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February 7, 2023

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Dr.
College Park, MD 20740

Attention: Dr. Susan Carlson
Re: GRAS Notification – *Bacillus subtilis BS50*

Dear Dr. Carlson:

GRAS Associates, LLC, acting as the Agent for BIO-CAT Microbials, LLC (689 Canterbury Rd., Shakopee, MN 55379), is submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS Notification for *Bacillus subtilis BS50*. Along with BIO-CAT Microbials, LLC's determination of safety for the intended use in a wide variety of foods, including baked goods and baking mixes, beverages and beverage bases (including carbonated and flavored waters, sports and nutritional drinks), breakfast cereals, cheese, chewing gum, coffee and tea, confections and frostings, dairy product analogs, frozen desserts (dairy, non-dairy and ices), gelatins, puddings and fillings, grain products and pastas, hard candy and cough drops, milk products, plant protein products, processed fruits and fruit juices, processed vegetables and vegetable juices, snack foods and soft candy at a maximum level of $2x10^9$ CFU per serving. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email. I also authorize Amy Mozingo (amozingo@gras-associates.com), VP US Nutra Regulatory Sciences, GRAS Associates LLC to lead communications related to this submission.

We look forward to your feedback.

Sincerely,

[redacted]

William J. Rowe

Agent for BIO-CAT Microbials, LLC

President, CEO
GRAS Associates, LLC
1810 Grand Park Ave, Suite 500
North Bethesda, MD 20852
wrowe@nutrasource.ca
Enclosure: GRAS Notification for BIO-CAT Microbials, LLC – Bacillus subtilis BS50

Safety Evaluation Dossier Supporting the Generally Recognized as Safe (GRAS) Conclusion

of

Bacillus subtilis BS50

Prepared By:

GRAS Associates
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

Prepared For:

BIO-CAT Microbials, LLC
689 Canterbury Rd
Shakopee, MN 55379

February 7, 2023

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FOREWORD

BIO-CAT Microbials, LLC (BIO-CAT) based this Generally Recognized as Safe (GRAS) assessment of *Bacillus subtilis* BS50 (also referred to as *B. subtilis* BS50 or BS50 or commercially as OPTIBIOME® BS50), on the composite safety information, e.g., scientific procedures with corroboration from history of use. The safety/toxicity of Bacillus subtilis BS50, history of use of Bacillus subtilis BS50, and compositional details, specifications, and method of preparation of the subject ingredient were reviewed. In addition, a search of the scientific and regulatory literature was conducted through January 10, 2023, with particular attention paid to adverse reports, as well as those that supported conclusions of safety. Those references that were deemed pertinent to this review are listed in Part 7. The composite safety/toxicity studies, in concert with dietary exposure information, ultimately provide the specific scientific foundation for the GRAS conclusion.

BIO-CAT based its GRAS assessment on the large body of information that addressed the safety/toxicity/use(s) of *Bacillus subtilis* BS50 and other *Bacillus subtilis* strains, history of use of *Bacillus subtilis*, and compositional details, specifications, and method of preparation of the subject ingredient. Safety/toxicity studies performed with animals and human clinical trials were noted to have value. The totality of information about the composition, safety/toxicity/use(s) and dietary exposure ultimately provide the specific scientific foundation for the GRAS conclusion. BIO-CAT Microbials, LLC has asked GA to act as Agent for the submission of this GRAS notification.

PART 1. SIGNED STATEMENTS AND CERTIFICATION

BIO-CAT has concluded that *Bacillus subtilis* BS50 (also referred to as *B. subtilis* BS50 or BS50 or commercially as OPTIBIOME® BS50), which meets the specifications described below, is GRAS in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act (FD&C Act). The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the intended conditions of food use for the designated *B. subtilis* BS50 preparation.

This signed statement and certification has been prepared in accordance with the requirements of 21 CFR 170.225.

(a) This certification is signed by a responsible official of GRAS Associates, LLC acting as agent for BIO-CAT.

(b) This Part 1 of the GRAS notification does not include any confidential information;

(c) (1) This GRAS Assessment was conducted in accordance with Subpart E of 21 CFR Part 170;

(c) (2) Names and addresses of organizations;

Sponsoring Party:

BIO-CAT Microbials, LLC
689 Canterbury Rd
Shakopee, MN 55379
U.S.A.

Agent:

GRAS Associates, LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

(c) (3) The name of the ingredient is *Bacillus subtilis* BS50.

(c) (4) The ingredient will be used as an ingredient in a wide variety of foods (baked goods and baking mixes, beverages and beverage bases (including carbonated and flavored waters, sports and nutritional drinks), breakfast cereals, cheese, chewing gum, coffee and tea, confections and frostings, dairy product analogs, frozen desserts (dairy, non-dairy and ices), gelatins, puddings and fillings, grain products and pastas, hard candy and cough drops, milk products, plant protein products, processed fruits and fruit juices, processed vegetables and vegetable juices, snack foods and soft candy) at levels up to 2×10^9 CFU/serving.

(c) (5) The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with § 170.30(a) and (b).

(c) (6) It is our view that the ingredient is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

(c) (7) If FDA were to ask to see the data and information that are the basis for our conclusion of GRAS status, either during or after FDA evaluation of this notice, we agree to:

(i) make the data and information available to FDA; and

(ii) agree to both of the following procedures for making the data and information available to FDA:

(A) Upon FDA's request, we will allow FDA to review and copy the data and information during customary business hours at our address specified where these data and information will be available; and

(B) Upon request by FDA, we will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for their evaluation or on paper.

(c) (8) None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552 (e.g., as trade secret or as commercial or financial information that is privileged or confidential).

(c) (9) We certify that, to the best of our knowledge, this GRAS Assessment is a complete, representative, and balanced review that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(c) (10) BIO-CAT does not intend to add *Bacillus subtilis* BS50 to any meat and/or poultry products that come under FSIS/USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

(c) (11) Signature

William Rowe
President
GRAS Associates, LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

Date: 2/6/2023

PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

A. Notified Substance *Bacillus subtilis* BS50 Identification

1. Common or Usual Name

The name of the ingredient is *Bacillus subtilis* BS50 (also referred to as *B. subtilis* BS50 or BS50 or commercially as OPTIBIOME® BS50). The taxonomy is shown in **Table 1**.

Table 1. Taxonomy of *B. subtilis*

Super Kingdom	**Bacteria**
Clade	Terrabacteria group
Phylum	Bacillota (syn. Firmicutes)*
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species Group	*Bacillus subtilis* group

NCBI Genome (2022); NCBI Taxonomy (2022); UniProt Taxonomy (2022)
* Oren and Garrity (2021)

2. Characterization

B. subtilis was discovered in 1835 by Christian Gottfried Ehrenberg and named *Vibrio subtilis*. It was subsequently renamed *B. subtilis* by Ferdinand Cohn in 1872 (Ehrenberg, 1835; Cohn, 1872). The BIO-CAT Microbials, LLC (BIO-CAT) *B. subtilis* that is the subject of this safety evaluation / GRAS determination is a proprietary preparation of a *B. subtilis* strain isolated from soil samples from Gallatin County, Montana, USA and designated as *B. subtilis* BS50. *B. subtilis* BS50 is a non-toxigenic and non-pathogenic organism that has a patent deposit with the ATCC (BS50 PTA-127287). *B. subtilis* BS50 is periodically monitored for genetic drift. Identification is confirmed annually through 16S and the entire genome will be re-sequenced and compared to the previous sequence for any nucleotide changes every three years to detect genetic mutations or drift.

The organism is a gram-positive, spore-forming rod that is a facultative aerobe. The length typically ranges from 2 to 6 micrometers long (standard for *B. subtilis*). The diameter is <1 micrometer. It is not genetically modified in any manner.

3. DNA Ribotyping Analysis and Full Genome Sequence Analysis

BIO-CAT completed a whole genome sequence (WGS) for *B. subtilis* BS50. The WGS is available upon request. An individual stock vial of the strain was streaked to tryptic soy agar plates to generate isolated colonies. Plates were incubated at 35°C overnight in aerobic conditions. Liquid

growth media was inoculated with isolated colonies. Cultures were grown at 35°C with shaking overnight in aerobic conditions. Genomic DNA was purified from pure culture.

The WGS was performed on MinIOn FlowCells FLO-MIN106D over 48-72h (Oxford Nanopore Technologies, Oxford, United Kingdom). The genome was assembled with Flye using default settings (Kolmogorov et al., 2019) and annotated using prokka 1.13.7.[1]

B. subtilis BS50 has a 4,150,844 base pair genome. No plasmids were detected. The genome most closely aligned to the strain was *B. subtilis* subsp. *subtilis*.

a. Phylogenetic Tree Analysis

Results of genotyping have been published (Brutscher et al., 2022). BLAST+ command line software (Camacho et al., 2009) and the BLASTn algorithm (Altschul et al., 1990) were used to identify nucleotide sequences in the BS50 genome and 20 other *B. subtilis* genomes that aligned with six genes from the genome of *B. subtilis* subspecies *subtilis*, strain 168, one of the longest existing and most extensively studied strains of *B. subtilis* (type strain Marburg derived) (Burkholder and Giles Jr, 1947; Zeigler et al., 2008): rpoB (GeneID: 936335), purH (GeneID: 936053), gyrA (GeneID: 940002), groEL (GeneID: 938045), polC (GeneID: 939620), and 16S rRNA (GeneID: 936895). These genes are standard “housekeeping” genes for *Bacillus* species and commonly used for phylogenetic analysis of *Bacillus* species (Kubo et al., 2011). For each strain, the sequences representing these six genes were then concatenated into single nucleotide sequences.

Multiple sequence alignment of the concatenated sequences for each *Bacillus* strain was performed using Multiple Alignment using Fast Fourier Transform (MAFFT) (Katoh et al., 2019). The multiple sequence alignment file produced by MAFFT was then input into MEGA X for phylogenetic tree construction (Kumar et al., 2018). Evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). Data was bootstrapped 50 times. The tree with the highest log likelihood (-30362.06) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 15,093 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

In order to assess the similarity of *B. subtilis* BS50 to other *Bacillus* genomes on a whole genome basis, pairwise whole genome alignments were performed between BS50 and the other *Bacillus* strains using BLASTn (Altschul et al., 1990) (default settings). Strain genomes sharing at least

[1] https://github.com/tseemann/prokka

95% average nucleotide identity are generally accepted as belonging to the same species (Konstantinidis and Tiedje, 2005; Richter and Rosselló-Móra, 2009). BLASTn outputs are shown in Table 2 and the phylogenetic tree is shown in Figure 1.The results show that *B. subtilis* BS50 aligns closely with other common *B. subtilis* strains including *B. subtilis* type strain 168 (99.0% similar) and *B. subtills* MB40 (98.5% similar), a strain already on the market. *B. subtilis* BS50 also closely aligns with *B. subtilis* subsp. Natto (*B subtilis*-BEST195, 99.0% similar), a *B. subtilis* strain commonly found in Japanese fermented natto beans.

Table 2. Listing of the BLASTn Outputs from the Whole Genome Sequence Alignments to *Bacillus subtilis* BS50

Description	Total Score	Query	E Value	Ident	Accession
B. subtilis strain MB8_B10	8.48E+06	98%	0	99.6%	GCA_009662195.1
B. subtilis strain ms-2	8.54E+06	98%	0	99.3%	GCA_008831405.1
B. subtilis strain HJ0-6	8.54E+06	97%	0	99.3%	CP013984.1
B. subtilis strain QB61	8.12E+06	96%	0	98.9%	GCA_003148355.2
B. subtilis strain SEM-9	8.06E+06	96%	0	99.2%	GCA_006165085.1
B. subtilis strain MB40	5.77E+06	96%	0	98.5%	N/A
B. subtilis strain NCIB 3610	7.94E+06	95%	0	99.0%	GCA_002055965.1
B. subtilis strain LBUM979	7.94E+06	95%	0	99.0%	GCA_016065415.1
B. subtilis strain subsp. subtilis strain 168	7.94E+06	95%	0	99.0%	NC_000964.3
B. subtilis strain SH1	7.90E+06	95%	0	98.9%	GCA_015654205.1
B. subtilis strain ATCC 11774	7.61E+06	92%	0	99.3%	GCA_004101945.1
B. subtilis strain SRCM103612	7.82E+06	92%	0	98.0%	GCA_004119775.1
B. subtilis strain BJ3-2	7.24E+06	92%	0	95.9%	GCA_002893805.1
B. subtilis strain CW14	6.68E+06	92%	0	94.3%	GCA_002163815.1
B. subtilis strain JAAA	7.57E+06	91%	0	98.8%	GCA_009363835.1
B. subtilis strain BL-01	5.77E+06	90%	0	94.2%	GCA_013393725.1
B. inaquosorum strain DE111	5.77E+06	90%	0	94.2%	GCA_001534785.1
B. subtilis strain KH2	7.38E+06	89%	0	99.0%	GCA_001890405.1
B. subtilis strain ATCC 21228	7.36E+06	89%	0	98.9%	GCA_002982175.1
B. subtilis subsp. natto strain BEST195	7.35E+06	89%	0	99.0%	GCA_000209795.2

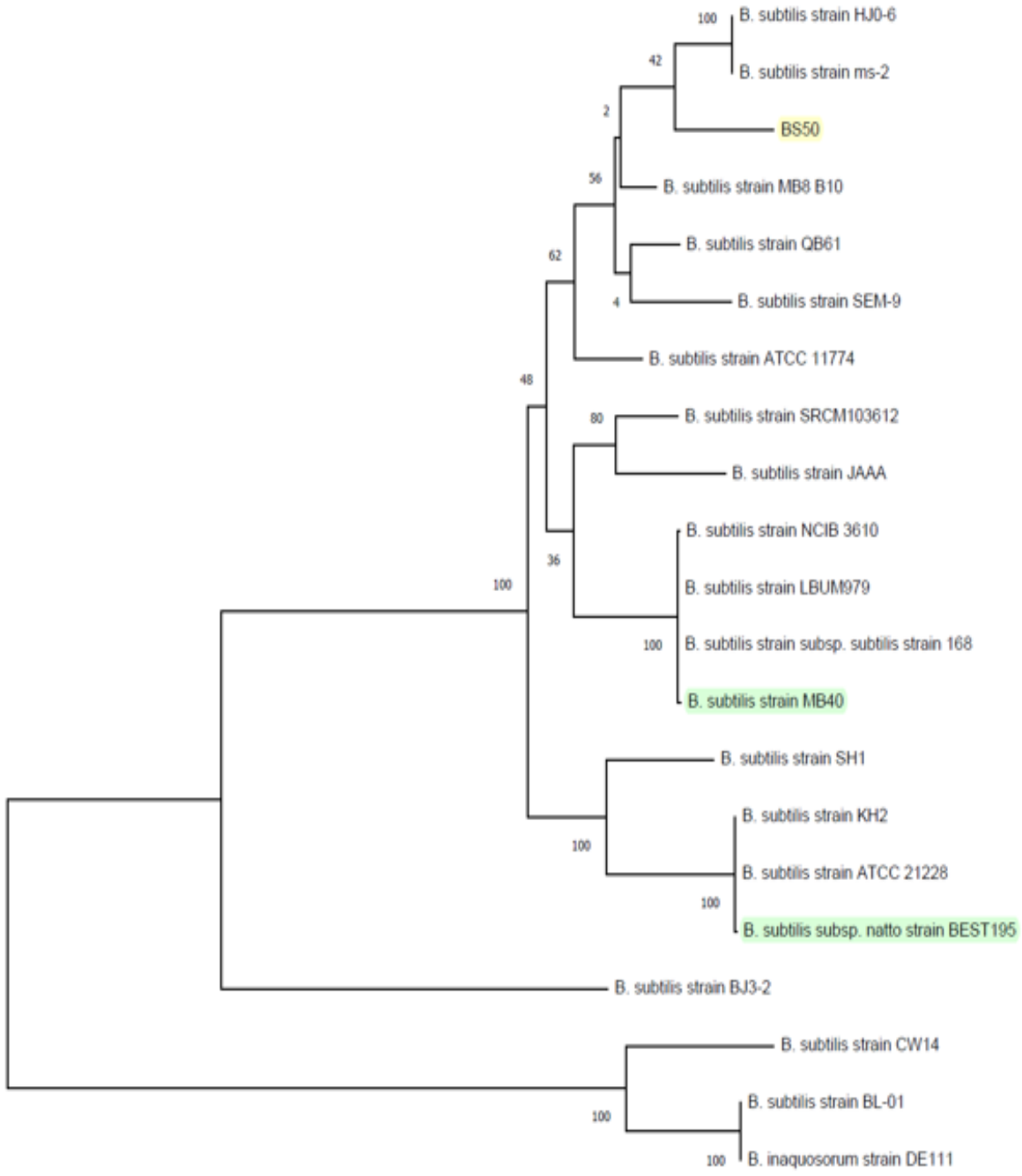

Figure 1. Speciation of BS50*

*The phylogenetic tree was constructed using the maximum likelihood method with sequences containing the concatenation of the rpoB, purH, gyrA, groEL, polC, and 16S rRNA genes. The numbers along the branches indicate bootstrap percentages. BS50 is highlighted in yellow. *Bacillus subtilis* MB40 and *Bacillus subtilis* natto, two safe-for-human-use Bacillus strains are highlighted in green.

B. Method of Manufacture of *Bacillus subtilis* BS50

B. subtilis BS50 is produced consistent with current Good Manufacturing Practices (cGMP) as a pure spore culture consisting of only fermentation medium and *B. subtilis* BS50 spores. BIO-CAT manufacturing adheres to a food safety plan consistent with Safe Quality Food (SQF) requirements for Hazard Analysis Critical Control Point (HACCP) preventive controls. The pure spore culture is concentrated via centrifugation. The concentrated liquid is then blended with enough maltodextrin, so the total solids is up to 10% and then spray dried. The preparation is then blended with additional maltodextrin to achieve the finished formulation. Other safe and suitable carriers/processing aids such as dextrin, tapioca maltodextrin, etc. meeting the requirements under 21 CFR 184.1277 may be used as an alternative to maltodextrin.

The final powdered product of *B. subtilis* BS50 is as close to 100% spores as can be measured. After fermentation, acid is added to the culture to drop the pH to 4.5. At this pH, spores will survive but any vegetative cells will not. Additionally, the stabilized culture is concentrated via centrifugation and then spray dried. Any possible remaining vegetative cells will not survive during the spray drying process. To ensure the final preparation is entirely spores, a total aerobic enumeration is compared to an aerobic enumeration that has been heat treated (80^{o}C for 5 min). Only spores will survive the heat treatment. If the total aerobic count and the heat-treated spore count are the same, the preparation is 100% spores. If the total aerobic count is higher than the spore count, then there are vegetative cells present in the preparation.

A manufacturing process diagram for *B. subtilis* BS50 is provided in Figure 2.

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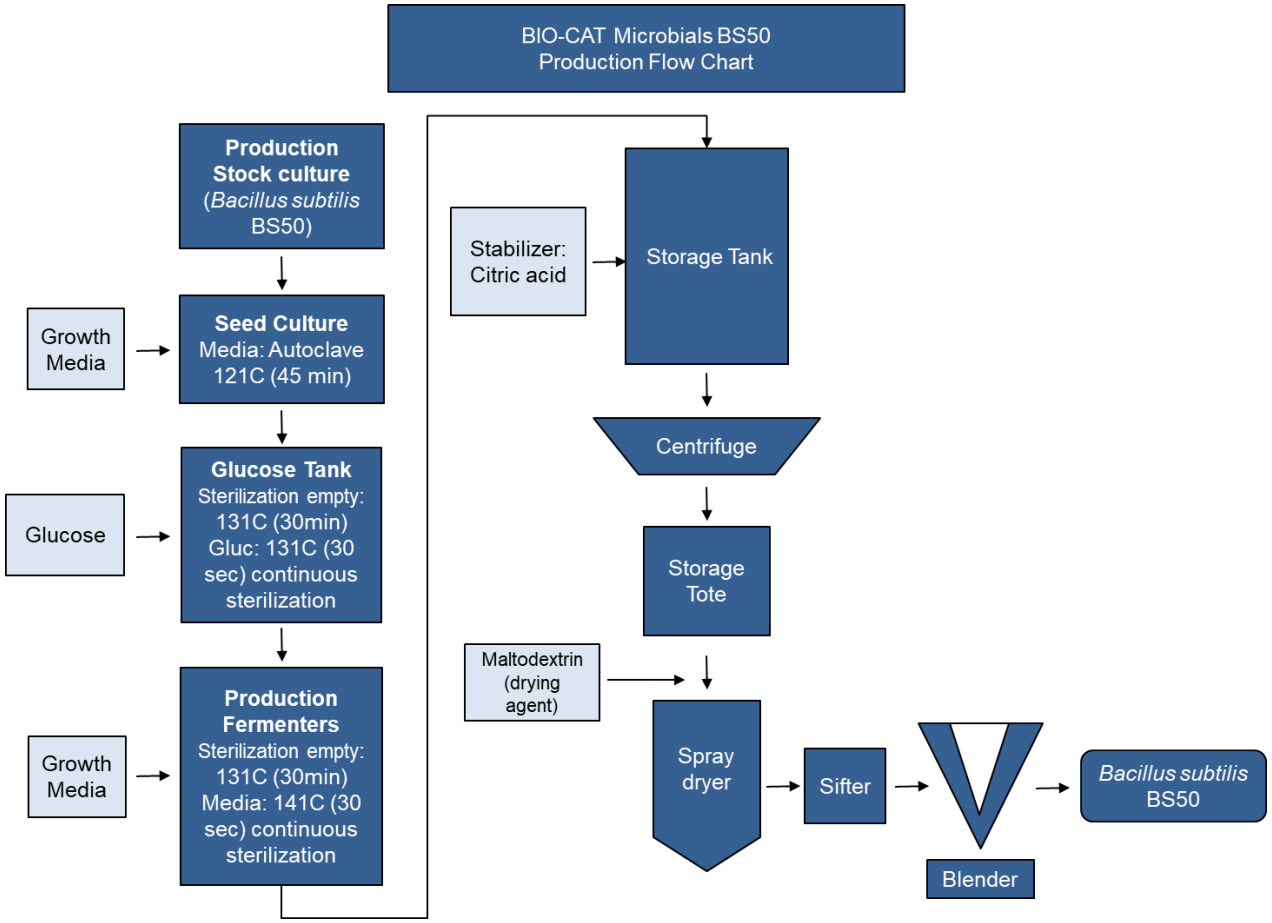

Figure 2. Method of Manufacture of *B. subtilis* BS50

Finished product is packaged in a liner bag in an additional liner bag within a cardboard box. The bags are made of polyethylene[2] film approved by FDA and USDA for food contact.

