use a sterile solution of 0.1% peptone in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Dispense into sterile containers as needed for preparing samples.

Tryptone soy agar (TSA) medium: Mix the reagents shown in [Table 1](#) in a 1-L conical flask. Heat the mixture to boiling (with stirring) on a hot plate and allow the mixture to boil until the bacteriological agar is completely dissolved. Add additional water to bring the volume in the flask to 1000 mL. Cover the flask with aluminum foil and sterilize it in an autoclave at 121° for NLT 15 min. Once the autoclave can safely be opened, remove the flask and incubate in a water bath at 45 ± 2° until needed for plating.

Table 1

Reagent	Quantity
Casein peptone (pancreatic)	15.0 g
Soy peptone (papainic)	5.0 g
Sodium chloride	5.0 g
Bacteriological agar	15.0 g
Water	900 mL

Sample preparation: Transfer 2.00 g of probiotic powder into a sterile blender jar. Add 198 mL of sterile *Peptone diluent* to the jar and blend for 10 s on a high liquefy setting, ensuring that all product is off the glass sides. Soak for 20 min to allow cultures and carriers to hydrate. Blend for 60 s on a high liquefy setting, again ensuring that all product is off the glass sides. Immediately transfer 1 mL from the blender to a sterile test tube containing 9 mL of *Peptone diluent*; this is considered a 10:3 dilution. Mix thoroughly by vortexing the test tube or shaking in the diluent shaker.⁹ Repeat dilutions in a succession until the final dilution is expected to contain between 25 and 250 cfu/mL. The final 3 dilutions will be used in the *Analysis*. [NOTE—The *Sample preparation* should be performed in duplicate. Take care to plate the *Sample preparation* dilutions within 10–20 min of preparation.]

Analysis: For each *Sample preparation* tube to be plated, prepare Petri plates as follows. Aseptically transfer 1.0 mL of the *Sample preparation* separately into 3 sterile 15-mm × 100-mm Petri plates, and then pour 15–20 mL of the molten *TSA medium* into each plate. Place the lid on each plate after adding the molten *TSA medium*, and then gently swirl the plates to mix. [NOTE—3M Petrifilm Aerobic Count Plates¹⁰ can be used for quicker plating and better visualization of colonies.] Repeat this procedure for the additional 2 dilutions of the *Sample preparation* (and all duplicate tubes). Prepare 1 negative control plate that contains only *TSA medium* and a second negative control plate that contains 1.0 mL of *Peptone diluent* and *TSA medium* mixture. Allow the plates to sit at room temperature until the *TSA medium* solidifies, and then invert the plates and incubate them at 37 ± 2° for 24 h. Count the colonies on the prepared plates, including both blank plates. Plates containing between 25 and 250 colonies are considered ideal for counting. Only count colonies with the following appearance. Surface colonies should be 1–5 mm in diameter, circular, fuzzy white to slightly brown in color, with jagged edges. Colonies inside the *TSA medium* should be 0.5–1 mm in diameter and should appear as pinpoints. [NOTE—Colonies on 3M Petrifilm Aerobic Count Plates appear as pinkish red pinpoints.] After the incubation period, count the colonies and record the results as viable cfu/g, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in cfu/g. The presence of any colonies not conforming to the previous description suggests a contaminated sample that must be investigated. Depending on the outcome of the investigation, the test may be rejected or repeated. Both blank plates should be entirely free of any type of colonies. [NOTE—When blank plates contain colonies, the entire procedure must be repeated, potentially including the preparation of the *Peptone diluent* and the *TSA medium*, depending on which plate(s) contain colonies.]

Acceptance criteria: NLT 100% of the labeled viable count, in cfu/g, of the specified strain of *Bacillus inaquosorum*

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]