In an effort to prevent cross-contamination of allergens prior to fermentation batches of *B. subtilis* BS50, all fermentation equipment (tanks, lines) undergoes clean-in-place (CIP), sanitization and sterilization processes and all ancillary equipment (totes, separator, spray dryer, screens, and blenders) are cleaned and sanitized. Post cleaning and sanitation, the equipment is swabbed for microbes and allergens utilizing ATP technology. Should any equipment fail ATP testing, cleaning and sanitization is repeated then subsequently retested prior to use. While *B. subtilis* BS50 is manufactured using allergen-free media, BIO-CAT Microbials manufactures other *Bacillus* strains that utilize dairy and soy ingredients as growth media in fermentation. Therefore, due to shared equipment, the statement “although this product is not made with milk or soy, it may contain trace amounts due to manufacturing methods” is placed on labeling.

[2] 21 CFR 177.1520

The substances used in production of *B. subtilis* BS50 are listed in Table 3. All substances used in production of the ingredient are food-grade (Appendix 1).

Table 3. Substances Used in Production of *B. subtilis* BS50

MATERIAL	PURPOSE
Yeast extract	Fermentation
Dextrose	Fermentation
Disodium phosphate	Fermentation
Monosodium phosphate	Fermentation
Magnesium sulfate	Fermentation
Manganese sulfate	Fermentation
Calcium chloride	Fermentation
Sodium chloride	Fermentation
Antifoam AF-100 FG	Foam control
Sodium hydroxide	pH control
Citric acid	pH control
Maltodextrin	Drying

C. Product Specifications

The food grade specifications for the finished *B. subtilis* BS50 product are summarized in Table 4. Conformance to specifications and consistency of *B. subtilis* BS50 manufacturing is demonstrated by the analyses of four non-consecutive lots of commercially representative *B. subtilis* BS50 with the results summarized in Table 5. The certificates of analysis (COA) are provided in Appendix 2.

The specific methods were selected because they represent current industry standards for the analysis. Internal validations of all Standard Operating Procedures (SOPs), media, and other testing materials are performed to ensure they are functioning as expected.

The collection of these reports demonstrates that the substance is well characterized and meets the established purity criteria.

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Table 4. Food Grade Specification for *Bacillus subtilis* BS50

Physical and Chemical Parameters	Specification (Acceptable Target/Range)	Test Method
Color	Light Tan to Tan	Visual
Visual Inspection	Visually free of foreign material	Visual
Texture	Crystalline, free flowing powder	Organoleptic
Odor	Strong fermentation	Organoleptic
Identity*	>98% homology	16S Sequencing
Activity (CFU/g), Total viable spore count	NLT 100 Billion	FDA BAM Chapter 3
Moisture Content (%)	<10	Ohaus MB-45
Heavy Metals**		
Lead (ppm)	<0.5	ICP
Mercury (ppm)	<0.1	ICP
Cadmium (ppm)	<0.5	ICP
Arsenic (ppm)	<0.3	ICP
Microbiological Limits		
Yeast and Mold (CFU/g)	≤300	FDA BAM Chapter 18
Salmonella (per 25 g)	Negative	FDA BAM Chapter 5
Coliforms (CFU/g)	≤30	AOAC 991.14
***E. coli* (per 25 g)**	Negative	AOAC 991.14
Listeria (per 25 g)***	Negative	FDA BAM Chapter 10
***S. aureus* (CFU/g)**	< 10	FDA BAM Chapter 12

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing the first 5 lots and thereafter, a minimum of very 5th lot. The analyses are performed by a contract lab that uses an internally validated method.
*** The Listeria assay used will identify the presence of the genus Listeria which includes *Listeria monocytogenes* but is not limited to only this species.
AOAC – Association for Official and Analytical Chemists; BAM – Bacteriological Analytical Manual; CFU – Colony Forming Units; FDA – Food and Drug Administration; g – gram; ICP – Inductively Coupled Plasma; NLT – not less than; ppm – parts per million

Table 5. Analytical Results for *Bacillus subtilis* BS50

Physical and Chemical Parameters	Acceptable Target/Range	*Bacillus subtilis* BS50 Batch Results			
		Lot No. OPTIBS50-OE27-1	Lot No. OPTIBS50-PE01-1	Lot No. OPTIBS50-PE01-3	Lot No. OPTIBS50-PE02-1
Color	Light Tan to Tan	Light Tan	Light Tan	Light Tan	Light Tan
Visual Inspection	Visually free of foreign material	Pass	Pass	Pass	Pass
Texture	Crystalline, free flowing powder	Pass	Pass	Pass	Pass
Odor	Strong fermentation	Pass	Pass	Pass	Pass
Identity*	>98% homology	Pass	Pass	Pass	Pass
Activity (CFU/g), Total viable spore count	NLT 100 Billion	111 Billion	106 Billion	113 Billion	105 Billion

Physical and Chemical Parameters	Acceptable Target/Range	*Bacillus subtilis* BS50 Batch Results			
		Lot No. OPTIBS50-OE27-1	Lot No. OPTIBS50-PE01-1	Lot No. OPTIBS50-PE01-3	Lot No. OPTIBS50-PE02-1
Moisture Content (%)	<10	4.84	5.30	5.31	5.60
Heavy Metals**					
Lead (ppm)	<0.5	0.27	0.11	0.14	0.12
Mercury (ppm)	<0.1	<0.01	<0.01	0.02	0.01
Cadmium (ppm)	<0.5	<0.01	<0.01	<0.01	<0.01
Arsenic (ppm)	<0.3	0.03	<0.03	0.03	<0.03
Microbiological Limits					
Yeast and Mold (CFU/g)	≤300	<10	<10	<10	<10
Salmonella (per 25 g)	Negative	Negative	Negative	Negative	Negative
Coliforms (CFU/g)	≤30	< 10	< 10	< 10	< 10
***E. coli* (per 25 g)**	Negative	Negative	Negative	Negative	Negative
Listeria (per 25 g)***	Negative	Negative	Negative	Negative	Negative
***S. aureus* (CFU/g)**	<10	<10	<10	<10	<10

*Results determined from testing of Bacillus subtilis raw material
**Results determined from testing the first 5 lots and thereafter, a minimum of every 5th lot. The analyses are performed by a contract lab that uses an internally validated method.
*** The Listeria assay used will identify the presence of the genus Listeria which includes *Listeria monocytogenes* but is not limited to only this species.
CFU – Colony Forming Units; g – gram; NLT– not less than; ppm – parts per million

D. Function

B. subtilis BS50 is intended for use as an ingredient in a wide variety of foods.

E. Stability Data

Shelf-life stability of one lot (OPTIBS50-OE27-1) of the manufactured product has been determined for up to 18 months at 30 °C/65% Relative Humidity (RH) (Figure 3) and 25°C/60% RH (Figure 4). The results show that *B. subtilis* BS50 is stable under both conditions over an 18-month period.

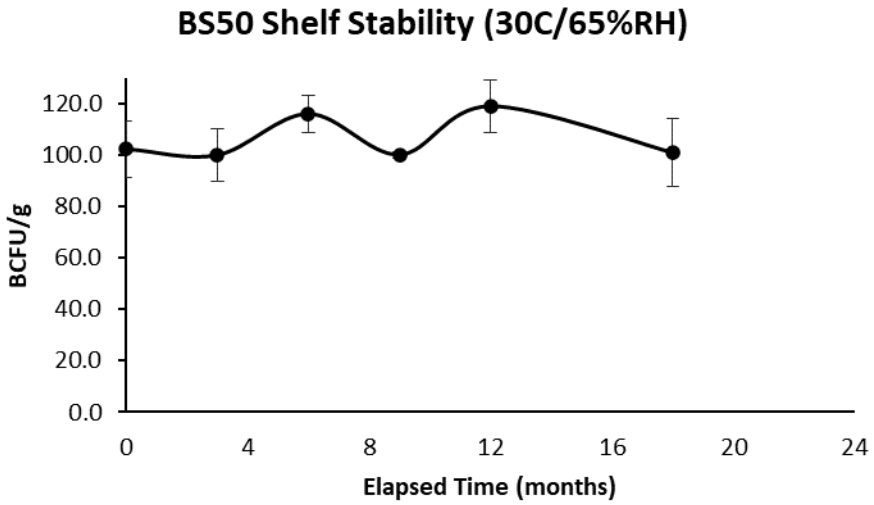

Figure 3. Stability of *Bacillus subtilis* BS50 at 30°C/65% Relative Humidity*
*BCFU = billion CFU/g

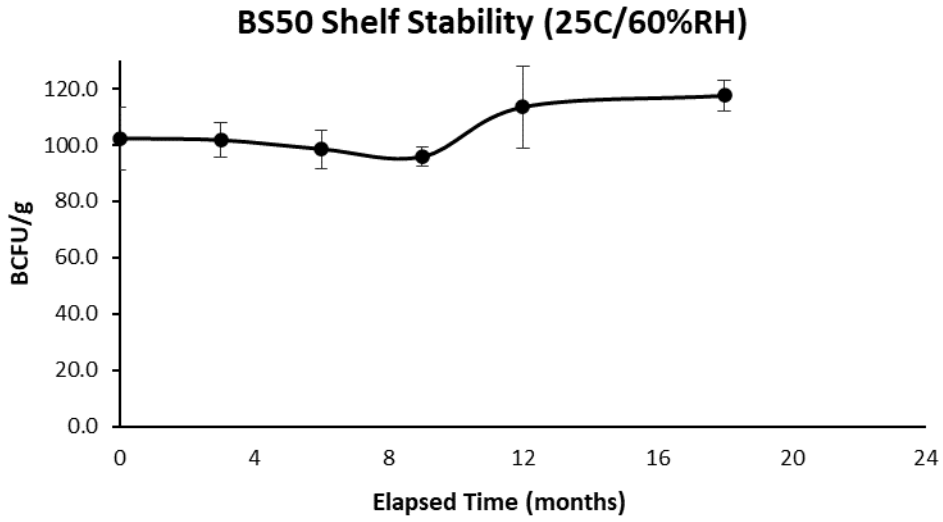

Figure 4. Stability of *Bacillus subtilis* BS50 at 25°C/60% Relative Humidity*
*BCFU = billion CFU/g

PART 3. DIETARY EXPOSURE

A. Estimated Dietary Exposure

1. Proposed Uses

B. subtilis BS50 is intended for use as an ingredient in a wide variety of foods, including baked goods and baking mixes, beverages and beverage bases (including carbonated and flavored waters, sports and nutritional drinks), breakfast cereals, cheese, chewing gum, coffee and tea, confections and frostings, dairy product analogs, frozen desserts (dairy, non-dairy and ices), gelatins, puddings and fillings, grain products and pastas, hard candy and cough drops, milk products, plant protein products, processed fruits and fruit juices, processed vegetables and vegetable juices, snack foods and soft candy. *Bacillus subtilis* BS50 will be added to foods at a maximum level of 2 x 10^9 CFU/serving. The food categories, as defined in 21 CFR 170.3(n), to which *B. subtilis* BS50 will be added are listed in Table 6.

Table 6. Proposed Food Uses of *Bacillus subtilis* BS50

Food Category
(1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving.
(3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee

Food Category
substitutes, and fruit and vegetable flavored gelatin drinks.
(4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals.
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(6) Chewing gum, including all forms.
(7) Coffee and tea, including regular, decaffeinated, and instant types.
(9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars.
(10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products.
(20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties.
(21) Fruit and water ices, including all frozen fruit and water ices.
(22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads.
(23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables.
(25) Hard candy and cough drops, including all hard type candies.
(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products*.
(33) Plant protein products, including the National Academy of Sciences/ National Research Council "reconstituted vegetable protein" category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins.
(35) Processed fruits and fruit juices, including all commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, "ades", and drink substitutes made therefrom
(36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends.
(37) Snack foods, including chips, pretzels, and other novelty snacks.
(38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies

**Bacillus subtilis* BS50 is not intended for use in infant formula.
***Bacillus subtilis* BS50 is not intended for use in any product that that come under FSIS/USDA jurisdiction.

2. Estimated Dietary Intake (EDI)

Consumer exposure to *Bacillus subtilis* BS50 was estimated using the methods described in GRN 399 (FDA, 2011) and GRN 955 (FDA, 2021a) which utilized data from the USDA Nutrition Insights publication of the USDA Center for Nutrition Policy and Promotion (Basiotis et al., 2000). According to this report, males, aged 51 or older, consume the greatest number of servings of food per day, estimated as 18.2 servings of food/day, from the following categories: grains, fruits, vegetables, milk, meat and other (fats, oils, sweets). Therefore, using this upper intake level of 18.2 servings of food/day and assuming that *Bacillus subtilis* BS50 is added to every category of food outlined above, at the maximum use level of 2×10^9 CFU/ serving, the maximum estimated daily intake (EDI) is calculated as 3.64×10^{10} CFU/day (approximately 36 billion CFU/day or 5.2×10^8 CFU/kg bw/day for a 70 kg human).

3. Acceptable Daily Intake (ADI)

Consumption of *Bacillus subtilis* BS50 and for the related strain *Bacillus subtilis* MB40 is well tolerated by humans at 2 x 10^9 CFU/day, 5 x 10^9 CFU/day, and 1 x 10^{10} CFU/day (highest dose tested) (see Part 6).

In addition, the acceptable daily intake (ADI) of *Bacillus subtilis* BS50 was calculated using the methodology employed for a similar microbial ingredient with GRAS status as a food ingredient, as described in GRN 399 (Ganeden, 2011) and Endres et al. (2011). Based on the No-Observed-Adverse-Effect-Level (NOAEL) of 2000 mg/kg bw/day (equivalent to 3.7 x 10^{11} CFU/kg bw/day or 8.51 x 10^{10} CFU/day) in the 14-day toxicity study in rats with the related strain *Bacillus subtilis* MB40 (Spears et al., 2021) (See Part 6), and conservative 100-fold safety factor for inter- and intra-species differences, the ADI of *Bacillus subtilis* BS50 in humans is calculated as 3.7 x 10^9 CFU/kg bw/day (or 2.6 x 10^{11} (260 billion) CFU/day for a 70 kg person). Clinical and nonclinical studies with other *Bacillus subtilis* strains and a GRAS Notice for a different *Bacillus subtilis* strain (GRN 831) support the safety and appropriateness of the ADI for *Bacillus subtilis* BS50.

B. Estimated Dietary Exposure to Any Other Substance That is Expected to be Formed in or on Food

No other substance(s) is/are expected to be formed during the production process.

C. Dietary Exposure to Contaminants, Byproducts and Other Bioactives

Potential contaminants of BIO-CAT's *B. subtilis* BS50 include microbes and heavy metals. The specifications set for BIO-CAT's *B. subtilis* BS50 place limits on the maximum permissible levels of these impurities to assure an acceptable final product. The batch data for four different lots document quality control of the final product such that it meets these specifications (Table 5).

PART 4. SELF-LIMITING LEVELS OF USE

There are no inherent self-limiting levels of use for *B. subtilis* BS50.

PART 5. EXPERIENCE BASED ON COMMON FOOD USE IN FOOD BEFORE 1958

The statutory basis for the conclusion of GRAS status of *B. subtilis* BS50 in this document is not based on common use in food before 1958. The GRAS conclusion is based on scientific procedures.

PART 6. NARRATIVE

A. Information on Dietary Exposure to *Bacillus subtilis*

Humans are inherently exposed to *B. subtilis*, given that the microbe can be isolated from water, soil, air and decomposing plant matter (Lefevre et al., 2017). Bacilli are reported to occur at population levels of 10^6 to 10^7 per gram of soil with 60-100% being in its inactive spore state (EPA, 1997). *Bacillus* counts of 10^6 CFU/g have been found in wheat, grain, and whole meal (Sorokulova, 2013). *B. subtilis* spores have been found in the gastrointestinal (GI) tract of humans who have not intentionally consumed *B. subtilis*-containing food or supplements (Tam et al., 2006; Hong et al., 2009; Fakhry et al., 2008).

B. subtilis has a long history of use in the food industry, especially in fermented food products marketed in Asian and African regions. Alkaline-fermented foods generated by bacterial cultures containing *B. subtilis* include Thai thua-nao and kinema from cooked soybeans, dawadawa from African locust beans, ugba from African oil beans, and orgiri from melon seeds (Wang and Fung, 1996). The traditional Japanese food “nattō” (fermented soybean) is made from soybeans fermented by *B. subtilis*. Nattō is believed to have been a component of the Japanese diet as early as the year 1450 (Shurtleff, 2012). At least three kinds of commercial nattō starter strains are available in Japan (Nishito et al., 2010). There are up to 1 x 10^9 viable spores of *B. subtilis*/gram of nattō product, the consumption of which has a long history of safe use and is associated with beneficial health effects (Cutting, 2011; Homma et al., 2006). The USDA nutrient databank (USDA, SR-28) states that there are 175 g in a serving of nattō. A person consuming one serving of nattō/day would therefore consume 1.75 x 10^{11} CFU *B. subtilis*/day (175 billion CFU/day) from this source only (USDA, 2019).

It is expected that exposure to *B. subtilis* from foods that have not been supplemented with the bacteria is low relative to the amount of *B. subtilis* that will be added to food per this GRAS determination, with the possible exception of consumers of nattō. It is unlikely, however, that a daily consumer of nattō would also be consuming foods containing *B. subtilis* BS50 at 90th percentile levels of intake.

B. Regulatory History

1. United States

a. GRAS Inventory

Five GRAS notifications for the use of *B. subtilis* in food have received no questions letters from FDA. One is pending (GRN 1007), and one was withdrawn (GRN 562) (Table 7). The highest estimated intake of *B. subtilis* in the successfully notified GRAS Determinations was up to 2.78 x 10^{11} CFU/day, for *B. subtilis* “Bss-19” spore preparation (GRN 969).

The GRAS status of carbohydrase and protease enzyme preparations sourced from nonpathogenic and nontoxigenic strains of *B. subtilis* was affirmed in 1997 (21 CFR 184.1148 and 21 CFR 184.1150). Subsequently, several enzyme preparations sourced from *B. subtilis* have been notified as GRAS for use in foods based on scientific procedures.

Table 7. Summary of *Bacillus subtilis* in FDA GRAS Inventory

Substance	GRN # / Closure Date	Intended Use	Use Rate	Company/ Reference	FDA Response
***Bacillus subtilis* strain R0179**	GRN 1007	For use in baked goods and baking mixes, beverage and beverage bases, breakfast cereals, chewing gum, confections and frostings, dairy product analogs, fruit and water ices, nuts and nut products, plant protein products, processed fruits and fruit juices, and snack food	Up to 1 x 10^{10} CFU/serving	Lallemand Health Solutions FDA (2022)	Pending
***Bacillus subtilis* "Bss-19" spore preparation ATCC SD-7780)**	GRN 969 Oct 6, 2021	For use in a number of conventional foods, excluding infant formula or USDA-regulated foods	Up to 1 x 10^{10} CFU/serving	Danisco USA, Inc. FDA (2021c)	FDA had no questions
***Bacillus subtilis* PLSSC (ATCC SD-7280)** (also known as BioSEB BS and SEBtilis)	GRN 956 Aug 18, 2021	For use in a number of conventional foods	Up to 6 x 10^{9} CFU/serving	Advanced Enzyme Technologies Ltd FDA (2021b)	FDA had no questions
***Bacillus subtilis* strain BS-MB40 PTA-122264 spore preparation**	GRN 955 Mar 26, 2021	For use in a number of conventional foods, excluding infant formula or USDA-regulated foods	Up to 2 x 10^{9} CFU/serving	BIO-CAT Microbials, LLC FDA (2021a)	FDA had no questions
***Bacillus subtilis* SG188 (DSM 32444)**	GRN 905 June 8, 2020	For use as an ingredient in beverages, such as milk drinks, protein high energy sports drinks, hot beverages and juices; and dry and shelf-stable products such as cereals, cookies, gums and confectionary	Up to 1 x 10^{9} spores per serving	SporeGen Ltd FDA (2020b)	FDA had no questions
Bacillus subtilis* DE111	GRN 831 Aug 13, 2019	For use in conventional foods and infant formula	Up to 1 x 10^{10} CFU/serving in foods intended for adults Up to 1 x 10^{9} CFU/serving in foods intended for children aged 2-12 Up to 2 X 10^{8} CFU/100 mL infant formula	Deerland Probiotics and Enzymes FDA (2019)	FDA had no questions
Bacillus subtilis	GRN 562	For use in post-harvest processing of bananas as an	6.3 x 10^{2} CFU/mL to 1.9 x 10^{3} CFU/mL	BiOWiSH Technologies,	FDA ceased to evaluate at

Substance	GRN # / Closure Date	Intended Use	Use Rate	Company/ Reference	FDA Response
		ingredient added to wash water		Inc. FDA (2014b)	notifier's request
Substances Produced via *B. subtilis*					
Pullulanase from *Bacillus deramificans* produced in *Bacillus subtilis*	GRN 861 July 21, 2020	For use as an enzyme in the saccharification of liquified starch in the production of dextrose and maltose syrups	Up to 186 mg TOS/kg raw material	GenScript/ Bestzyme FDA (2020a)	FDA had no questions
Maltogenic alpha-amylase from *Bacillus stearothermophilus* produced in *Bacillus subtilis*	GRN 751 July 31, 2018	For use in processing starch in food manufacturing	Up to 49.5 mg TOS/kg starch raw material	Novozymes North America, Inc. FDA (2018c)	FDA had no questions
Maltogenic amylase from *Geobacillus stearothermophilus* produced in *Bacillus subtilis*	GRN 746 June 13, 2018	For use in baking processes	Up to 20 mg Total TOS/kg flour	AB Enzymes FDA (2018b)	FDA had no questions
Subtilisin from *Bacillus amyloliquefaciens* produced in *Bacillus subtilis*	GRN 714 Feb 6, 2018	For use in the processing of protein at to facilitate protein hydrolysis	58-369 mg TOS/kg substrate	Danisco US Inc. FDA (2018a)	FDA had no questions
β-galactosidase enzyme preparation from *Bacillus circulans* produced in *Bacillus subtilis*	GRN 649 Nov 28, 2016	For use as a processing aid in the production of galacto-oligosaccharides (GOS)	Up to 0.3% of the lactose starting material	GenoFocus, Inc. FDA (2016b)	FDA had no questions
β-glucanase from *Bacillus subtilis*	GRN 592 Oct 7, 2015	For use as a processing aid in brewing and potable alcohol production	36.56 mg TOS/kg grist	Danisco US Inc. FDA (2015b)	FDA had no questions
Lactase from *Bifidobacterium bifidum* produced in *Bacillus subtilis*	GRN 579 Nov 5, 2015	For use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products	1.1 mg TOS/g milk 1.3 mg TOS/g GOS for use in infant formula	Danisco US Inc. FDA (2015a)	FDA had no questions
Asparaginase enzyme preparation produced by genetically modified *Bacillus subtilis*	GRN 476 Feb 3, 2014	As an enzyme in bread, potato, cereals, coffee and chocolate products, at a level of up to 20 milligram Total Organic Solids per kilogram of food	Up to 20 mg TOS/kg food	Novozymes North America, Inc. FDA (2014a)	FDA had no questions
1,4-α-glucan branching enzyme preparation from *Bacillus subtilis* strain 168 expressing the glucan branching enzyme gene from *Aquifex aeolicus* strain VF5	GRN 406 Sep 11, 2012	As an enzyme in the production of cyclic dextran and enzymatically-synthesized glycogen	0.07 mg TOS/ g substrate for cyclic dextran production 0.67 mg TOS/g substrate for glycogen production	Ezaki Glico Co., Ltd. FDA (2012a)	FDA had no questions
Branching glycosyltransferase enzyme preparation from *Bacillus subtilis* expressing a branching glycosyltransferase gene from *Rhodothermus obamensis*	GRN 274 Jun 25, 2009	As an enzyme in the starch industry to obtain dextrins with improved physical properties, such as higher solubility, lower viscosity, and reduced retrogradation	Up to 4%	Novozymes North America, Inc. FDA (2009)	FDA had no questions

Substance	GRN # / Closure Date	Intended Use	Use Rate	Company/ Reference	FDA Response
Pullulanase enzyme preparation from *Bacillus subtilis* expressing the pullulanase gene from *B. acidopullulyticus*	GRN 205 Dec. 4, 2006	As an enzyme in the brewing industry (to hydrolyze 1-6-alpha-D-glucosidic linkages in pullulan, amylopectin, and glycogen	Up to 25 L/ton of starch dry substance	Novozymes North America, Inc. FDA (2006a)	FDA had no questions
Pectate lyase enzyme preparation from *Bacillus subtilis*	GRN 114 Jan. 27, 2003	Use in fruit and vegetable purees and concentrates as an enzyme	0.5-1.0 % by weight	Japan Cellfoods Co., Ltd. FDA (2003)	FDA had no questions
Pullulanase derived from *Bacillus subtilis* carrying a gene encoding pullulanase from *Bacillus naganoensis*	GRN 20 Sep. 30, 1999	Use in hydrolyzing starch and starch-related compounds in the production of corn sweeteners, baked goods, and alcoholic beverages at minimum levels necessary to accomplish the intended effect in accordance with current good manufacturing practices	Minimum levels necessary to accomplish the intended effect in accordance with cGMP	Enzyme Bio-Systems Ltd. FDA (1999)	FDA had no questions

* Originally a member of the Bacillus subtilis subsp. *inaquosorum* group. This group has been reclassified to Bacillus *inaquosorum* (Oren and Garrity, 2020).
CFU – colony forming units; cGMP – current Good Manufacturing Practices; GOS – galacto-oligosaccharides; kg – kilogram; mg – milligram; mL – milliliter; TOS – total organic solids

b. New Dietary Ingredient Notifications

Seven New Dietary Ingredient Notifications (NDINs) for various *B. subtilis* strains have been submitted to FDA, one which (combined with *B. clausii*) has been accepted after initially being rejected at a higher usage rate (Table 8). In several cases, FDA was unable to establish the safety of the ingredient. Reasons cited for lack of approval include lack of information about identity, consumption, antibiotic resistance, colonization in the gastrointestinal tract, effect on normal gut flora, metabolites known to be produced by the particular strain, or potential for allergy.

A total of 290 dietary supplement products containing *B. subtilis* are mentioned on the National Institutes of Health (NIH) Dietary Supplement Label Database (National Institutes of Health, 2021). The majority of these products were blends that did not mention the recommended use levels of *B. subtilis*; however, where mentioned they ranged from 1×10^9 CFU/day to 5.0×10^9 CFU/day. A number of dietary supplements containing *B. subtilis* in combination with other live microbials are available for sale on the internet, and a few contained only *B. subtilis*. Recommended usage rates of two additional supplements containing only *B. subtilis* that were found on websites are 3.1×10^9 CFU/day and 1×10^{10} CFU/day (Life IRL, 2021; Simply Nutrients, 2021).

Table 8. Summary of *Bacillus subtilis* in FDA NDI Inventory

Substance	NDIN # / Date of FDA's Response	Recommended Daily Dose	Company/ Reference	FDA Response
Bacillus clausii and Bacillus subtilis, under tradename LiveSpo® COLON	NDI 1167 Dec. 15, 2020	3 billion CFU/day	Ana Bio Research & Development JSC FDA (2020e)	Notification accepted for filing
Bacillus subtilis	NDI 1159 Sept. 10, 2020	25 x 10^9 CFU/serving/day	Danisco USA, Inc. FDA (2020d)	Insufficient evidence to conclude that the strain was a dietary ingredient
Bacillus clausii and Bacillus subtilis	NDI 1138 March 23, 2020	6 billion CFU/day	Ana Bio Research & Development JSC FDA (2020c)	FDA was unable to establish the safety of the ingredient
Bacillus Subtilis Strain PB6 ATCC PTA-673	NDI 741 Jan. 30, 2012	95 billion CFU /serving/day	Kemin Pharma FDA (2012b)	FDA was unable to establish the safety of the ingredient
Bacillus subtilis PB6	NDI 477 July 31, 2008	1 x 10^9 to 1 x 10^{10} CFU/serving/day	Kemin Industries, L.C. FDA (2008)	FDA was unable to establish the safety of the ingredient
Bacillus Subtilis Strain DB9001	NDI 324 March 3, 2006	7.5 x 10^8 CFU/serving/day	BAU Inc. FDA (2006b)	FDA was unable to establish the safety of the ingredient
***Bacillus subtilis* DB9011**	NDI 277 May 27, 2005	16.5 mg/capsule 1-3 capsules/day	BAU Inc. FDA (2005)	FDA was unable to establish the safety of the ingredient

CFU – colony forming unit; NDI – New Dietary Ingredient; NDIN – New Dietary Ingredient Notification

c. Animal Feed

Under section 36.14 of the 2019 Association of American Feed Control Officials (AAFCO) Official Publication, *B. subtilis* is listed as a microorganism that was reviewed by the Food and Drug Administration, Center for Veterinary Medicine and found to present no safety concerns when used in direct-fed microbial products (AAFCO, 2021).

d. Pesticides

Several *B. subtilis* strains have been approved for use as biocides by the Environmental Protection Agency (EPA), and have been exempted from tolerances in food crops, including GB03; FMCH002, BU1814; MBI 600; CX-9060, QST 713, and QST 713 variant soil (EPA, 2008; EPA, 2017; EPA, 2018; EPA, 2009; 2012a; EPA, 2012b). In the Federal Register notice for the QST 713 variant soil exemption, the EPA stated that *B. subtilis* is not considered to be toxic or pathogenic to humans, animals or plants (EPA, 2012b).

2. Europe

The European Food Safety Authority (EFSA) confirmed a Qualified Presumption of Safety (QPS) Determination for the use of *B. subtilis* as an animal feed additive based on the absence of toxigenic potential (EFSA, 2013). The QPS approach requires the identity of the strain to be conclusively established, evidence that the strain is not toxigenic and that it does not show resistance to antibiotics of human and veterinary importance (EFSA, 2019a).

Using the QPS approach, the EFSA FEEDAP Panel concluded that *B. subtilis* DSM 28343 can be presumed safe for pigs for fattening, consumers of products derived from animals fed the additive and the environment, and consequently approved an intake of 2 x 10^8 CFU/kg complete feed for this species (EFSA, 2019a). The EFSA Panel also concluded that *B. subtilis* DSM 25841 is safe for use of feed for piglets (suckling and weaned), pigs for fattening, and sows by the same rationale. The approved usage rate for this strain is 5 x10^8 CFU/kg complete feed or 1.7 x 10^8 CFU/L of drinking water in all cases (EFSA, 2019b). In 2021, EFSA also used the QPS approach to approve *B. subtilis* DSM 32324 and 32325 for all animal species at a minimum inclusion level of 1 x 10^8 CFU/kg complete feeding stuff and *B. subtilis* CNCM I-4606, CNCM I-5043, and CNCM I-4607 spores at 1 x 10^9 CFU/kg dry feed (or liter liquid feed) (EFSA, 2021b; EFSA, 2021c; EFSA, 2021a).

3. Canada

B. subtilis is recognized by the Natural and Non-Prescription Health Products Directorate (NNHPD) of Health Canada as a Natural Health Product (NHP) ingredient under Schedule 1, Item 1 (bacterium) of the *Natural Health Product Regulations*. In order to sell NHPs in Canada, a Product License in the form of an eight digit Natural Product Number (NPN) must be issued by Health Canada. Thus, submission of a Product License Application (PLA) to the NNHPD is required. Only once Health Canada has reviewed and approved a PLA for safety, efficacy, and quality, is an NPN granted. This unique identifier (i.e., 8000XXXX) must appear on the label's Principal Display Panel (PDP). The Master File pathway precedes the PLA process and is a mechanism which enables manufacturers of raw materials or finished products to protect safety, efficacy, manufacturing, packaging, processing, and/or quality data. This proprietary information is held on file with the Government, preventing direct disclosure to the customer/Clinical Trial or Product License Applicant, while still permitting efficient investigation, approval, and registration.

Although the Master File is specific to NHP use, it should be noted that food enzymes produced by various strains of *B. subtilis* are also recognized as food additives in Canada (Government of Canada, 2021).

C. *Bacillus subtilis* Safety Evaluation (Other Strains)

B. subtilis is not considered pathogenic or toxigenic to humans, animals, or plants (EPA, 1997). Based on a review of literature citing human infections with *B. subtilis* (de Boer and Diderichsen, 1991), almost all cases of *B. subtilis* infection were related to drug abusers or debilitated patients.

In general, there was no evidence of any pathogenic potential of *B. subtilis* to healthy humans and very few examples of *B. subtilis* strains as confirmed causes of food poisoning.

In the FDA comments section for GRN 905 (FDA, 2020b), the authors stated “occasionally there are documented reports of what, prima facie, appears as a genuine [Bacillus subtilis] infection. For example, Jeon et al. (2017) describe a case of bacteremia following an esophageal perforation caused by *B. subtilis* and *B. licheniformis*. Similarly, a recent report (Gu et al., 2019) identified a strain of *B. subtilis* isolated from a deep-sea hydrothermal vent that has virulence potential in animals. In this case the precise mechanism whereby *B. subtilis* can invade vertebrate cells was not identified. As discussed by Harwood et al. (2018), *Bacillus* species can secrete molecules that have cytotoxic potential.” Harwood et al. (2018) also mentions, “although widely used commercial strains of *B. subtilis* and *B. licheniformis* produce well-characterized secondary metabolites (PKs and NRPs) and AMPs, there are no well-authenticated reports of human or animal toxicity associated with these compounds. Indeed, each year the Japanese consume ∼7 billion helpings of natto, a soybean-based food fermented using a surfactin producing natto variant of *B. subtilis*.” The ability of *B. subtilis* BS50 to produce secondary metabolites identified by Harwood et al. (2018) is discussed in Part 6D.

The authors of GRN 905 also stated “while it is possible that the strains involved may have carried unique features enabling pathogenicity, it does illustrate that even non-pathogenic microorganisms can under some occasions participate in potentially lethal infection requiring clinical treatment. Most importantly, these studies demonstrate the need to conduct safety analysis on a strain-by-strain basis.” As shown in Part 6D, results of *in silico* tests and a clinical study support the safety of *B. subtilis* BS50.

In a case report of two patients presenting with cholestatic hepatitis, pruritus, and/or cirrhosis after consumption of Herbalife® preparations, samples of the Herbalife® products ingested by both patients showed growth of *B. subtilis* (identified via sequencing of 16S rRNA and *gyrB* genes), likely from contamination by an environmental source (Stickel et al., 2009). Although causality between consumption of Herbalife® products and disease was scored ‘‘probable” in both cases, Gram-positive bacteria are extremely rare causes for liver injury. Further, the NIH has examined 50 cases of liver injury attributed to Herbalife® products and opined that the mechanism is unexplained (NIH, 2018). The clinical safety of preparations containing *B. subtilis* (discussed below) also supports the conclusion that the isolated case reports of hepatoxicity from *B. subtilis*-contaminated Herbalife® preparations do not give rise to safety concerns regarding the intended use of *B. subtilis* BS50.

1. Toxicology Data on *Bacillus subtilis* (Other Strains)

a. Safety studies in experimental animals

Oral toxicity studies with *B. subtilis* in rats, mice, rabbits, and piglets confirm the lack of adverse effects associated with repeated exposures to *B. subtilis*. The results of these studies support the

safety of several *B. subtilis* species, some of which have documented histories of commercial applications (Sorokulova et al., 2008) at anticipated consumer exposure levels from use as an ingredient in foods.

1. Studies in rats

Results of an unpublished 90-day oral toxicity study of *B. subtilis* PLSSC (SD-7280) spore preparation are described in pending GRN 956 (FDA, 2021b). In this Organization for Economic Cooperation and Development (OECD) Guideline 408 study, four groups of 7-8 week old Wistar rats (10/sex), were assigned to receive daily gavage doses of 0, 250, 500, and 1000 mg spore preparation/kg bw (providing 0, 0.41, 0.81, and 1.62 $x10^{11}$ spores/kg bw) for 90 days. Five rats/sex receiving 0 or 1000 mg spore preparation/kg bw/day were assigned to 28-day recovery groups. There was no mortality and no clinical abnormalities in rats given any dose of *B. subtilis* PLSSC. There was no effect of the test material on ophthalmology, sensory reactivity, grip strength, motor activity, feed intake or body weight gain, hematology, clinical chemistry, urinalysis, absolute or relative organ weights, or gross or histopathology. The NOAEL was 1000 mg/kg bw/day (the highest dose tested), providing 1.62 x 10^{11} spores/kg bw/day.

A 90-day repeated-dose oral toxicity study conducted according to Good Laboratory Practice and OECD Guideline 408 was performed in rats administered lyophilized spores of genetically modified strain *B. subtilis* ZB183 (Appala Naidu et al., 2019). The strain was created from parental strain *B. subtilis* PY79 and contained genes for acetaldehyde dehydrogenase from *C. necat*. Lyophilized spores at doses of 10^9, 10^{10}, and 10^{11} CFU/kg bw/day were administered by gavage to Wistar rats (10/sex/group) until study termination. *B. subtilis* ZB183 had no effects on clinical signs, mortality, ophthalmology, functional observational battery, body weight, body weight gains and food consumption in both sexes. There were no test item-related changes in hematology, coagulation, urinalysis, thyroid hormones, organ weights, gross pathology, or histopathology. The NOAEL was defined at the highest dose of 10^{11} CFU/kg bw/day for lyophilized *B. subtilis* ZB183 spores under the test conditions employed.

A 28-day oral (gavage) toxicity study of *B. subtilis* R0179 in rats was reported by Tompkins et al. (2008). *B. subtilis* R0179 was administered to 15 male and 15 female Sprague- Dawley albino rats at a single dose of 2 x 10^9 CFU/kg bw/day (vehicle not reported). A control group received an equal volume of the vehicle. Animals were monitored daily for mortality, morbidity, and clinical signs of toxicity. Body mass, food consumption, anatomic pathology, intestinal colonization, and infection were evaluated. The sensory reactivity to auditory, visual and proprioceptive stimuli, grip strength, and motor activity were also assessed. At the end of the treatment period, all animals were sacrificed and select organs (liver, kidneys, spleen, heart, and lungs) were subjected to histopathological and microbiological examination. Terminal portions of the small and large intestine from 4 animals/sex/group were removed for microbial examination of intestinal contents.

No clinical signs of toxicity or oral intolerance were reported in the study. There were no variations in body mass, food consumption, or mortality compared to the vehicle control group. There were no gross

lesions at necropsy or changes in organ weights with the exception of lower absolute heart weights reported for test article-treated females only; heart weights relative to body weight were not affected. The intestinal contents collected from treated animals were found to contain high levels of *B. subtilis*. The authors concluded that the results of this study in combination with the observations of clinical studies in both infants and adults indicate that these microbes are safe for use and pose low risk to the consumer (Tompkins et al., 2008).

The toxicity of *B. subtilis* MB40 was evaluated in a 14-day oral dose study in Sprague Dawley [Crl:CD(SD)] rats (Spears et al., 2021). Groups of 10 male and 10 female rats were administered *B. subtilis* MB40 (supplied as a spray-dried powder at an activity level of 1.85 x 10^{11} CFU/g) by gavage at doses of 500, 1000, and 2000 mg/kg bw/day using concentrations of 50, 100, and 200 mg/mL prepared in deionized water. The doses were equivalent to 9.25 x 10^{10}, 1.85 x 10^{11} and 3.7 x 10^{11} CFU/kg bw/day. Based on average initial body weights, the doses in terms of CFU/day were 2.18 x 10^{10}, 4.33 x 10^{10}, and 8.51 x 10^{10}. A vehicle control group was concurrently administered deionized water on the same daily dosing regimen as the test article-treated groups. Test article formulations were prepared daily. The protocol was designed in general accordance with FDA Redbook 2000 Testing Guideline IV.C.3.a, *Short-Term Toxicity Studies with Rodents*.

Animals were evaluated twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. Clinical pathology evaluations (hematology, coagulation, serum chemistry, and urinalysis) were performed on all rats at the scheduled termination. The animals were fasted overnight prior to blood collection while in metabolism cages for urine collection. Complete necropsies were conducted, and organ weights were measured for preselected organs. A standard listing of tissues and organs were collected from all animals for potential microscopic examination.

No mortality and no test article-related effects were reported for any of the aforementioned evaluated parameters at any dose of *B. subtilis* MB40. Some statistically significant differences in hematology, coagulation, and serum chemistry parameters were reported when the control and test article-treated groups were compared but were considered non-test-article related because they were not dose-dependent and were generally within the laboratory's historical range. Some statistically significant differences in organ weights, absolute and/or relative, were reported when the control and test article-treated groups were compared but were considered non-test article-related because of the lack of a dose-response and because group means were within the laboratory's historical control range. The NOAEL for *B. subtilis* MB40 in this study was 2000 mg/kg bw/day (equivalent to 3.7 x 10^{11} CFU/kg bw/day or 8.51 x 10^{10} CFU/day), the highest dose tested.

Recently, the acute toxicity of *B. subtilis* IDCC 1101 was tested in female SD rats (9 or 10 weeks old). The rats (3/group/age) were administered 3.09 x 10^{10} CFU/ kg bw or 2.06 x 10^{11} CFU/ kg bw orally (presumably by gavage) or after a 16 hr fast and were weighed and observed for clinical signs for 14 days (Kim et al., 2022). After 14 days, all animals were fasted for 12 hr, humanely euthanized and examined for gross pathological changes. There was no effect of the test material on body weight, gross pathology or clinical condition.

2. Studies in other species

The effect of *B. subtilis* 18 (BS-18) on intestinal health of 15-day old mice was studied by Li et al. (2019). Groups of 10 KM mice (5/sex) were necropsied after administration of 0 or 1×10^9 CFU/day BS-18 for 18 days and the intestine (duodenum, jejunum, ileum, and cecum), liver, spleen, and kidney were analyzed macroscopically and microscopically. The diversity of bacteria in the intestine was also examined. The mice exhibited no abnormal behavior during the treatment period and no pathological lesions were observed in tissues that were examined after necropsy. There also were no adverse effects on the microbiome of the intestine or on body weight.

A 10-day oral (gavage) toxicity study of *B. subtilis* VKPM B2335 (BS3) was conducted in male BALB/c mice, male New Zealand white rabbits, newborn piglets (strain and sex not reported) (n=10/species) and a separate 30-day study was performed with rabbits (n=20) (Sorokulova et al., 2008). *B. subtilis* VKPM B2335 (BS3) was administered at a single dose (1.0×10^6 CFU/day for mice; 1.0×10^9 CFU/day for rabbits and piglets) in sterile phosphate buffered saline. An additional 10 animals/species received the vehicle alone in each study. The animals were observed for activity and behavior and histopathological evaluation of select tissues and organs was conducted after euthanasia. Blood samples were collected from rabbits by cardiac puncture on days 10 and 30 and evaluated for hematology parameters. Leukocytes were counted to determine the differential percentages of white blood cells (lymphocyte, monocytes, eosinophils, and heterophils). Total red blood cells, sedimentation rate and hemoglobin concentration were determined. Hematology parameters were not evaluated in mice or piglets.

There were no adverse effects on the general health status of the animals, and no changes in the organs and tissues of treated animals were reported. There were no differences in the hematological indexes measured in the blood from control and treated rabbits. The authors concluded that the test strain of *B. subtilis* (VKPM B2335; BS3) "may therefore be considered as non-pathogenic and safe for human consumption" (Sorokulova et al., 2008).

Hong et al. (2008) conducted a 30-day gavage study of *B. subtilis* Nattō in six male New Zealand White rabbits at a single dose of 1.0×10^9 CFU/day. A naïve control group received the vehicle (saline) at the same volume (1 mL/day). Blood samples for hematological evaluation (total red blood cells, leucocytes, hemoglobin concentration, and differential percentages of white blood cells) were collected by cardiac puncture from anaesthetized animals on day 30 and select tissues and organs were collected for histopathological examination after euthanasia, including liver, kidneys, spleen, small intestine, and mesenteric lymph nodes. In a separate acute single-dose study conducted by the same authors, groups of 5 male and female Harley Dunkin guinea pigs were administered a 1 ml dose of *B. subtilis* Nattō at 1.0×10^{12} CFU or the vehicle (saline) and observed for 14 days. Animals were observed daily for behavior, appearance, activity and feces. Body weights were recorded on days 0, 7, 14, and 17. On day 17, blood was drawn (by cardiac puncture from anaesthetized animals) for hematological analysis (same parameters as 30-day study). Select

tissues and organs were collected for histopathological examination after euthanasia including liver, kidneys, spleen, small intestine, and mesenteric lymph nodes.

There were no reported adverse effects on the general health status or feed intake of rabbits administered *B. subtilis* Nattō at 1.0 x 10^9 CFU/day for 30 days. No changes in selected visceral organs and tissues were reported and no significant differences in the hematological indexes were reported in treated rabbits compared to controls. In the acute toxicity study, a statistically significant higher weight gain in female guinea pigs administered 1.0 x 10^{12} CFU *B. subtilis* Nattō was reported on day 14 (but not days 7 or 17), while feed intake was unaffected in both males and females. Histological analysis of organs and tissues revealed no signs of inflammation or pathological changes and no differences in the hematological indices between control and treated groups. The authors concluded that "*Bacillus subtilis* appeared to show no sign of toxicity or virulence using *in vivo* assessments" (Hong et al., 2008).

b. Feeding studies in livestock

Several studies have been conducted in pigs and rabbits to assess the effect of *B. subtilis* on performance. The results show that up to 1.3 x 10^8 CFU/day *B. subtilis* has no effect on performance of rabbits, that up to 1.8 x 10^9 CFU/day during gestation and 6.2 x 10^9 CFU/day during lactation has no effect on reproduction or development of pigs, and that up to 3.1 x 10^8 CFU/day or 1.1 x 10^9 CFU/day has no effect on the performance of piglets or fattening pigs, respectively. Results of these studies are summarized in Table 9.

Table 9. Results of *Bacillus subtilis* Studies in Livestock

Species	Concentration/ Dose/Duration	Endpoints Measured	Results	Reference
Weaned piglets experimentally infected with an enterotoxigenic *E. coli*	*Bacillus subtilis* DSM 32540 (1 × 10^9 CFU/kg feed, approx. 7.2 × 10^8 CFU/day based on overall ADFI of 742 g/day) for 28 days in infected animals	BW, ADG, ADFI, GTF, diarrhea score, total and differential WBC, TNF-α and haptoglobin, intestinal morphology, bacterial translocation to mesenteric lymph nodes and spleen, microbial count in intestine hemolytic coliforms	No adverse effect on any parameter measured compared to infected controls	He et al. (2020a)
Weaned piglets experimentally infected with an enterotoxigenic *E. coli*	*Bacillus subtilis* DSM 32540 (2.56 × 10^9 CFU/kg feed, approx. 1.5 × 10^9 CFU/day based on overall ADFI of 598 g/day) for 28 days in infected animals	BW, ADG, ADFI, GTF, diarrhea score, alertness score, hematology, TNF-α and haptoglobin, intestinal morphology; bacterial translocation to mesenteric lymph nodes and spleen, microbial count in intestinal hemolytic coliforms	No adverse effect on any parameter measured compared to infected controls	He et al. (2020b)

Species	Concentration/ Dose/Duration	Endpoints Measured	Results	Reference
Pigs (sucking)	*Bacillus subtilis* PB6 0 or 2 × 10^9 CFU/kg formula powder[3] (3.1×10^8 CFU/day)[1] 21 days	BW, ADG, ADMI, FCR, intestinal morphology, weight of heart, liver, spleen, kidney, pancreas and intestine, differential white blood cell count, plasma immunoglobulins and cytokines, digestive enzyme activities, bacteria in colonic digesta, expression of genes associated with innate immunity in ileal tissue	No adverse effect on any parameter measured	Hu et al. (2017)
Pigs (pregnant sows and offspring)	*Bacillus subtilis* C-3102 0 or 3×10^5 CFU/g feed (8.4×10^8 CFU/day)[2]	Reproductive performance for two generations, body condition, feed consumption, BW, fecal bacteria	No adverse effect on any parameter measured	Kritas et al. (2015)
Weaned piglets	Control diet or diet containing a multi-strain *B. subtilis*-based DFM (CDFM) (United Animal Health, Sheridan, IN) comprised of a dried spore preparation Dose at least 7.35 × 104 CFU/g of complete feed for 42 days	Mortality, ADG, ADFI, GTF, individual amino acid digestibility in ileum, jejunum and ascending colon, GE, nitrogen and total amino acid digestibility among segments of the GI tract, colonic pH	No adverse effect on any parameter measured	Lewton et al. (2021)
Pigs (pregnant sows and offspring)	*Bacillus subtilis* C-3102 0 or 5×10^5 CFU/g gestation feed plus 1×10^6 CFU/g lactation feed 0 or 5×10^5 CFU/g nursery feed (1.2×10^9 CFU/day during gestation, 6.2×10^9 CFU/day during lactation and 3×10^8 CFU/day during the nursery period)[3]	ADG, ADFI, BW, fecal consistency, fecal microbes, litter size and weight, number of piglets total born, born alive, stillborn, and mummies, pre-wean mortality	No adverse effect on any parameter measured with the exception of ↓ ADG and ADFI in late nursery period in piglets born from treated sows. There was, however, no effect of sow dietary treatment on piglet gain: feed during this period.	Menegat et al. (2019)

Species	Concentration/ Dose/Duration	Endpoints Measured	Results	Reference
Weaned piglets	*Bacillus subtilis* plus *Bacillus licheniformis* (strains not stated) 0.01% in feed or control feed (20/group) for 14 days. Dose of *Bacillus subtilis* in terms of CFU/day cannot be determined from available information	BW, ADG, ADFI, GTF, diarrhea score, inflammatory cytokines, WBC, histology of GI tract	No adverse effect of test substance on any parameter measured.	Mun et al. (2021)
Weaned piglets experimentally infected with an enterotoxigenic *E. coli*	*Bacillus subtilis* DSM 32540 (1.3×10^6 CFU/g feed, approx. 5.2×10^8 CFU/day based on overall ADFI of 598 g/day) for 21 days in infected animals	BW, ADG, ADFI, GTF, fecal score, frequency of diarrhea, intestinal morphology, liver, stomach, small intestine, cecum, colon and spleen weight, pH and VFA content of cecal digesta	No adverse effect on any parameter measured compared to infected controls	Park et al. (2020)
Fattening pigs	*Bacillus subtilis* DSM 5750 spores plus *Bacillus licheniformis* DSM 5749 (1×10^9 CFU/g each strain) at 400 g/tonne (1000 kg) from 78 days of age until marketing to the slaughter plant. Dose of *Bacillus subtilis* is 1.1×10^9 CFU/day based on ADFI of 2.48 kg/day	Mortality, FCR, ADG, ADFI, length of fattening period, carcass characteristics	No adverse effect of test substance on any parameter measured. The results of microbiological analyses of colon sections from the pigs did not indicate any presence of pathogenic or potentially pathogenic microorganisms.	Rybarczyk et al. (2021)
Pigs (pregnant sows and offspring)	*Bacillus subtilis* 541 (5×10^8 CFU/kg feed) or control. Dose of *Bacillus subtilis* is 1.8×10^9 CFU/day and 2.9×10^9 CFU/day during gestation and lactation, respectively, based on ADFI of 2.6 kg/day and 5.8	Sows: ADFI, BW, and backfat thickness, litter size, numbers of born alive and weaned piglets, milk composition, immunoglobulin concentrations in milk and serum Offspring: BW (birth, after cross-fostering, at weaning), ADG, mortality rate, creep feed intake, loss rate	No adverse effect of test substance on any parameter measured	Saladrigas-García et al. (2022)

Species	Concentration/ Dose/Duration	Endpoints Measured	Results	Reference
	kg/day during these periods. Respective diets were tested over three reproductive cycles			
Weaned piglets	Control diet or diet containing 0.2% lactylate, 0.05% *Bacillus subtilis* strain mixture (B. 747 + B. 1999 from Certillus™, Waukesha, WI) or lactylate plus *Bacillus subtilis* for 42 days	BW, ADG, ADFI, GTF, fecal Clostridium, *E. coli,* and *Streptococcus suis*, plasma lactylates, complete blood cell count	No adverse effect of *B. subtills* on any parameter measured. Increased lymphocytes in *B. subtilis* group compared to control. No significant clinical symptoms of diseases were observed throughout the study except for minor postweaning scours (diarrhea). Scours were not associated with any of the treatments. Two pigs (none from the *B. subtills* group) were removed from the trial due to illness	Wang et al. (2021)
Rabbits (8 weeks old)	4×10^9 CFU/g 0, 200, 400 g /ton feed (5×10^7 or 1.3×10^8 CFU/day)[4] 56 days	FC, BW, BW gain, FCR, carcass characteristics, serum cholesterol, hemoglobin, RBC, platelets, cell-mediated immunity	No adverse effect on any parameter measured	Fathi et al. (2017)
Rabbits (28 days old)	0 or 1×10^6 CFU/g feed (5×10^7 CFU/day)[5] 42 days	ADFI, BWG, FCR, performance index, fecal score, intestinal bacteria and VFA, feed digestibility	No adverse effect on any parameter measured	Phuoc and Jamikorn (2017)

[1] Calculated using stated CFU/kg powder, average initial body weight (2.69 kg) and average daily dry matter intake from Days 1-7 (154 g/day)

[2] Calculated using stated CFU/kg feed and feed consumption of 2.8 kg/day from 65th day of gestation to farrowing

[3] Calculated using stated CFU/kg feed and ADFI in sows of 2.4 kg/day during weaning and 6.2 kg/day during lactation and overall ADFI in offspring of 600 g/day during nursery period

[4] Calculated using 907 kg/ton feed, stated CFU/g microbial, g feed consumed over study (3193.1 and 3987.1 g feed consumed in low and high dose groups, and 56 study days

[5] Calculated using stated CFU/g feed and ADFI of 48.42 g/day from Days 28-42.

ADFI – average daily feed intake; ADG – average daily gain, ADMI – average daily dry matter intake; BW – body weight, CFU – colony forming units; FC – feed consumption; FCR – feed conversion ratio; GE – gross energy; GI – gastrointestinal; GTF – gain to feed ratio; RBC – red blood cell count; TNF-α – tumor necrosis factor alpha; VFA – volatile fatty acids; WBC – white blood cell count

2. Clinical Safety Data on *Bacillus subtilis* (other strains)

A number of clinical studies have been performed with *B. subtilis*, and, for the purpose of this dossier, we have focused on any discussion of potential adverse effects associated with their intake. The results of the studies show that *B. subtilis* is safe in humans at up to 4.8×10^{10} CFU/day for 28 days (the longest period administered in a clinical study at this highest dose studied).

a. *B. subtilis* MB40

In a single-blind, placebo lead-in study, the safety and tolerability of OPTIBIOME™ *B. subtilis* MB40 was evaluated in normal, healthy adult volunteers (Spears et al., 2021). Thirty subjects were enrolled, and 27 subjects (12 males and 15 females) completed the study. Subjects were initially given two placebo capsules per day for 7 days (placebo: 250 mg capsules containing only maltodextrin and other excipients). Subjects then received two test capsules per day for 21 days (study product: 250 mg capsules containing 20 billion CFU/g of *B. subtilis* MB40 [5 billion CFU/capsule] with maltodextrin and excipients). The total daily dose of *B. subtilis* MB40 during the treatment period was 10 x 10^9 (10 billion) CFU/day. Subjects received a total of 42 doses of the study product, *B. subtilis* MB40, throughout the duration of the study. Three subjects discontinued participation from the study after week 1 (two subjects) and week 2 (one subject) due to non-compliance with test product and completion of the study forms. The overall test product compliance of the subjects that completed the study was 99.2% ± 3.3%.

There were no clinically significant changes as a result of study product administration based on physical exam findings, clinical laboratory tests, and vital signs and no Serious Adverse Events (SAE) were reported during the study. There were five reported adverse events (AE) during the study, all graded as mild. Three cases of viral upper respiratory infection were reported by three different subjects and ascribed as not likely related to the administration of the study product. Two AE, a case of vomiting and chills both reported by the same subject, were ascribed as likely related to the administration of the study product; however, these transient symptoms occurred during the middle of the 21-day treatment period and resolved within 31 hours. There were no significant changes in the total number of bowel movements per subject per week between the placebo week (average of 11.1 ± 4.6) and the three subsequent treatment weeks (week 2: 10.7 ± 3.6; week 3: 10.7 ± 3.8; week 4: 11.2 ± 4.3) with *B. subtilis* MB40 administration, and each subject's Bristol Stool Chart was consistent across all of the study weeks. Symptoms reported on daily GI Questionnaires during the treatment period generally occurred with similar or lower incidence and severity compared to the placebo week. The administration of OPTIBIOME™ *B. subtilis* MB40 at 10 x 10^9 (10 billion) CFU/day for 21 days to 27 healthy volunteer subjects was concluded to be safe and well tolerated.

In a multi-center, randomized, double-blind, placebo-controlled, parallel-arm study, the efficacy and safety of OPTIBIOME™ *B. subtilis* MB40 on abdominal discomfort, gas and bloating was evaluated in a healthy adult population (Penet et al., 2021). Following a two-week run-in period, participants received either a single capsule containing 5 x 10^9 CFU of *B. subtilis* MB40 plus excipients

(maltodextrin, magnesium stearate, gelatin and silicon dioxide) or a single placebo capsule containing only the excipients, once daily for 28 days. One hundred participants with an age range of 18-75 years were enrolled, and 75% of the participants were female. Data from 99 participants were analyzed in the Intent-to-Treat population (ITT; MB40, n=50; placebo, n=49), data and from 91 participants were analyzed in the Protocol Compliant Population (MB40, n=45; placebo, n=46). None of the subjects withdrew because of adverse effects of treatment. Overall product compliance was 100%.

The OPTIBIOME™ *B. subtilis* MB40 product was tolerated well among study participants. There were no adverse effects of treatment on any GI parameter evaluated. With respect to the safety analysis, all laboratory measures of complete blood count with differential, hematology, electrolyte count, liver and kidney function tests, and vitals remained within clinically normal levels during this study. Thirty AE were reported by 22 participants in this study. Of these, 13 were reported by participants in the MB40 group and 17 were reported by participants in the placebo group. Of the 13 AE reported by those in the MB40 group, 8 were possibly related to the product: abdominal discomfort (1), constipation (3), diarrhea (1), dry mouth (1), flatulence (1), and increased appetite (1). All other AE were assessed as unlikely or not related to the product. Of the 17 AE reported by those in the placebo group, five were possibly related to the product: abdominal discomfort (1), constipation (2), infrequent bowel movements (1), and paresthesia (1). All other AE were assessed as either unlikely or not related to the product. All AE resolved before the end-of-study.

b. *B. subtilis* R0179

In a randomized, double-blind, placebo-controlled trial, healthy adults (n=81; 18-50 years old) received *B. subtilis* R0179 at doses of 0.1, 1.0 or 10 x 10^9 CFU/capsule daily for four weeks (Hanifi et al., 2015). The test article was comprised of 75% *B. subtilis* R0179 in spore form and 25% in vegetative form. Participants were instructed to consume one capsule/day at the end of a meal. General wellness was assessed using a daily questionnaire evaluating GI, cephalic, ear-nose-throat, behavioral, emetic, and epidermal symptoms. GI symptoms were further evaluated using a weekly gastrointestinal symptom rating scale (GSRS). GI transit viability of *B. subtilis* R0179 was assessed by plating and microbiota analysis by 16S rRNA at baseline, week 4 of the intervention and washout.

There were no reported AE related to consumption of the study product. General wellness and GI function were not affected by oral consumption of *B. subtilis* R0179 at any dose. Daily questionnaire syndrome scores were not different from baseline and did not exceed a clinically significant score of 1. GSRS syndrome scores were not different from baseline and ranged from 1.1±0.1 to 1.9±0.2. Fecal viable counts of *B. subtilis* R0179 were statistically significantly higher compared to the placebo group and demonstrated a dose response. The authors concluded that "*Bacillus subtilis* R0179 survives passage through the human GI tract and is well tolerated by healthy adults at intakes from 0.1 to 10 x 10^9 CFU/day".

In an 18-week, randomized, double-blind, crossover study, healthy adults (n = 114, 53±8 years) with a high waist circumference underwent a 1-week pre-baseline period and were then randomized to receive 1 capsule/ day of *B. subtilis* R0179 (2.5×10^9 CFU/capsule; n=39), *Lactobacillus plantarum* HA-119 (5×10^9 CFU/capsule; n=38), *Bifidobacterium animalis* subsp. *lactis* B94 (5×10^9 CFU/capsule; n=37) or placebo for 6 weeks (n = 18-20 per group) (Culpepper et al., 2019). There were six groups – one group per strain and three placebo groups (one per strain). Following a 3-week washout and second pre-baseline week, participants were crossed to the other intervention (strain or placebo) for 6 weeks followed by a 1-week post-intervention period. Participants who received a strain during the first intervention were crossed over to the placebo and those who received the placebo initially were crossed over to their respective strain. Blood and stool samples were collected at the beginning and end of each intervention to measure bile acids, serum lipid profiles, and glucose and insulin levels. Data from the placebo intervention were combined for all participants for analyses. In a subgroup of participants with body mass index (BMI) ≥30, but not the total study population, the difference (final-baseline) in the sum of deconjugated plasma bile acids was greater with consumption of *B. subtilis* (691±378 nmol/l, *P*=0.01) and *B. lactis* (380±165 nmol/l, *P*=0.04) than with placebo (98±176 nmol/l, n=57). No significant differences were observed for any group for stool bile acids, serum lipids, blood glucose, insulin or white blood cell numbers or percentages. Differences in GI symptoms measured by a GSRS or bowel movement frequency were not observed between any of the three interventions compared with the placebo group. On average (mean ± standard error of the mean), nausea, vomiting, or stomach upset was reported by 6.7±1.8 participants on 16.7±4.7 days when on placebo and by 6.0±1.5 participants on 16.0±2.0 days when on the intervention. Headaches were reported by 9 participants during the placebo arms and by one participant receiving *B. subtilis*. The reason for the effect of *B. subtilis* on deconjugated bile acids in obese individuals is unclear but there was no mention by the study authors that this effect was adverse.

Tompkins et al. (2010) published a review of 24 clinical investigations and 3 case studies with Medilac® formulations containing *B. subtilis* R0179 and *E. faecium* R0026 (ratio 1:9). Male and female study participants with ulcerative colitis, diarrhea, irritable bowel syndrome, and other gastrointestinal conditions were included in these studies. No adverse reactions were directly linked to the use of Medilac® formulations.

Total enrollment ranged from 34 to 352 subjects in each study with an overall median enrollment of 56 subjects. The median age in the treatment groups ranged from 27 to 65 years. The dose regimen in nearly all of the reviewed studies was two capsules three times/day, resulting in approximately 3.0×10^9 CFU/day for 5 days to 12 weeks, with the exception of one study in which the subjects received 1.5×10^9 CFU/day for 2 weeks. The basis for the selection of doses administered in the 27 studies reviewed was not described in this publication; however, all studies were reported to be investigator or institution-initiated, post-market clinical trials evaluating efficacy of supplementation (Tompkins et al., 2010).

A study in critically ill patients performed after publication of the Tompkins review reported no adverse effects of three times/day treatment with one capsule of Medilac-S® (total dose of *B. subtilis*

and *E. faecium*,1.35 x10^{10} and 1.5 x10^{9} CFU/day, respectively) for up to 14 days (Zeng et al., 2016).

c. *B. subtilis* C-3012

A double-blind, randomized, placebo-controlled trial was conducted to assess the effect of *B. subtilis* C-3102 on chronic diarrhea in healthy volunteers with loose stools (Hatanaka et al., 2018). The subjects (n=44/group) received three tablets/day of a placebo or *B. subtilis* C-3102 spores (total of 2.2×10^{9} CFU/day) for a total of eight weeks. Evaluations included Bristol stool scale, a physician-conducted GSRS, a subject's perception of general health questionnaire, and water and microbial analyses of feces. Two subjects in the placebo group and four in the *B. subtilis* C-3102 group dropped out of the study – none for intolerance to their designated treatment. Compliance was good – 99.4% in the placebo group and 99.7% in the *B. subtilis* C-3102 group. There were no adverse effects of treatment on any parameter measured in the study, and there was no mention of any adverse events.

Hatanaka et al. (2020) recently performed a double-blind, randomized, placebo-controlled trial to determine whether ingestion of 4.8×10^{10} CFU/day *B. subtilis* C-3102 for 28 days was safe for healthy adults. The subjects (n=44) were equally divided into the treatment and placebo groups. Safety parameters, including physical examination, urinalysis, hematology, clinical chemistry, and bone mineral density (BMD) were measured at baseline, 2 and 4 weeks. Adverse events were recorded in a medical questionnaire administered by a clinical trial physician and daily reports written by the subjects. All subjects completed the study without violating the protocol and their rates of consumption were >90 %. There were no statistically significant differences in urinalysis, BMD or adverse events between groups. Statistically significant differences were noted in values of some parameters between the *B. subtilis* C-3102 and placebo groups; however, they were not considered toxicologically relevant because they were transient and/or within stated reference ranges. These include increases in systolic blood pressure and mean corpuscular hemoglobin level and decreases in body fat percentage, cholinesterase, total cholesterol, and triglyceride levels at two weeks and an increase in direct bilirubin and a decrease in total cholesterol at 4 weeks. It is altogether possible that the statistically significant differences in blood pressure denoted at 2 weeks and direct bilirubin at 4 weeks are erroneous, because the values for systolic blood pressure in the two groups differed by less than 1 mm Hg (117.1 ± 14.8 mm Hg in the treatment group versus 116.4 ± 18.0 mm Hg in the placebo group) and the values for direct bilirubin were equal (0.1 ± 0.0 mg/dL in both groups).

d. *B. subtilis* CU1

The effect of *B. subtilis* CU1 on immune stimulation and resistance to common infectious disease episodes was tested in healthy, free-living seniors (age 60-74) in a randomized, double-blind, placebo-controlled, parallel-arm study (Lefevre et al., 2015). Results of safety tests are reported in a different publication (Lefevre et al., 2017). Subjects (50/group) consumed either the placebo or the test material (2.1x 10^{9} *B. subtilis* CU1 spores daily) for 10 days, followed by 18 days without

consumption of the study products (break period). This scheme was repeated four times during the 16-week study. Blood was collected at baseline (1-2 weeks before the start of the study) and at week 16 for hematology and evaluation of liver and kidney markers. Hemodynamic parameters, including arterial pressure and heart rate, were evaluated on the first day of the study (prior to test material consumption), halfway through the study (Day 56), and at the end of the study. Symptoms of gastrointestinal and upper/lower respiratory tract infections were recorded daily by the subjects. Blood, saliva and stool samples were collected in a predefined subset of the first forty-four subjects enrolled in the study (22/group) for analysis of Immunoglobulin A (all samples) and cytokines (blood only). *B. subtilis* CU1 was found in stool of treated, but not control subjects. None of the subjects withdrew from the study after treatment start. There were no differences between groups in the number of subjects experiencing at least one adverse event or the likelihood of the adverse events being associated with study participation. Three events in the treatment group were possibly associated with participation in the study (2 incidents of nasal obstruction episodes in the same subject and one report of headache in another subject), and one event was likely related (mild pain for about 10 min after test capsule consumption) but remained an isolated event. In the placebo group, one event (a headache that appeared minutes after taking the test product and disappeared over the course of the day) was possibly related to study participation. All adverse events related to treatment in both groups were mild in severity. There was no effect of treatment with the test material on hematology, markers of liver or kidney toxicity or hemodynamics. The authors concluded that the test material was safe and well tolerated.

e. *B. subtilis* MY02

Wauters (2021) published results of a randomized, double-blind, placebo-controlled trial of a 1:1 combination of *B. coagulans* MY01 and *B. subtilis* MY02 (5×10^9 CFU total/day) in subjects (≥ 18 years of age) with functional dyspepsia as part of a Doctoral Dissertation. Subjects consumed the *B. subtilis* (n=32) or placebo (n=36) for 8 weeks under the double-blind procedure, followed by an open-label extension phase of 8 weeks with the microbial combination. Symptoms (daily diary), immune activation and fecal microbiota were determined. There were no adverse effects of *B. subtilis* on the outcome measures. The number of patients with adverse events was lower in the *B. subtilis* (5/32 (16%)) than the placebo (12/36 (33%)) group. Two serious adverse events occurring during the open-label phase (appendicitis and syncope) were assessed as unlikely related to the study product.

D. *Bacillus subtilis* BS50 Safety Evaluation

Using *in silico* analyses, the *B. subtilis* BS50 genome has been analyzed for the ability to produce secondary metabolites, secreted proteins, virulence factors, toxins, mobile elements, as well as genes coding for antibiotic resistance. The strain also has been tested for antibiotic resistance and the ability to cause cytotoxicity. The results of these studies have been published (Brutscher et al., 2022) and are discussed below.

1. Complete Genome Sequencing and Strain Lineage

Complete genome sequencing was conducted on isolated *B. subtilis* BS50 colonies in order to perform DNA sequence-based testing for potential risk ranging from antibiotic resistance to toxin production. As discussed in Part 3, the complete genome sequence is available upon request and could be provided electronically.

2. Genome Analysis - Production of Secondary Metabolites

To determine if *B. subtilis* BS50 has the capacity to produce secondary metabolites, the *B. subtilis* BS50 genome was inputted into the online database antiSMASH bacterial database (version 6.0.1) (accessed January 18, 2022). It was predicted that *B. subtilis* BS50 can produce seven different secondary metabolites, three of which (surfactin, fengycin and bacilysin) are produced by *B. subtilis* MB40, which has been determined GRAS (Table 10). As mentioned in Harwood et al. (2018), surfactin, plipastatin/fengycin, bacillibactin, bacillaene and bacilysin are produced by 99%, 97%, 99%, 77% and 93% of *B. subtilis* strains tested. Subtilosin A is also produced by several *B. subtilis* strains, including Strain 22a, a wild type strain of *B. subtilis* isolated from a fermented soybean product (Stein et al., 2004; Zheng et al., 1999). As shown in Bolocan et al. (2017), all four strains of *B. subtilis* and no other species isolated from a mushroom substrate (including *Lactococcus lactis*, *B. licheniformis* and *B. sonorensis*) produce subtilomycin. Because all secondary metabolites produced by *B. subtilis* BS50 are produced by other species of *B. subtilis* this property should be considered intrinsic. It is recognized that the ability of *B. subtilis* to produce secondary metabolites (some of which have antibiotic activity) contributes to their survival in their natural environment (Stein, 2005).

Table 10. Summary of BS50 Secondary Metabolites Predicted via antiSMASH

Cluster type	Most similar known cluster	Similarity
NRPS	Surfactin	78%
NRPS	Fengycin	100%
NRPS	Bacillibactin	100%
Other	Bacilysin	100%
Polyketide + NRP	Bacillaene	100%
RiPP: Thiopeptide	Subtilosin A	100%
RiPP: Thiopeptide	Subtilomycin	100%

NRP – Non-ribosomal peptide; NRPS- Non-ribosomal peptide synthase; RiPP - Ribosomally synthesized and posttranslationally modified peptide

B. subtilis is reported to produce 66 antibiotics, with 4-5% of its genome devoted to antibiotic synthesis (Sorokulova, 2013; Stein, 2005). Lantibiotics (peptide antibiotics) are among the many antimicrobial substances produced by members of the *Bacillus* genus (Lee and Kim, 2011; Mora et al., 2011). Lantibiotics are used in food preservation, but not orally administered as a treatment in human or veterinary medicine due to a lack of functional stability. These peptides are rapidly degraded through the digestive process rendering them of little use when orally administered

(Edwards et al., 1999; Hansen, 1994). There is no indication that *B. subtilis* produces antimicrobial substances that are used in medical or veterinary medicine and could potentially disrupt the normal intestinal microflora (Pariza et al., 2015).

A cross-streak screening experiment was performed to determine if *Bacillus subtilis* BS50 could affect growth of the common gut bacteria *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* (*Lacticasesibacillus rhamnosus*), and *Lactobacillus plantarum* (*Lactiplantibacillus plantarum*) (Figure 5). All strains were suspended in water to the equivalent of a 0.5 McFarland turbidity standard. *Bacillus subtilis* BS50 was streaked down the center of the agar plate, and the plate was incubated at 35°C to allow the organism to grow. The other organism(s) were then streaked perpendicular to the middle streak starting at the middle streak and the plate was incubated again. If any of the substances produced by the *Bacillus subtilis* BS50 (the middle streak) were deleterious to the growth of the other organisms, clear inhibition zones adjacent to the middle streak would be observed. Because the perpendicular streaks grew all the way up to the BS50 middle streak, there was no inhibition. The results confirm that growth of common gut bacteria is not inhibited by the presence of *Bacillus subtilis* BS50.

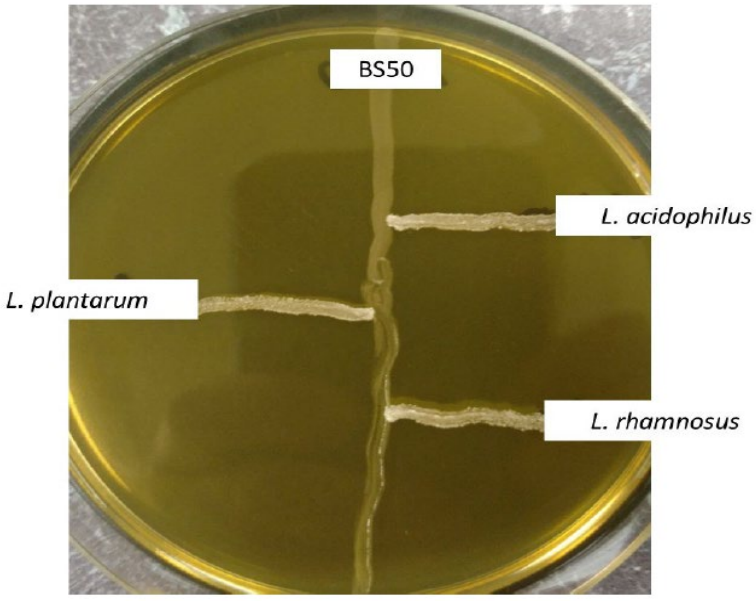

Figure 5. Cross-streak Plate with BS50 and Common Gut Bacteria

3. Genome Analysis – Virulence Factors

To assess if *B. subtilis* BS50 encodes for virulence factors (VF), the virulence factor database[5] was accessed online (January 17, 2022) and the "full dataset" of VF protein sequences was downloaded. The "full dataset" includes amino acid sequences for both verified and predicted VFs, whereas the "core dataset" only includes sequences of verified VFs. Using the BLASTx algorithm[6] with local Blast+ command line software[7], the *B. subtilis* BS50 genome was translated and screened against the VF dataset. Hits that had <20% coverage were excluded from analysis and multiple hits that matched the same region of the *B. subtilis* BS50 genome were screened for the hit with the highest bitscore. There were 12 distinct hits for VF factors in the *B. subtilis* BS50 genome. Ten of the VF factors (5 Bacillibactrins, 4 capsule/immune modulation factors and 1 stress survival factor) had ≥ 98.519% homology with strain *B. subtilis* 168 and are therefore intrinsic to the species. The other two ((tufA) elongation factor Tu, an adherence factor, and (hlyIII) putative membrane hydrolase, an exotoxin), had 72.629% homology with *L. monocytogenes* EGD-e and 74.707% homology with *Francisella,* respectively.

4. Genome Analysis - Toxin Screening

Using the genome sequence, the potential of *B. subtilis* BS50 to produce the major enterotoxins (hemolysin (Hbl), non-haemolytic enterotoxin (Nhe), cytotoxin K (CytK), enterotoxin FM (entFM), and *Bacillus cereus* bceT enterotoxin (BceT)) found in other disease/illness related *Bacillus* species was investigated using three *in silico* methods: BLASTn[3], BLASTx[4], and Virtual PCR[5] (Altschul et al., 1990; Sayers et al., 2010; Gish and States, 1993; San Millán et al., 2013). The accession dates were June 2, 2021 for the BLAST assays and June 4, 2021 for the virtual PCR. In the BLASTn investigation, entire individual toxin genes were compared to the complete *B. subtilis* BS50 genome. In the BLASTx investigation, the protein sequences of individual toxins were compared to the complete translated *B. subtilis* BS50 genome. For the virtual PCR analysis, primers previously identified for the purpose of assessing the presence of *Bacillus* toxins were used to virtually detect the presence of toxin gene(s) within the entire *B. subtilis* BS50 genome. Any potential gene sequence(s) found (or "amplified") were then compared to the sequence of each specific toxin of interest.

The BLASTn search was completed via the National Library of Medicine, National Center for Biotechnology Information (NCBI) website[6] to determine the presence/absence of toxin genes

[3] A nucleotide Basic Local Alignment Search Tool (BLASTn) is a bioinformatics tool used to compare a given nucleotide sequence to a database of sequences. A series of algorithms determines similarity between the sequences by identifying short matches in the sequences. Matches and gaps between those matches are scored and the end result is a statistical output that tells the user how similar the sequences are.
[4] Translated nucleotide BLAST (BLASTx) is a bioinformatics tool used to translate a query nucleotide sequence into six different reading frames and compare those protein sequences to a database of protein sequences. A series of algorithms determines similarity between the sequences by identifying short matches in the sequences. Matches and gaps between those matches are scored and the end result is a statistical output that tells the user how similar the protein sequences are.
[5] Virtual PCR, similar to a standard lab bench-based polymerase chain reaction (PCR), utilizes a specific set of DNA primers that have been designed to detect a particular nucleotide sequence within a genome through primer annealing and elongation (amplification) of the targeted nucleotide sequence.
[6] http://blast.ncbi.nlm.nih.gov/Blast.cgi

commonly associated with the *Bacillus* genus. The BLASTn procedure involved three steps. First, positive control genes were identified: *B. subtilis* glutamyl-tRNA(Gln) amidotransferase subunit and *B. cereus* methionyl-tRNA synthetase. These genes were used as a query against the *B. subtilis* BS50 genome to demonstrate the BLASTn algorithm was able to generate a match both within and across species when one existed. Second, each toxin DNA sequence was identified using NCBI gene[7]. Finally, each toxin gene DNA sequence was used as a query against the subject sequence *B. subtilis* BS50 genome. All nucleotide alignments were run using default BLASTn parameters.

The control genes, gatA and metG, yielded positive matches of 98% identity with 100% sequence coverage and 71% identity with 95% sequence coverage, respectively. The metG gene from *B. cereus* was used as a control for cross species sequence matches to ensure that BLASTn could identify matches within *B. subtilis* BS50 when a gene from a different species was used as the input. Because *B. subtilis* and *B. cereus* are different species, a high identity is not expected and thus 71% identity with 95% sequence coverage satisfies its use as a control gene for cross-species matches. No significant similarities were found between the query toxin sequences and the *B. subtilis* BS50 genome. The matches identified, including HblA, entFM, cytK, and NheA, -B, and -C from *B. cereus* and NheA, -B, and -C from *B. weihenstephanensis* were only partial matches which covered less than 25% of the toxin sequences. The *B. subtilis* BS50 genome was also blasted against a single nucleotide sequence of the *B. cereus* cereulide gene cluster (cesHPTABCD) from the 270 kb plasmid pCER270 sequence (NC_010924.1, location: 15094 to 38668). Only 50% coverage and 79% sequence identity were achieved, suggesting an incomplete cereulide gene cluster in *B. subtilis* BS50 (Table 11).

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[7] http://www.ncbi.nlm.nih.gov/gene

The GRAS status of carbohydrase and protease enzyme preparations sourced from nonpathogenic and nontoxigenic strains of *B. subtilis* was affirmed in 1997 (21 CFR 184.1148 and 21 CFR 184.1150). Subsequently, several enzyme preparations sourced from *B. subtilis* have been notified as GRAS for use in foods based on scientific procedures.

Table 7. Summary of *Bacillus subtilis* in FDA GRAS Inventory

Substance	GRN # / Closure Date	Intended Use	Use Rate	Company/ Reference	FDA Response
***Bacillus subtilis* strain R0179**	GRN 1007	For use in baked goods and baking mixes, beverage and beverage bases, breakfast cereals, chewing gum, confections and frostings, dairy product analogs, fruit and water ices, nuts and nut products, plant protein products, processed fruits and fruit juices, and snack food	Up to 1 x 10^{10} CFU/serving	Lallemand Health Solutions FDA (2022)	Pending
***Bacillus subtilis* "Bss-19" spore preparation ATCC SD-7780)**	GRN 969 Oct 6, 2021	For use in a number of conventional foods, excluding infant formula or USDA-regulated foods	Up to 1 x 10^{10} CFU/serving	Danisco USA, Inc. FDA (2021c)	FDA had no questions
***Bacillus subtilis* PLSSC (ATCC SD-7280)** (also known as BioSEB BS and SEBtilis)	GRN 956 Aug 18, 2021	For use in a number of conventional foods	Up to 6 x 10^{9} CFU/serving	Advanced Enzyme Technologies Ltd FDA (2021b)	FDA had no questions
***Bacillus subtilis* strain BS-MB40 PTA-122264 spore preparation**	GRN 955 Mar 26, 2021	For use in a number of conventional foods, excluding infant formula or USDA-regulated foods	Up to 2 x 10^{9} CFU/serving	BIO-CAT Microbials, LLC FDA (2021a)	FDA had no questions
***Bacillus subtilis* SG188 (DSM 32444)**	GRN 905 June 8, 2020	For use as an ingredient in beverages, such as milk drinks, protein high energy sports drinks, hot beverages and juices; and dry and shelf-stable products such as cereals, cookies, gums and confectionary	Up to 1 x 10^{9} spores per serving	SporeGen Ltd FDA (2020b)	FDA had no questions
Bacillus subtilis* DE111	GRN 831 Aug 13, 2019	For use in conventional foods and infant formula	Up to 1 x 10^{10} CFU/serving in foods intended for adults Up to 1 x 10^{9} CFU/serving in foods intended for children aged 2-12 Up to 2 X 10^{8} CFU/100 mL infant formula	Deerland Probiotics and Enzymes FDA (2019)	FDA had no questions
Bacillus subtilis	GRN 562	For use in post-harvest processing of bananas as an	6.3 x 10^{2} CFU/mL to 1.9 x 10^{3} CFU/mL	BiOWiSH Technologies,	FDA ceased to evaluate at

BS50 genome and the protein sequences of CesA, CesB, CesC, CesH, CesP, and CesT; all of which were less than 40% identical. There were no significant matches with CesD. Given the absence of CesD in the *B. subtilis* BS50 genome, the low sequence identity and ubiquity of proteins belonging to the same protein families as CesC, CesH, CesP, and CesT throughout prokaryotic genomes, there is sufficient evidence to conclude *B. subtilis* BS50 does not contain a functioning homologous cereulide synthase cluster.

Virtual PCR[10] was used to search the *B. subtilis* BS50 genome for toxins via gene primer matches. Ten sets of sequence primers for toxin DNA amplification were identified through primary literature sources and used to complete the virtual PCR (Agata et al., 1995; Asano et al., 1997; Mäntynen and Lindström, 1998). The following parameters were used to closely mimic an actual PCR run: 2 mismatches allowed, no mismatch allowed in the last nucleotide of the 3' end, and a maximum band length of 10,000 nucleotides. As a positive control for the primers the same set of primers were run against the *B. cereus* genome generating matches in all cases. As a control for the virtual PCR protocol, primers for 16S RNA were used to show that the program would find a match when one was present.

The virtual PCR only yielded matches using the positive control 16S and spoIVA primers. No toxin genes (including bceT) were found in the *B. subtilis* BS50 genome during the virtual PCR. The ability of this tool to identify the presence of the control sequences both in *B. subtilis* BS50 as well as in the *B. cereus* genome indicates a functional tool and thus the lack of toxin matches found in *B. subtilis* BS50 indicates an absence of such genes in the genome.

Altogether, the results of the nucleotide and protein BLAST (BLASTn and BLASTx, respectively) analyses revealed no significant or in-frame matches to known toxins such as hemolysin (Hbl), non-hemolytic enterotoxin (Nhe), or cereulide in the *B. subtilis* BS50 genome. Additionally, no bands corresponding to known *Bacillus* toxins were amplified during virtual PCR. Taken together, genomic analysis supports the absence of known *Bacillus* toxins in *B. subtilis* BS50.

5. Antibiotic Resistance

Three different methods were used to test *B. subtilis* BS50 for antibiotic resistance. The Resistance Gene Identifier (RGI) (part of the Comprehensive Antibiotic Resistance Database (CARD), which utilizes BLAST to predict of complete resistomes from genomic and metagenomic data) (Alcock et al., 2020; McArthur et al., 2013), and the Clinical and Laboratory Standards Institute's (CLSI) minimal inhibitory concentration (MIC) and disk diffusion tests. The accession dates for the *in silico* analyses were April 23, 2021 and April 24, 2021, respectively.

For the RGI test, the *B. subtilis* BS50 genome sequence was submitted to the RGI CARD webserver using the following criteria: Perfect, Strict, complete genes only, 95% identity nudge used. The 'Perfect' algorithm detects antimicrobial resistance (AMR) proteins with an exact (100%)

[10] http://insilico.ehu.eus/user_seqs/PCR/

match to a CARD reference sequence. The 'Strict' algorithm is more flexible, allowing for variation from the CARD reference sequence as long as the sequence falls within the curated BLAST bit score cut-offs, and is useful for detecting previously unknown variants of AMR genes or antibiotic targets altered via mutation. Identity nudge allows any loose hit with at least 95% identity to be scored as a strict hit. RGI identified 1 perfect, 3 strict, and 275 loose hits. Of the 275 loose hits, 12 hits had at least a 95% identity and were nudged to strict hits. In total, there were 16 potential resistance hits, but only 7 that covered more than 90% of the reference gene sequence (Table 12). Based on the presence of a gene with roughly 98% identity to *aadK*, an aminoglycoside 6-adenylyltransferase that is part of the ANT6 gene family, *B. subtilis* BS50 is predicted to be resistant to streptomycin. *B. subtilis* BS50 is also predicted to be resistant to the macrolides spiramycin and telithromycin due to the presence of *mph(K)*, a macrolide phosphotransferase. Additionally, *B. subtilis* BS50 is predicted to be resistant to tetracycline due to the presence of a tetracycline efflux pump. Other hits with > 90% identity over the length of the reference sequence are genes associated with resistance to phenicol, lincosamide, fluoroquinolone, acridine dye, and peptide antibiotics. The possibility of the strain being resistant to aminoglycosides (including streptomycin), glycopeptides, macrolides, lincosamides and tetracyclines was examined further (see below). Acridine dyes are mutagenic, and as such their use has been supplanted by safer alternatives. Use of fluoroquinolones also is limited due to toxicity. FDA has determined that use of fluoroquinolones for treatment of sinusitis, bronchitis and urinary tract infections should be reserved to cases that have no alternative treatment options, but the benefits of fluroquinolones outweigh the risks for treatment of bacterial pneumonia, anthrax and plague (FDA, 2016a). Possible fluroquinolone resistance by people consuming *B. subtilis* BS50 is not expected to be a health concern by the majority of people but could be for individuals with serious health conditions that are refractive to other antibiotics.

Table 12. Resistance Gene Identifier (RGI) Results for *Bacillus subtilis* BS50 for Antibiotic Resistance

ARO Term (gene)	AMR Gene Family	Drug Class	Resistance mechanism	% Identity of Matching Region	% Length of Ref. Seq.	RGI Criteria
ykkD	small multidrug resistance (SMR) antibiotic efflux pump	aminoglycoside antibiotic, tetracycline antibiotic, phenicol antibiotic	antibiotic efflux	100	101.9	Strict
ImrB	ATP-binding cassette (ABC) antibiotic efflux pump	lincosamide antibiotic	antibiotic efflux	96.65	100.42	Strict
ykkC	small multidrug resistance (SMR)	aminoglycoside antibiotic, tetracycline	antibiotic efflux	100	100	Perfect

ARO Term (gene)	AMR Gene Family	Drug Class	Resistance mechanism	% Identity of Matching Region	% Length of Ref. Seq.	RGI Criteria
	antibiotic efflux pump	antibiotic, phenicol antibiotic				
tet(45)	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	75.82	100	Strict
mphK	macrolide phosphotransferase (MPH)	macrolide antibiotic	antibiotic inactivation	97.71	100	Strict
blt	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic, acridine dye	antibiotic efflux	99.75	98.5	Strict
Bacillus subtilis pgsA with mutation conferring resistance to daptomycin	daptomycin resistant pgsA	peptide antibiotic	antibiotic target alteration	99.71	90.53	Strict
Bacillus subtilis mprF	defensin resistant mprF	peptide antibiotic	antibiotic target alteration	99.69	76.87	Strict
vmlR	ABC-F ATP-binding cassette ribosomal protection protein	macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic, tetracycline antibiotic, oxazolidinone antibiotic, phenicol antibiotic, pleuromutilin antibiotic	antibiotic target protection	98.54	75.5	Strict
aadK	ANT(6)	aminoglycoside antibiotic	antibiotic inactivation	97.74	63.03	Strict
bmr	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic, nucleoside antibiotic, acridine dye, phenicol antibiotic	antibiotic efflux	100	47.3	Strict
tmrB	tunicamycin resistance protein	nucleoside antibiotic	reduced permeability to antibiotic	97.59	42.13	Strict
aadK	ANT(6)	aminoglycoside antibiotic	antibiotic inactivation	97.22	39.44	Strict

ARO Term (gene)	AMR Gene Family	Drug Class	Resistance mechanism	% Identity of Matching Region	% Length of Ref. Seq.	RGI Criteria
vmlR	ABC-F ATP-binding cassette ribosomal protection protein	macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic, tetracycline antibiotic, oxazolidinone antibiotic, phenicol antibiotic, pleuromutilin antibiotic	antibiotic target protection	96.4	27.24	Strict
tmrB	tunicamycin resistance protein	nucleoside antibiotic	reduced permeability to antibiotic	100	26.9	Strict
Bacillus subtilis mprF	defensin resistant mprF	peptide antibiotic	antibiotic target alteration	100	16.36	Strict

AMR- antimicrobial resistance; ARO- antibiotic resistance ontology

Potential resistance to eight antibiotics (chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, oxytetracycline, and vancomycin was tested in the minimum inhibitory concentration (MIC) test. The MIC of each antibiotic was determined based upon the methodology described by CLSI (CLSI, 2018a). The antibiotics tested were representative of most of the classes of concern identified by the *in silico* analyses. *B. subtilis* BS50 cells (3.93×10^6 CFU/mL per well) were exposed to each of 10 different dilutions of each antibiotic in sterile nutrient broth. Following an appropriate incubation period, the MIC of each antibiotic was determined visually and documented. *Enterococcus faecalis* (ATCC Accession No. 29212) and *Staphylococcus aureus* (ATCC #29213) (2.96×10^6 and 8.25×10^5 CFU/mL per well, respectively) were tested in tandem with *B. subtilis* BS50 to verify the methodology performed in this study. These microbes exhibited MICs within the CLSI quality control range. *B. subtilis* BS50 was susceptible to seven of eight antibiotics but exhibited resistance against streptomycin (Table 13).

Table 13. Results of Antibiotic Susceptibility Testing (MIC) for *Bacillus subtilis* BS50

Test Group	MIC (µg/mL)	Interpretation
Aminoglycosides		
Gentamicin	0.5	S

Test Group	MIC (µg/mL)	Interpretation
Streptomycin	125	R
Kanamycin	2	S
Glycopeptides		
Vancomycin	0.25	S
Macrolides, Lincosamides		
Clindamycin	0.5	S
Erythromycin	<0.0625	S
Phenicols		
Chloramphenicol	2	S
Tetracyclines		
Oxytetracycline	8	S

MIC- minimal inhibitory concentration; R – resistant; S- susceptible

For the CLSI zone inhibition test, published guidelines established by the organization were followed for all procedures (CLSI, 2018b). *B. subtilis* BS50 inoculum was plated onto trypticase soy agar plates and incubated at 35±2°C for 18-24 hours. At least three typical, well-isolated colonies were selected and suspended in Butterfield's Buffer. The suspension turbidity was adjusted to a 0.5 McFarland standard equivalent by comparing to a commercially available 0.5 McFarland standard (Hardy Diagnostics). The cell suspension was used to inoculate room-temperature, 100 mm Mueller-Hinton Agar (MHA) plates (Hardy Diagnostics). The MHA plates were inoculated by covering the entire plate with the standardized BS inoculum using repeating sweeping swabbing motions as described by the CLSI standard M02 to ensure an even covering of bacterial suspension over the entire plate. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control organisms. All tests were performed in duplicate, and all zone measurements reported are an average of at least two trials.

Antibiotic discs, stored at 4°C, were brought to room temperature before use. BBL™ Sensi-Disc™ discs containing the prescribed amount of antibiotic were loaded into a BBL™Sensi-Disc™ dispenser (Becton Dickinson and Company) and dispensed onto each inoculated plate within 15 minutes of inoculation. Four discs were evenly placed on each 100mm plate. The plates were then inverted and incubated at 35±2°C for 16-18 hours. Inhibition zones were detected and measured to the nearest millimeter using InterScience Scan500 and accompanying software. Zone measurements were visually inspected and confirmed. If no zone was present, 6mm, the diameter of the disc, was recorded. All tests were performed in duplicate, and all zone measurements reported are an average of at least two trials.

B. subtilis BS50 was susceptible to the majority of the antibiotics to which it was exposed (7 of 8), which included members of classes to which the *in silico* analysis predicted resistance (Table 14).

B. subtilis BS50 was neither susceptible nor resistant to clindamycin. There is some difficulty in assessing the antibiotic susceptibility of *B. subtilis* using the CLSI inhibition zone method because there is no given range for data interpretation for this genus and species. If *B. subtilis* BS50 behaves similar to a *Staphylococcus* strain, it should be considered neither susceptible nor resistant to clindamycin; however, if *B. subtilis* BS50 behaves more similar to a *streptococcus* strain, then it should be considered susceptible to clindamycin. The results of the MIC test showed that *B. subtilis* BS50 is susceptible to clindamycin.

Table 14. Results of Antibiotic Susceptibility Testing (Zone Inhibition) for *Bacillus subtilis* BS50

Test Group	DISC Code	Zone (mm)	Zone Interpretation
Aminoglycosides			
Gentamicin	GM 10	26 ± 0.5	S
Streptomycin	S 10	18 ± 0.5	S
β-lactams: Penicillins			
Penicillin	P 10	34 ± 0.4	S
Glycopeptides			
Vancomycin	Va 5	20 ± 0.4	S
Fluroquinolones			
Ciprofloxacin	CIP 5	29 ± 0.6	S
Levofloxacin	LVX5	29 ± 0.6	S
Norfloxacin	NOR5	27 ± 0.3	S
Macrolides, Lincosamides			
Clindamycin	CC 2	20 ± 1.1	S/Int*
Erythromycin	E 15	35 ± 1.3	S
Phenicols			
Chloramphenicol	C 30	28 ± 0.4	S
Tetracyclines			
Tetracycline	Te 30	20 ± 0.4	S

Int – neither susceptible nor resistant; S – susceptible
* Susceptible if *Bacillus subtilis* BS50 behaves similar to a *Streptococcus* strain, and Int if *Bacillus subtilis* BS50 behaves similar to a *Staphylococcus* strain.

In conclusion, *B. subtilis* BS50 was found to encode 16 antibiotic resistance genes, and *in vitro* susceptibility tests determined that *B. subtilis* BS50 was susceptible to all antibiotics tested, with the exception of streptomycin (MIC test only). Resistance genes for streptomycin such as aadK (ANT6 gene family) are well established in the *Bacillus* genus (Ohmiya et al., 1989; Adimpong et al., 2012; Jeon et al., 2017) and should be considered intrinsic.

6. Genomic Analysis - Mobile Elements

The presence of insertional sequences and/or mobile elements can be problematic because transferable elements can move from one bacterium to neighboring bacteria and transfer genetic material such as antibiotic resistance genes to the recipient bacteria. It is important that bacterial

strains do not harbor mobile elements in the vicinity of genes conferring antibiotic resistance or other potential toxin genes that may be passed to host microbiome organisms. To determine if *B. subtilis* BS50 contains mobile elements, two databases containing known mobile elements were used: ISfinder and the "A CLAssification of Mobile genetic Elements" (ACLAME). The *B. subtilis* BS50 genome was screened for known insertion sequences using the online program ISfinder, which utilizes the BLASTn algorithm to search for query nucleotide sequences that match insertion sequences (Siguier et al., 2006; Altschul et al., 1990). Default settings were used. The ACLAME database version 0.4 contains 125,190 nucleotide sequences of known mobile gene elements from prophages, virus, and bacterial plasmids (Leplae et al., 2010). The database was downloaded and blasted against the *B. subtilis* BS50 genome using the BLASTn command with local Blast+ software under default parameters (Camacho et al., 2009; Altschul et al., 1990).

The results of the ISfinder analysis showed no matches between the *B. subtilis* BS50 genome and any known insertional sequences with coverages greater than 15%. The ACLAME analysis showed that there were 122 unique loci in the *B. subtilis* BS50 genome that aligned with known mobile genetic element sequences with greater than 50% coverage, e-values less than 1.3 e-11, and bit scores greater than 65. Out of the 122 loci containing mobile genetic elements, the prophage-associated gene YrkC2 (GeneID: 3099880) was detected 1,641 bp upstream of the CARD-identified antibiotic resistance encoding gene blt (start position: 3,686,740; stop position 3,687,924). However, the YrkC2 sequence only aligned to the *B. subtilis* BS50 genome with 80.3% similarity and 67% coverage, for which the 5' region, including the start codon did not align, indicating that this gene is non-functional or would yield a truncated protein.

In order to assess if these putative mobile genetic elements could play a role in AMR gene transfer, the loci of sequences in the *B. subtilis* BS50 genome matching mobile genetic elements were then compared to loci that were determined to have AMR via CARD (Alcock et al., 2020). Insertion sequences that were not within 5Kb of AMR genes were not considered to play a role in AMR gene transfer. Out of the 122 loci that aligned to mobile genetic elements from the ACLAME database (4.0), one was found within five kb of an antibiotic resistance gene. The nucleotide sequence for the cupin domain-containing protein (NC_006322.1 (1,461,102-1,461,695)) was detected 1,641 bp upstream of the blt-encoding gene (start position: 3,686,740; stop position 3,687,924). However, the nucleotide sequence for the cupin domain-containing protein only aligned to the BS50 genome with 80.3% similarity and 67% coverage. Furthermore, 174 nt of the 5' region of the sequence encoding for the cupin-domain-containing protein did not align to the BS50 genome, suggesting that this gene is non-functional and/or truncated. Thus, BS50 is at low risk of transferring antibiotic resistance including resistance to streptomycin.

7. Production of Biogenic Amines

Bacterially-produced biogenic amines such as histamine and tyramine can have negative clinical effects when consumed (Barbieri et al., 2019; Comas-Basté et al., 2020). Decarboxylases are the primary bacterial enzymes responsible for converting amino acids to biogenic amines and can be the cause of food spoilage (Barbieri et al., 2019).

In order to assess the potential of *B. subtilis* BS50 for production of biogenic amines, its genome was screened for the nucleotide and amino acid sequences of decarboxlyases, including those from common lactic acid bacteria involved in food spoilage. Nucleotide and amino acid sequence analyses were performed using BLASTn and BLASTx, respectively, under default settings using the NCBI website (Altschul et al., 1990).[8,9] The accession date was June 22, 2022.

The BLASTn analyses did not yield any significant matches between the *B. subtilis* BS50 genome and the gene sequences for histidine decarboxylase, tyrosine decarboxylase, agmatine deiminase, ornithine decarboxylase or lysine decarboxylase. BLASTx aligned the translated *B. subtilis* BS50 genome with decarboxylase amino acid sequences for ornithine decarboxylase or lysine decarboxylase only, but each match shared less than 30% sequence identity, which is insufficient to support homology or shared protein function. In addition, the alignment coverage for all of the decarboxylase amino acid sequences was less than 50%, further confirming that *B. subtilis* BS50 does not encode for proteins involved in biogenic amine production.

Higher polyamines such as spermine and spermidine are synthesized from putrescine (Bardócz, 1995; Kalac and Krausová, 2005). Since *B. subtilis* BS50 does not encode for any decarboxylases (ornithine or agmatine) involved in putrescine production, it is likely that the organism cannot produce spermine and spermidine.

8. Cytotoxicity Evaluation

The effects of *B. subtilis* BS50 lysate on the integrity of Caco-2 monolayer was tested using two different methods – intracellular ATP and transepithelial electrical resistance (TEER). To generate the cell lysate, *B. subtilis* BS50 cells were harvested from overnight bacterial cultures and washed. The cells were lysed via enzymatic and mechanical bead-based processes. The final lysate was filtered through a 0.2 µM filter to remove any remaining cells. A “blank” sample (sterile, uninoculated media) was used as a process control sample for the lysate production method. For the ATP assay, established Caco-2 cells were seeded into a 96-well plate and allowed to adhere overnight followed by exposure to *B. subtilis* BS50 lysate (top dose of 1:5) in triplicate for 48 hours. Controls included Caco-2 cells that were left untreated and Caco-2 cells that were fully lysed at the time of treatment. After 48 hours, the Caco-2 cells were lysed and the ATP levels (in µM) were determined using a standard curve. *B. subtilis* BS50 lysate did not negatively impact the Caco-2 cells. ATP levels in the process blank and lysate treatments were equal and similar to the ATP levels in the untreated cells (Figure 6).

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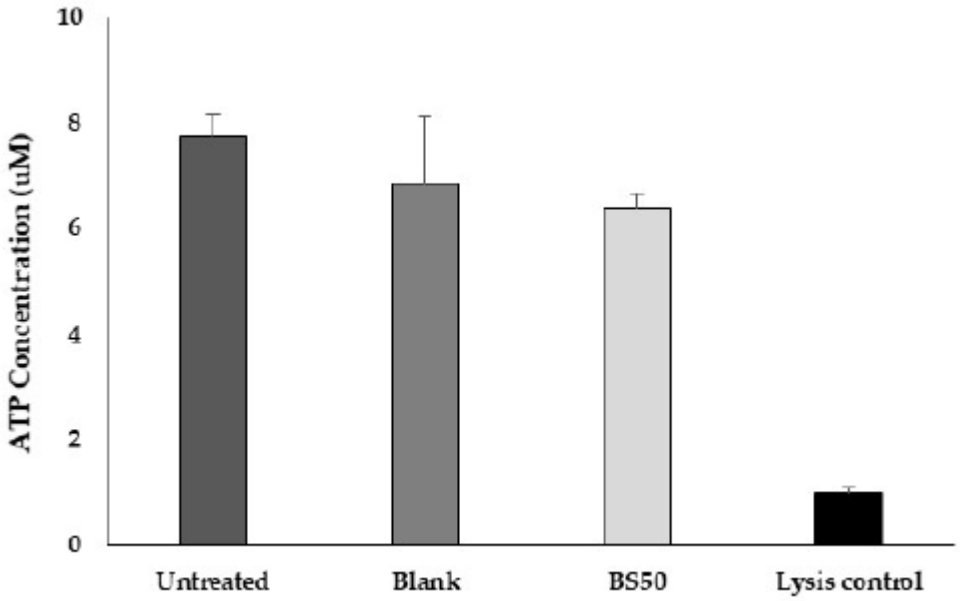

Figure 6. Effect of the *B. subtilis* BS50 Lysates on Caco-2 Cell Viability
Data are expressed as mean ± standard error (SEM) of technical triplicates.

For the TEER assay, Caco-2 cells were seeded on Transwell inserts over 14 days. At day 14, the polarized Caco-2 monolayers were treated with a 1:5 dilution of *B. subtilis* BS50 lysate, sterile medium, or lipopolysaccharide (LPS) stimulation for 48 hours. There also was a non-treatment control. TEER was measured before treatment (0 hours) and at 2, 4, 6, 24 and 48 hours after treatment. Two separate trials (A and B) were conducted with separate lysate preparations. Due to variations in the initial TEER measurements across wells, fold-changes relative to 0 hr from both trials were combined for statistical analysis. There were no significant differences in TEER fold-change values between the untreated control, blank process control, and cells treated with *B. subtilis* BS50 lysate at both 24 hr and 48 hr post-treatment ($p > 0.2$), whereas the LPS control lowered TEER compared to all other treatments at 24 hr ($p < 0.006$) (Figure 7).

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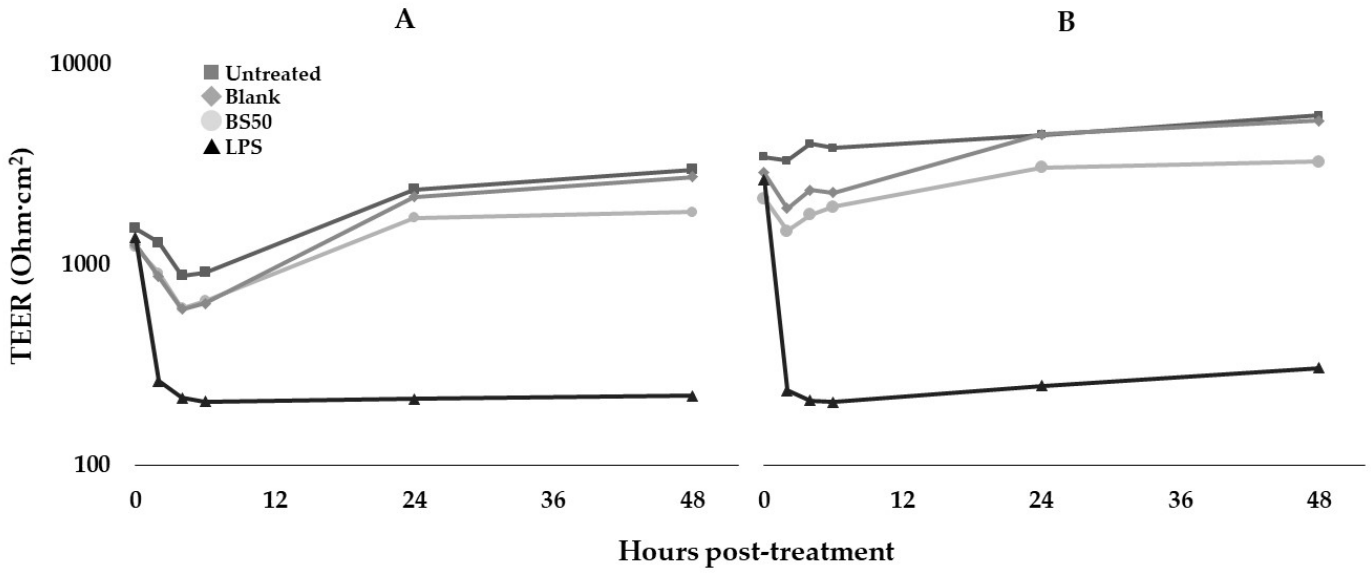

Figure 7: Effect of *B. subtilis* BS50 Lysate on Caco-2 Cell Monolayer TEER

B. subtilis BS50 was streaked onto sheep blood agar plates to assess its ability to lyse blood cells. After incubation overnight, the agar was inspected for alpha- or beta-hemolysis. Alpha-hemolysis, or incomplete hemolysis, is indicated by a discolored, darkened, or green medium color after test culture growth. Beta-hemolysis, or complete hemolysis, is indicated by clearly colorless medium after growth. An indiscernible change in the color of the agar indicates that no hemolysis occurred (i.e., gamma-hemolysis). The agar displayed a greenish hue surrounding the streaks where *B. subtilis* BS50 colonies grew, indicating that the organism exhibits alpha-hemolysis. Hemolytic activity has been detected throughout several *Bacillus* strains isolated from commercially available products (Deng et al., 2021). While this may present a safety concern if *B. subtilis* BS50 comes into contact with the bloodstream, the likelihood of the strain translocating through the intestinal barrier into the bloodstream is small based on results of the *in silico* and cytotoxicity studies. The results of the Caco-2 cell studies indicate that *B. subtilis* BS50 will not be toxic to enterocytes in the human intestine or affect gut barrier integrity, supporting that this strain will have low potential for translocation to the bloodstream.

9. Human Clinical Safety and Tolerability Studies with *Bacillus subtilis* BS50

In a randomized, double-blind, placebo controlled, parallel-arm study, the safety and tolerability of *B. subtilis* BS50 was evaluated in normal, healthy adult volunteers (ClinicalTrials.gov Identifier: NCT05004454) (Garvey et al., 2022). Seventy-six subjects were given placebo or *B. subtilis* BS50 (n=38/group). All subjects completed the study. The subjects were healthy men (n=18 and n=16 in *B. subtilis* BS50 and placebo groups, respectively) and women (n=20 and n=22 in *B. subtilis* BS50 and placebo groups, respectively), aged 30 to 65 years (inclusive), who had a body mass index (BMI) 18.0-31.9 kg/m^2 (inclusive) and a combined weekly total symptom score for flatulence,

abdominal bloating, and burping of ≥3 as assessed using an 8-item Gastrointestinal Tolerance Questionnaire (GITQ).

Subjects consumed one test capsule per day for 42 days. Each BS50 capsule contained 2 x 10^9 CFU of *B. subtilis* BS50. Each placebo capsule contained maltodextrin. Subjects were directed to consume the study product once a day with their meal that is typically the largest of the day. Before supplementation, and throughout the 42-day supplementation period, GI symptoms were captured using the GITQ. Per the 24-hour recall GITQ, subjects reported daily occurrence and severity of each of flatulence, burping, abdominal distention/bloating, nausea, vomiting, abdominal cramping, borborygmus/stomach rumbling, and reflux/heartburn. Additionally, sleep quality and presence and duration of any respiratory infection were assessed before supplementation and weekly during the 42-day supplementation period using a Sleep Quality and Respiratory Infection Questionnaire. Before and at the end of the 42-day supplementation period, body weight and vital signs were measured. Fasting blood samples were collected for chemistry (albumin, globulin, albumin/globulin ratio, albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, anion gap, total bilirubin, blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, total calcium, chloride, glucose, potassium, sodium, total protein, CO_2, and osmolality), hematology (red blood cells, white blood cells, differential white blood cells, hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, platelets, red blood cell distribution width), lipid profiling (triglyceride, total cholesterol, low-density lipoprotein cholesterol, and high density lipoprotein cholesterol), measurement of GI permeability marker concentrations (zonulin, occludin, and lipopolysaccharide binding protein), and measurement of inflammatory marker concentrations (C-reactive protein, interleukin (IL)-8, IL-6, IL-10, interferon-γ, and tumor necrosis factor-α) before and after 42 days of once daily BS50 or placebo supplementation.

Per the GITQ, very few incidences of the individual symptoms of nausea, abdominal cramping, vomiting, borborygmus/stomach rumbling, and reflux were reported throughout the course of 42 days of supplementation with either BS50 or placebo, with no statistically significant differences within or between groups. Four subjects experienced a total of five adverse events (none serious) and only one (daily headaches in placebo group) was possibly related to treatment. There were no adverse effects of *B. subtilis* BS50 on blood chemistry or hematology, blood pressure, body weight, plasma lipids, inflammation markers, intestinal permeability markers, sleep, or rate of respiratory infection. Thus, the results indicate that *B. subtilis* BS50 at 2x10^9 CFU/day is safe in healthy adults.

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10. Safety Assessment of *Bacillus subtilis* BS50

The safety of *B. Subtilis* BS50 has been evaluated utilizing scientific procedures as outlined by Pariza et al. (2015) (Figure 8). Based on the outcome of the decision tree for determining the safety of microbial cultures for consumption by humans and animals including strain characterization and genome sequencing, screening for undesirable attributes and metabolites, and experimental evidence of safety by in appropriately designed safety evaluation studies, it was concluded that *B. subtilis* BS50 is deemed to be safe for human consumption.

Question 1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?
YES; *B. subtilis* BS50 is unambiguously characterized as *Bacillus subtilis* through nanopore technology, prokka 1.13.7 annotation, MAFFT and BLAST comparison to other *B. subtilis* strains; see Part 3.3.
(The evaluation proceeded to Question 2.)

Question 2. Has the strain genome been sequenced?
YES; The genome of *B. subtilis* BS50 was sequenced and typed to be 98.5% similar to *B. subtilis* MB40 and 99% similar to *B. subtilis* subsp. Natto BEST195; see Part 3.3.
(The evaluation proceeded to Question 3.)

Question 3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?
YES; Using either nucleotide BLASTn®, BLASTx® or virtual PCR analysis of *B. subtilis* BS50, no in-frame complete matches to the major enterotoxins found in other disease/illness related *Bacillus* species (i.e. HblA, cytK, entFM, bceT or NHEA, -B and -C) were generated; see Parts 6D3 and 6D4.
(The evaluation proceeded to Question 4.)

Question 4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?
YES; *B. subtilis* BS50 contains no plasmid DNA which is typically associated with the transfer of antibiotic resistance genes. In an *in vivo* Antibiotic Sensitivity Test, *B. subtilis* BS50 was susceptible to all antibiotics to which it was exposed, except for streptomycin. None of the loci containing mobile gene elements corresponded to the flanking regions of any antimicrobial genes, indicating that the risk of BS50 transferring any potential antibiotic resistance (including resistance to streptomycin) is minimal; See Part 6D5.
(The evaluation proceeded to Question 5.)

Question 5. Does the strain produce antimicrobial substances?
NO; The antimicrobial active compounds produced by members of the *Bacillus* genus are primarily lantibiotics and lantibiotic-like peptides which are rapidly degraded through the digestive process and not suitable for oral administration as a treatment in human or veterinary medicine; see Part 6D2
(The evaluation proceeded to Question 6.)

Question 6. Has the strain been genetically modified using rDNA techniques?
NO; *Bacillus subtilis* BS50 has not been genetically modified.
(The evaluation proceeded to Question 8a.)

Question 8a. For strains to be used in human food: Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?
NO; *B. subtilis* BS50 was isolated from the soil. However, it should be noted that *Bacillus subtilis* BS50 shares the same genus and species of *B. subtilis var. nattō* used in the fermentation of soybeans into "nattō, a traditional Japanese food.
(The evaluation proceeded to Question to 13a.)

Question 13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
NO. Results of Caco-2 cell studies indicate that *B. subtilis* BS50 will not be toxic to enterocytes in the human intestine or affect gut barrier integrity, supporting that this strain will have low potential for translocation to the bloodstream. The safety and tolerance of *B. subtilis* BS50

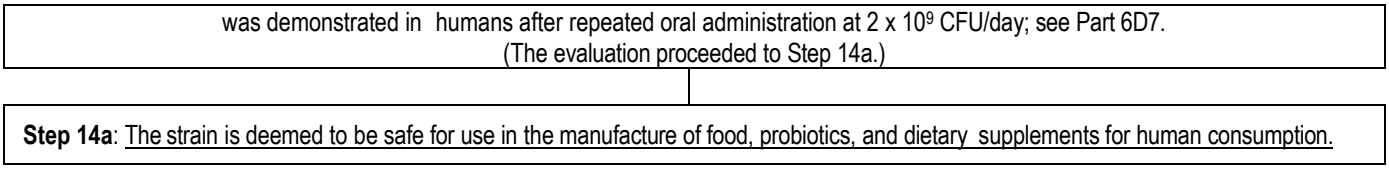

Figure 8. Pariza et al. (2015) Decision Tree Analysis of *Bacillus subtilis* BS50

E. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "…reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use."[11]

Amplification is provided in that the conclusion of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that[11]:

> "…General recognition of safety requires common knowledge, throughout the expert scientific community knowledgeable about the safety of substances directly or indirectly added to food, that there is reasonable certainty that the substance is not harmful under the conditions of its intended use."
>
> "'Common knowledge' can be based on either "scientific procedures" or on experience based on common use of a substance in food prior to January 1, 1958."

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles,

[11] See 21 CFR 170.3 (e)(i) and 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed January 24, 2023).

textbooks, or compendia, or by obtaining opinions of expert or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive. General recognition of safety through scientific procedures shall be based upon the application of generally available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.

The apparent imprecision of the terms “appreciable,” “at the time,” and “reasonable certainty” demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; Renwick, 1990; Rulis and Levitt, 2009).

As noted below, this safety assessment to ascertain GRAS status for *B. subtilis* BS50 for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

F. Common Knowledge Elements for GRAS Conclusions

The first common knowledge element for a GRAS conclusion requires that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge element for a GRAS conclusion requires that consensus exists within the broader scientific community.

1. Public Availability of Scientific Information

The regulatory framework for determining whether a substance is generally recognized as safe (GRAS) is in 21 CFR 170.30, which states that GRAS status through scientific procedures shall ordinarily be based upon published studies, which may be corroborated by unpublished studies and other data and information. These criteria have been applied to the existing data for *B. subtilis* strain BS50. This GRAS evaluation satisfies the first common knowledge element, as the scientific information that is the basis of the GRAS determination for *B. subtilis* BS50 is publicly available.

The key evidence for safety of *B. subtilis* BS50 (*in silico* and *in vitro* safety studies with *B. subtilis* BS50**,** nonclinical studies with other *B. subtilis* strains, and clinical studies with *B. subtilis* BS50 and other *B. subtilis* strains) are publicly available. The No-Observed-Adverse-Effect-Level (NOAELs) for the related strain *B. subtilis* MB40 after oral administration to rats for 14 days is 2000 mg/kg bw/day (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day in rats), the highest dose tested (Spears et al., 2021). Results of a clinical study show that consumption of 2×10^{9} (2 billion) CFU Bacillus subtilis BS50/day for 42 days is safe in humans (Garvey et al., 2022).

Four GRAS notifications for the use of *B. subtilis* in food have received no questions letters from FDA (GRN 969,955, 905 and 831), and two are pending. EPA exemptions for *B. subtilis* strains GB03, FMCH002, BU1814, MBI 600; CX-9060, QST 713, and QST 713 variant soil from tolerances in food crops are available in the Federal Register (EPA, 2008; EPA, 2017; EPA, 2018; EPA, 2009; 2012a; EPA, 2012b). *B. subtilis* is recognized by the Natural and Non-Prescription Health Products Directorate (NNHPD) of Health Canada as a Natural Health Product (NHP) ingredient. The European Food Safety Authority (EFSA) confirmed a QPS Determination for the use of *B. subtilis* as an animal feed additive based on the absence of toxigenic potential.

2. Scientific Consensus

The second common knowledge element for a GRAS conclusion requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use. BIO-CAT Microbials intends to add its *B. subtilis* BS50 to a wide variety of foods. *Bacillus subtilis* BS50 will be added to foods at a maximum level of 2×10^9 CFU/serving, for a maximum estimated daily intake (EDI) of 36.4×10^9 (36.4 billion) CFU/day. This EDI does not present a safety concern to humans.

The traditional Japanese food “nattō” (fermented soybean) contains up to 1×10^9 viable spores of *B. subtilis*/gram of nattō product. Based on a serving size of 175 g, a daily consumer of nattō would therefore consume up to 1.75×10^{11} CFU *B. subtilis*/day (175 billion CFU/day) from this source only.

Ingestion of 4.8×10^{10} (48 billion) CFU/day *B. subtilis* C-3102 for 28 days is safe for healthy adults.

A significant number of animal studies, clinical studies, and reviews consistently support the safety of numerous *B. subtilis* strains. Also, four GRNs for *B. subtilis* have been reviewed by FDA with “no question” responses. The highest estimated intake of *B. subtilis* in the successfully notified GRAS Determinations was up to 2.78×10^{11} CFU/day, for *B. subtilis* “Bss-19” spore preparation (GRN 969).

One NDIN for *B. subtilis* combined with *B. clausii* (LiveSpo® COLON) has been accepted for filing by FDA. The total recommended daily dose of this preparation is 3×10^9 CFU/day (total). *B. subtilis* is present in some currently marketed dietary supplements, with recommended doses up to 10×10^9 CFU/day. Numerous *B. subtilis* strains are permitted for use on crops by EPA and are exempted from tolerances. The classification as a Natural Health Product by Health Canada and the QPS conclusion from EFSA also demonstrate the view of other regulatory authorities on the safe use of *B. subtilis.*

Based on a conservative 100-fold safety factor for inter-and intra-species differences, the ADI of the closely related species *B. subtilis* MB40 (a GRAS organism) in humans is 3.7×10^9 CFU/kg bw/day (or 2.6×10^{11} (260 billion) CFU/day for a 70 kg person).

In addition, the strain specific data available for *B, subtilis* BS50, based on *in silico/in vitro* and clinical data demonstrate a lack of safety concerns for this strain based on the following:

- *B. subtilis* BS50 is adequately characterized phenotypically and lacks known genetic elements for virulence factors/toxins associated with pathogenicity
- The antibiotic resistance profile is acceptable compared to species of *Bacillus* that are used in food
- Results of a clinical study show that consumption of 2 x 10^9 (2 billion) CFU *Bacillus subtilis* BS50/day for 42 days is safe in humans.
- The estimated daily intake of *B. subtilis* BS50 from proposed uses at potential maximum intakes is 3.64 x 10^{10} CFU/day (approximately 36 billion CFU/day or 5.2 x 10^8 CFU/kg bw/day for a 70 kg person).

Overall, the safety data for *B. subtilis* BS50 and the closely related and GRAS strain *B. subtilis* MB40 support the conclusion that *B. subtilis* BS50 is safe for human consumption.

BIO-CAT Microbials maintains that other well-qualified scientists would conclude that BIO-CAT Microbials' *B. subtilis* BS50 is generally recognized as safe for use in food given the regulatory and safety data available and using well accepted toxicological principles.

G. Discussion of Information Inconsistent with GRAS Conclusion

B. subtilis BS50 exhibits alpha-hemolysis. Hemolytic activity has been detected throughout several Bacillus strains isolated from commercially available products (Deng et al., 2021). While this may present a safety concern if *B. subtilis* BS50 comes into contact with the bloodstream, the likelihood of the strain translocating through the intestinal barrier into the bloodstream is small and based on results of *in silico* and cytotoxicity studies performed with the strain. Results of the Caco-2 cell studies (Figs. 6 & 7) indicate that *B. subtilis* BS50 will not be toxic to enterocytes in the human intestine or negatively affect gut barrier integrity, supporting that this strain will have low potential for translocation to the bloodstream. In the unlikely event that translocation does occur, *B. subtilis* BS50 is susceptible to commonly used antibiotics.

H. GRAS Conclusion

In consideration of the aggregate safety information available, BIO-CAT Microbials, LLC concludes that *B. subtilis* BS50 as defined in the subject notification is safe for use in a wide variety of foods, including baked goods and baking mixes, beverages and beverage bases (including carbonated and flavored waters, sports and nutritional drinks), breakfast cereals, cheese, chewing gum, coffee and tea, confections and frostings, dairy product analogs, frozen desserts (dairy, non-dairy and ices), gelatins, puddings and fillings, grain products and pastas, hard candy and cough drops, milk products, plant protein products, processed fruits and fruit juices, processed vegetables and

vegetable juices, snack foods and soft candy, with an intended use level of up to 2 x 10^9 CFU/serving.

The weight of the publicly available evidence from nonclinical and clinical studies with *B. subtilis* BS50 provides a basis upon which to conclude that the proposed uses of *B. subtilis* BS50, as described in this dossier, satisfy the safety standard of Reasonable Certainty of No Harm and is safe. Based on the pivotal, published data and information that are generally available, one may conclude that the proposed uses of *B. subtilis* BS50 as, produced consistent with current Good Manufactory Practice (cGMP) and meeting the food grade specifications presented above, are Generally Recognized As Safe (GRAS) based on scientific procedures.

Accordingly, *Bacillus subtilis* BS50 as produced by BIO-CAT Microbials, LLC, in accordance with FDA Good Manufacturing Practices, and, when it meets those specifications declared within the subject notification, meets FDA's definition of safety in that there is "reasonable certainty of no harm under the intended conditions of use" as described herein and, therefore, is generally recognized as safe (GRAS).

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PART 7. LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE

A. List of Acronyms and References

1. List of Acronyms

AAFCO	Association of American Feed Control Officials
ACLAME	A CLAssification of Mobile genetic Elements
ADFI	Average daily feed intake
ADG	Average daily gain
ADI	Acceptable Daily Intake
ADMI	Average daily dry matter intake
AE	Adverse Events
AMR	Antimicrobial resistance
AOAC	Association for Official and Analytical Chemists
BAM	Bacteriological Analytical Manual
BceT	*Bacillus cereus* bceT enterotoxin
BIO-CAT	BIO-CAT Microbials, LLC
BLASTn	nucleotide Basic Local Alignment Search Tool
BLASTx	translated nucleotide Basic Local Alignment Search Tool
BMD	Bone mineral density
bw	body weight
CARD	Comprehensive Antibiotic Resistance Database
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
cGMP	current Good Manufacturing Practice
CLSI	Clinical Laboratory Standards Institute
COA	Certificate of Analysis
CytK	Cytotoxin K
EDI	Estimated Dietary Intake
EFSA	European Food Safety Authority
entFM	Enterotoxin FM
EPA	US Environmental Protection Agency
FC	Feed consumption
FCR	Feed conversion ratio
FDA	US Food and Drug Administration
GI	Gastrointestinal
GITQ	Gastrointestinal Tolerance Questionnaire
GOS	galacto-oligosaccharides
GRAS	Generally Recognized as Safe
GSRS	Gastrointestinal Symptom Rating Scale
HACCP	Hazard Analysis Critical Control Point
Hbl	hemolysin
ICP	Inductively Coupled Plasma
IL	Interleukin
ITT	Intent-to-Treat
kg	kilogram
mg	milligram
MIC	Minimal Inhibitory Concentration

min	Minute
mL	milliliter
n	number
NCBI	National Center for Biotechnology Information
NDI	New Dietary Ingredient
NDINs	New Dietary Ingredient Notifications
Nhe	Non-haemolytic enterotoxin
NHP	Natural Health Product
NIH	National Institutes of Health
NLT	not less than
NNHPD	Natural and Non-Prescription Health Products Directorate
NOAEL	No-Observed-Adverse-Effect-Level
NPN	Natural Product Number
OECD	Organization for Economic Cooperation and Development
PCR	polymerase chain reaction
PDP	Principal Display Panel
PLA	Product Licence Application
ppm	parts per million
QPS	Qualified Presumption of Safety
RBC	Red blood cell count
RGI	Resistance Gene Identifier
SOPs	Standard Operating Procedures
TEER	Transepithelial electrical resistance
TOS	total organic solids
USDA	US Department of Agriculture
VF	Virulence factor
VFA	Volatile fatty acids
WGS	Whole genome sequencing

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B. Appendices

Appendix 1 Food Grade Statement

Advancing Microbial Solutions

BIO-CAT Microbials

PRODUCT INFORMATION

CUSTOMER:		**PRODUCT:**	BS50
DATE:	03/28/2022		

FOOD GRADE STATEMENT:
The food grade product listed above is manufactured in accordance with Current Good Manufacturing Practices and complies with the Federal Food Drug and Cosmetic Act and all other FDA regulations.

All ingredients from which this product is produced are in compliance with regulations and specifications from the FDA and Food Chemical Codex.

Regards,

[redacted]

Chris Drahota
QA Coordinator
cdrahota@bio-cat.com

Appendix 2 *Bacillus subtillis* BS50 Certificates of Analysis

Appendix 2.1. Lot No. OPTIBS50-OE27-1

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

Bacillus subtilis BS50

Lot Number: OPTIBS50-OE27-1
Date of Manufacture: 27-May-21

Test	Test Results	Product Acceptance Criteria	Method
Activity	111 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	4.84%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	AOAC 991.14
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.27 ppm	<0.5 ppm	ICP
Mercury	<0.01 ppm	<0.1 ppm	ICP
Cadmium	<0.01 ppm	<0.5 ppm	ICP
Arsenic	0.03 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s):	Non-genetically modified *Bacillus subtilis*
Country of Origin:	USA
Additional Ingredients:	Maltodextrin from waxy maize
Shelf Life:	24 Months
Storage:	Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

BIO-CAT Microbials
689 Canterbury Rd
Shakopee, MN 55379
P 952.445.4251
F 952.445.7233

[redacted] 6-8-21
Chris Drahota
Quality Assurance Coordinator

Appendix 2.2. Lot No. OPTIBS50-PE01-1

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

Bacillus subtilis BS50 100 Billion CFU/g

Lot Number: OPTIBS50-PE01-1
Date of Manufacture: 1-Jun-21

Test	Test Results	Product Acceptance Criteria	Method
Activity	106 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	5.30%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	AOAC 991.14
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.11 ppm	<0.5 ppm	ICP
Mercury	<0.01 ppm	<0.1 ppm	ICP
Cadmium	<0.01 ppm	<0.5 ppm	ICP
Arsenic	<0.03 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s):	Non-genetically modified *Bacillus subtilis*
Country of Origin:	USA
Additional Ingredients:	Maltodextrin from waxy maize
Shelf Life:	24 Months
Storage:	Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

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[redacted] 1/4/2022
Chris Drahota
Quality Assurance Coordinator

Appendix 2.3. Lot No. OPTIBS50-PE01-3

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

Bacillus subtilis BS50 100 BCFU/g

Lot Number: OPTIBS50-PE01-3
Date of Manufacture: 1-Jun-21

Test	Test Results	Product Acceptance Criteria	Method
Activity	113 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	5.31%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	AOAC 991.14
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.14 ppm	<0.5 ppm	ICP
Mercury	0.02 ppm	<0.1 ppm	ICP
Cadmium	<0.01 ppm	<0.5 ppm	ICP
Arsenic	0.03 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s): Non-genetically modified *Bacillus subtilis*
Country of Origin: USA
Additional Ingredients: Maltodextrin from waxy maize
Shelf Life: 24 Months
Storage: Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

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1.14.2022
Chris Drahota
Quality Assurance Coordinator

Appendix 2.4. Lot No. OPTIBS50-PE02-1

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

Bacillus subtilis BS50 100 BCFU/g

Lot Number: OPTIBS50-PE02-1
Date of Manufacture: 2-Jun-21

Test	Test Results	Product Acceptance Criteria	Method
Activity	105 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	5.60%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	AOAC 991.14
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.12 ppm	<0.5 ppm	ICP
Mercury	0.01 ppm	<0.1 ppm	ICP
Cadmium	<0.01 ppm	<0.5 ppm	ICP
Arsenic	<0.03 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s):	Non-genetically modified *Bacillus subtilis*
Country of Origin:	USA
Additional Ingredients:	Maltodextrin from waxy maize
Shelf Life:	24 Months
Storage:	Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

BIO-CAT Microbials
689 Canterbury Rd **P 952.445.4251**
Shakopee, MN 55379 **F 952.445.7233**

[redacted] -14-2022
Chris Drahota
Quality Assurance Coordinator

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001131	Feb 13, 2023
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Robert C. Boyd	Director of Compliance
Organization *(if applicable)*	
BIO-CAT Microbials, LLC	
Mailing Address *(number and street)*	
689 Canterbury Rd.	

City	State or Province	Zip Code/Postal Code	Country
Shakopee	Minnesota	55379	United States of America

Telephone Number	Fax Number	E-Mail Address
434-591-4661		rboyd@bio-cat.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
William Rowe	President
Organization *(if applicable)*	
GRAS Associates, LLC	
Mailing Address *(number and street)*	
11810 Grand Park Avenue, Suite 500	

City	State or Province	Zip Code/Postal Code	Country
North Bethesda	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
519-341-3660	1-888-531-3466	amozingo@gras-associates.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Bacillus subtilis BS50

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

1 CD

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8) and 170.250(d) and (e))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

B. subtilis BS50 is intended for use as an ingredient in a wide variety of foods, including baked goods and baking mixes, beverages and beverage bases (including carbonated and flavored waters, sports and nutritional drinks), breakfast cereals, cheese, chewing gum, coffee and tea, confections and frostings, dairy product analogs, frozen desserts (dairy, non-dairy and ices), gelatins, puddings and fillings, grain products and pastas, hard candy and cough drops, milk products, plant protein products, processed fruits and fruit juices, processed vegetables and vegetable juices, snack foods and soft candy. Maximum level of 2 x 109 CFU/serving.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that BIO-CAT Microbials, LLC

(name of notifier)

has concluded that the intended use*(s)* of Bacillus subtilis BS50

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. BIO-CAT Microbials, LLC *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

689 Canterbury Rd., Shakopee, MN 55379

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Amy Mozingo Digitally signed by Amy Mozingo Date: 2023.02.07 14:45:19 -05'00'	Amy Mozingo on behalf of William J. Rowe, President	02/06/2023

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form 3667_GRAS Notice_BS50 Transmittal Letter_BS50 BioCat_B.Subtilis BS50_GRAS Conslusion_Notification_Feb 2023	

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September 7, 2023

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety
Division of Petition Review
5001 Campus Drive
College Park, MD 20740-3835

Attention: Dr. Kaiping Deng
Re: GRN 001131—Response to Questions Posed in an FDA Letter Dated August 24, 2023

Dear Dr. Deng:

Per your request, GRAS Associates, LLC, acting as the agent for BIO-CAT Microbials, is providing a response to FDA's request.

FDA's Question for the Notifier and Responses

1. Please clarify whether the strain genome data is available in a public domain, e.g., what the NCBI accession number is.

> Response: The *Bacillus subtilis* PTA-127287 whole genome sequence data is not currently available in a public domain. The sequence data is on file at BIO-CAT Microbials and available upon request. Additionally, the strain is deposited with the ATCC (American Type Culture Collection) (Manassas, Virginia).

2. For the administrative record, please briefly specify how the purity of the *B. subtilis* PTA-127287 inoculum for the manufacturing process is ensured.

> Response: Seed inoculum preparation: To begin, a frozen working vial of strain PTA-127287 is streaked to tryptic soy agar (TSA). TSA plates are inverted and incubated at 35°C overnight. BS50 colonies on the TSA plate are checked for typical morphology, then two to three typical and well isolated colonies are used to inoculate sterile growth medium in a Fernbach style flask. This Fernbach flask is used as the seed inoculum for the commercial bioreactors.
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> All streaks and inoculations are completed in a level II biosafety cabinet. The biosafety cabinet is additionally located in a HEPA filtered ISO 8/Class 100,000 modular cleanroom whereby the only activity in that cleanroom is production seed inoculum preparation.
>
> We have molecular (PCR) based methods to detect sample purity as well. These methods are used to monitor purity throughout the commercial production as needed.

3. Please confirm that the fermentation process is continuously monitored for contaminants. In addition, please provide a statement that all materials used in the manufacture of *B. subtilis* PTA-127287 are

permitted for their respective uses under a current U.S. regulation, are the subject of an effective food contact notification, or are GRAS for their intended use.

> Response: The seed inoculum is sampled after inoculation and streaked to TSA as well as selective and differential media (Bismuth Sulfite Agar (BS), Hekoen Enteric Agar (HE), Xylose-Lysine Deoxycholate Agar (XLD) and MacConkey Sorbitol Agar (SMAC)) to ensure no microbial contamination was present at the time of inoculation. During the fermentation process, there is continuous monitoring of parameters such as temperature, air flow, pH, dissolved oxygen, and carbon dioxide production. These profiles are compared to previous runs in production and from development to assess overall growth and health of the culture. Unusual readings or patterns can be used to identify possible contamination as early as possible and drive further testing. Additionally, samples at multiple points during fermentation are viewed under phase contrast microscopy and plated directly to TSA, BS, HE, XLD, and SMAC to confirm the absence of any microbial contaminants.
>
> During centrifugation, multiple samples of the slurry and supernatant are plated directly to TSA to ensure purity. Additionally, the slurry is directly plated to BS, HE, XLD, and SMAC to ensure the absence of microbial contaminants. Samples of the final “raw” spray dried BS50 are incubated in Tetrathionate broth, MacConkey broth, and Buffered Listeria Enrichment Broth (BLE) for 24 hours and then plated on BS, HE, XLD, SMAC, and PALCAM to ensure the absence of gram negative specifically focusing on E. coli, Salmonella, and Listeria.
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> All enrichments, plating, and analysis are completed by trained lab personnel who have knowledge of typical and atypical results for all assays. Follow up testing using either internal PCR or 3rd party lab sequencing can be used to identify any suspect results.
>
> Bio-Cat Microbials confirms that all materials used in the manufacture of *B. subtilis* PTA-127287 are permitted for their respective uses under a current U.S. regulation, are the subject of an effective food contact notification, or are GRAS for their intended use.

4. We have the following questions/comments regarding the specifications and test methods listed in Table 4 (page 14) and the results of the batch analyses in Table 5 (pages 14 and 15):

a. You provide the specification for total viable spore count as not less than (NLT) 100 billion colony forming units (CFU)/g and list FDA BAM Chapter 3 as the test method. We note that the FDA BAM Chapter 3 is for aerobic plate count but not for total viable spore count. On page 11, you state that “a total aerobic enumeration is compared to an aerobic enumeration that has been heat treated (80 °C for 5 min)” to ensure that the final preparation consists entirely of spores (i.e., only spores will survive the heat treatment). Please clarify whether the specification for the total viable spore count in Table 4 is total aerobic enumeration after the final product has been heat treated.

> Response: The total viable spore counts listed in Table 5 are total aerobic counts without heat treatment. Heat treatment as described on page 11 of the dossier is applied to upstream samples to determine sporulation efficiency. Because only spores will survive the spray drying process, once the spray dried material is standardized on an appropriate diluent (maltodextrin for example), heat treatment is not applied to the final product aerobic enumeration.

b. You provide the specification for Staphylococcus aureus (S. aureus) as <10 CFU/g and list the FDA BAM Chapter 12 as the test method. We note that this test method is suitable for the analysis of foods

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in which more than 100 S. aureus cells/g may be expected. Please clarify the limit of detection (LOD) for testing S. aureus in the final product (i.e., whether the LOD is 10 or 100 CFU/g).

> Response: The FDA BAM Chapter 12 method does indicate that if plates of the lowest dilution have less than 20 colonies, these plates may be used for enumeration (see step D, part 2) and because our lowest dilution plated is 1:10, the LOD for a 1:10 dilution would be <10 CFU/g when plating 1 mL of said dilution.

c. You state that the analyses of heavy metal content of B. subtilis PTA-127287 are performed by a lab that uses an internally validated method. Please provide the LODs for the method used or specify if the LODs are the same as the "acceptable target/range" parameters for heavy metals listed in Table 5.

> Response: We use certified and accredited third-party labs such SORA and Eurofins for all heavy metal analysis. Within the scope of their accreditation, SORA lists limits of quantification (LOQ) as: lead 0.01 ppm, mercury 0.01 ppm, cadmium 0.01 ppm, and arsenic 0.03 ppm. These limits of quantification are appropriate for our current limits and the newly proposed limits in response 4. d. below.

d. Please discuss the source of lead in your GRAS subject. We note that we typically see lower levels for lead for ingredients produced using controlled fermentation and following current good manufacturing practices (Please see GRNs 001074 and 001075 that were recently posted on our GRAS Notice inventory). We also note that the provided results of the batch analyses indicate that low levels of arsenic and cadmium can be achieved. Keeping in line with FDA's Closer to Zero initiative that focuses on lowering dietary exposure to heavy metals, we recommend that you consider lowering the specification limits for lead, arsenic, and cadmium to be as low as possible.

> Response: Traces of lead may be present in each ingredient used in fermentation. Specifications for the heavy metals will be lowered as follows: lead <0.3 ppm, mercury<0.05 ppm; cadmium <0.1 ppm; arsenic <0.2 ppm.

e. Please confirm that all analytical methods used to analyze the batches for the specification parameters are validated for their respective uses.

> Response: We confirm that all analytical methods used to analyze batches of PTA-127287 have been validated for their respective uses. Internal methods have been validated via in-house studies and results have been supported via accredited third-party lab confirmation.

5. In Table 6 (pages 16 and 17), you list the food categories in which B. subtilis PTA-127287 is intended to be used.

a. Please clarify whether the intended food uses are limited to the food categories and specific foods listed in Table 6, or if the ingredient is intended to be used in all conventional foods except alcoholic beverages, infant formula, and products under the jurisdiction of the United States Department of Agriculture.

> Response: The intended uses are limited to food categories described in Table 6. The specific foods listed for the categories in Table 6 are as defined in 21 CFR 170.3(n). Intended use is not limited to those specific foods but for use in general for the food category. As noted in Table 6, the intended use does not include use in infant formula or products under the jurisdiction of the

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United States Department of Agriculture. Intended use does not include use in alcoholic beverages.

b. Please clarify whether the intended uses include the use in cough drops. We are aware that cough drops are listed as food category under 21 CFR 170.3(n)(25). However, given the fact that most cough drops are marketed as over-counter drugs, we recommend that cough drops were not among the intended uses for your ingredient.

Response: The intended use does not include use in over-the-counter drug products such as cough drops.

6. Among the Clinical Safety Data on Bacillus subtilis (other strains) (pages 33-37), the study with B. subtilis strain C-3102 had the longest duration (28 days) administered in a clinical study at the highest dose (4.8×1010 CFU/day) studied, as mentioned on p.33. The livestock safety studies with the strain B. subtilis C-3102 are used to support the safety of the current GRAS subject B. subtilis PTA-27287. Please provide information on the homology between the B. subtilis strains C-3102 and PTA-127287. We also note that there is a typo on the "B. subtilis C-3102" strain title on page 36 (i.e., the title is noted as B. subtilis C-3012).

Response:

Thank you for identifying the typo. We confirm the title "c. B. subtilis C-3012" is in error. It should be "c. B. subtilis C-3102".

To our knowledge, there is no publicly available sequence for strain C-3102 thus a direct comparison between nucleotides of *Bacillus subtilis* PTA-127287 genome sequence and strain C-3102 is not possible at the present time. During the time of the human clinical trials with C-3102, the strain was identified as a *Bacillus subtilis* strain and thus was included in this dossier as continued evidence of safety for *Bacillus subtilis* strains. However, with recent taxonomic classification changes, strain C-3102 is now noted to be classified as *Bacillus velezensis* (EFSA, 2021)[1]. It is likely that *Bacillus subtilis* PTA-127287 is more closely related to the other *Bacillus subtilis* strains listed in pages 33-37 than strain C-3102.

7. Using the upper intake level of 18.2 servings of food/day and the assumption that B. subtilis PTA-127287 is added to every food category proposed, at the maximum use level of 2 x 10^9 CFU/serving, the maximum estimated daily intake (EDI) is 3.64 x 10^{10} CFU/day (page 17). According to the results of human studies with the strain (pages 52-53), you conclude that B. subtilis PTA-127287 at 2 x 10^9 CFU/day is safe in healthy adults (p.53). Please justify how the level of 2 x 10^9 CFU/day in the human study supports the EDI level of 3.64 x 10^{10} CFU/day.

Response: We did not mean to imply that this study would support the EDI. Note that on page 58 of the dossier, the results of this study are mentioned under the following heading, which we believe to be truthful and not misleading: "In addition, the strain specific data available for *B. subtilis BS50*, based on in silico/in vitro and clinical data demonstrate a lack of safety concerns

[1] EFSA (2021). Safety and efficacy of a feed additive consisting of Bacillus velezensis DSM 15544 (Calsporin®) for piglets (suckling and weaned), pigs for fattening, sows in order to have benefit in piglets, ornamental fish, dogs and all avian species (Asahi Biocycle Co.). EFSA Journal 19(11): 6903.

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for this strain based on the following…". The clinical study with PTA-127287 does demonstrate a lack of safety concerns for the strain.

8. Note, there is no number 8 in the FDA query letter.

9. For supporting the safety conclusion, you list five GRAS notices (i.e., GRAS Nos. 000969, 000956, 000955, 000905 and 000831) related to B. subtilis strains that have received no question letters from FDA. As the information submitted for GRAS notices should support the safety of the GRN 001131 subject, please provide a brief paragraph summarizing the information pertaining to safety for each of these GRAS notices. We note that the notifier for GRN 001007 requested that FDA cease to evaluate the notice and therefore, it should not be included to support the safety conclusion in GRN 001131.

Response: The status of GRN 001007 is changed to "FDA ceased to evaluate" instead of pending as directed and is not used to provide evidence of safety of *B. subtilis* BS50. Also, information for GRN 831 is not relevant for *B. subtilis* strains as the strain has been reclassified to *B. inaquosorum* (*formerly B. subtills subsp. Inaquosorum*). All of the *B. subtilis* GRN mentioned above showed that the genomes did not code for toxins or virulence factors and were not resistant to clinically relevant antibiotics. The *B. subtilis* GRNs mentioned above also mention the QPS status and a safe history of use of *B. subtilis* in fermented food such as natto. Like the GRN for *B. subtilis* BS50, the strains associated with GRN 956, GRN 955, and GRN 905 successfully navigated through the Pariza decision tree. Further, like the GRN for *B. subtilis* BS50, GRN 969, 956, 905, and 831 also included results of testing for the ability of the strain to cause cytotoxicity and/or hemolysis. As stated in the dossier for *B. subtilis* BS50, the highest estimated intake of *B. subtilis* in the successfully notified GRAS Determinations was up to 2.78 $x10^{11}$ CFU/day, for *B. subtilis* "Bss-19" spore preparation (GRN 969). This dossier mentioned that FDA had accepted previous GRAS notices for an intake of *B. subtilis* inaquosorum strain DE111 at up to 1.3 $x10^{11}$ CFU/day (GRN 831) and *B. subtilis* DSM 32444 at up to 5.0 x 10^{9} CFU/day (GRN 905). GRN 969 and 955 also mentioned that serious adverse events were not reported in clinical studies with up to 1 x 10^{10} CFU/day "B. Subtilis". GRN 969 and 956 also reported results of acute toxicity studies in rats with *B. subtilis* Bss-19 and PLSSC which showed oral LD_{50} values of > 5000 mg/kg bw and > 2000 mg/kg bw/day, respectively. An unpublished 90-day oral toxicity study of *B. subtilis* PLSSC in rats was cited in GRN 956 which showed a NOAEL of 1000 mg/kg bw/day (providing 1.62 x 10^{11} spores/kg bw/day), and a 14-day repeated-dose oral toxicity study of *B. subtilis* MB40 in rats was cited in GRN 955 which showed a NOAEL of 2000 mg/kg bw/day (equivalent to 3.7 x 10^{11} CFU/kg bw/day). Both of these studies are cited in the GRN for *B. subtilis* BS50. Nonclinical or clinical safety studies were not performed with the strain of interest for GRN 905 (*B. subtilis* SG188 is also referred to as DSM 32444), however, the authors provided a discussion of available safety studies, animal feeding studies, and human clinical trials that supported safety of the species.

10. Please provide updated information on the literature searches performed to prepare the notice. This includes the date(s) (e.g., month and year) of the search, the resource databases used (e.g., PubMed), the principal search terms used, and the time period that the search spanned (e.g., 1/2000 to 7/2023).

Response: GRAS Notice and NDIN inventory websites through January 10, 2023 (search Bacillus subtilis) and pertinent references and safety information contained in the GRN and NDIN that were located.

PubMed (2 search strings):

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("Bacillus subtilis") and (human or humans or rat or rats or mouse or mice or hamster or hamsters or rabbit or rabbits or dog or dogs or cats or pig or pigs) and (safe* or toxic* or "adverse effect" or "adverse event" or "case report" or growth) Oct 9, 2019 (search confined to 541 hits over the previous 5 years)

("Bacillus subtilis") and (rat or rats or mouse or mice or dog or dogs or pig or pigs or rabbit or rabbits or human or humans) and (NOAEL or safe or safety or performance), January 1, 2019-January 10, 2023.

11. Comment (response not needed): On page 12, you state that “Post cleaning and sanitation, the equipment is swabbed for microbes and allergens utilizing ATP technology.” We note that ATP testing is a cleaning verification process to detect ATP in living cells such as microorganisms on a food processing surface. To verify the cleanliness of surfaces by detecting allergen residues, an allergen swab kit should be used because other Bacillus strains that utilize dairy and soy ingredients as growth media are manufactured in the same fermentation equipment.

Response: Noted. Additionally, since submitting this dossier, the ingredient containing dairy (casein) and soy has been removed from the facility completely. We no longer manufacture any Bacillus strains using dairy and soy; thus, there are no longer allergens used in the fermentation equipment.

Should there be any more questions or requests for information, please contact me directly.

Sincerely,

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September 21, 2023

Food and Drug Administration
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Attention: Dr. Kaiping Deng
Re: GRN 001131—Response to Questions Posed in an FDA Letter Dated September 19, 2023

Dear Dr. Deng:

Per your request, GRAS Associates, LLC, acting as the agent for BIO-CAT Microbials, is providing a response to FDA's request.

FDA's Question for the Notifier and Responses

Question:

In response to our request to lower the specifications for heavy metals, you lowered the specification for arsenic from <0.3 mg/kg to <0.2 mg/kg. We note that results from your batch analyses for arsenic are all ≤0.03 mg/kg. We request that you consider further lowering the arsenic specification to align with your batch analyses and our Closer to Zero initiative.
Should there be any more questions or requests for information, please contact me directly.

Response:
Thank you for the opportunity to address this. BIO-CAT Microbials agrees to lower the arsenic specification to ≤0.1 mg/kg. This specification aligns with the results of over a years' worth of lot analysis data in which arsenic levels ranged from ≤0.03 mg/kg to 0.08 mg/kg. Lowering the specification to ≤0.1 mg/kg is technologically feasible and aligns with the FDA's Closer to Zero initiative.

Sincerely,

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