JHeimbach LLC

June 4, 2020

Susan J. Carlson, Ph.D., Director
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

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, Advanced Enzymes Techologies, Ltd., through me as its agent, hereby provides notice of a claim that the addition of *Bacillus subtilis* strain PLSSC to conventional foods is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Advanced Enzymes Techologies, Ltd., has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

A CD is enclosed containing Form 3667, the GRAS monograph, and the signatures of members of the GRAS panel in a zip directory produced through COSM.

If you have any questions regarding this notification, please feel free to contact me at 202-320-3063 or jh@jheimbach.com.

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

1205 Prince Edward Street, Fredericksburg Virginia 22535, USA
tel. (+1) 804-742-5548 cell (+1) 202-320-3063 jh@jheimbach.com

GRAS NOTIFICATION

Bacillus subtilis PLSSC (ATCC SD 7280)

Advanced Enzyme Technologies Ltd.

5th Floor, 'A' wing, Sun Magnetica,

L.I.C. Service Road, Louiswadi,

Thane (W) – 400 064, INDIA

Table of Contents

Part 1. Signed Statements and Certifications

1.1 GRAS Notice Submission

Advanced Enzymes Technologies Ltd. submits this GRAS notice through its agent James T. Heimbach, president of JHeimbach LLC, in accordance with 21 CFR part 170, subpart E.

1.2 Name and Address of Notifier

APPLICANT

Name:	Advanced Enzyme Technologies Ltd.
Address:	5th Floor, 'A' wing, Sun Magnetica LIC Service Road, Louiswadi
Postal code and City:	Thane (W), India 400604
Country:	India
Tel. no:	+91 22 41703200
Fax no:	+91 22 25835159
E-mail:	info@advancedenzymes.com

MANUFACTURER

Name:	Advanced Enzyme Technologies Ltd.
Address:	5th Floor, 'A' wing, Sun Magnetica LIC Service Road, Louiswadi
Postal code and City:	Thane (W), India 400604
Country:	India
Tel. no:	+91 22 41703200
Fax no:	+91 22 25835159

PERSON RESPONSIBLE FOR THE DOSSIER

Name:	Dr. Anil Kumar Gupta VP – Research & Development
Address:	Advanced Enzyme Technologies Ltd. 5th Floor, 'A' wing, Sun Magnetica LIC Service Road, Louiswadi
Postal code and City:	Thane (W), India 400604
Country:	India
Tel. no:	+91 22 25830284
E-mail:	anil@advancedenzymes.com

US AGENT

Name:	James T. Heimbach, Ph.D., F.A.C.N. JHeimbach LLC
Address:	923 Water Street #66
Postal code and City:	Port Royal, Virginia 22535
County:	USA
Tel. no:	+1 804-742-5543
E-mail:	jh@jheimbach.com

1.3 Name of Notified Microorganism

Bacillus subtilis strain PLSSC is the designation of the proprietary *Bacillus subtilis* strain of Advanced Enzyme Technologies Ltd. The strain, originally isolated from soil, is deposited at the American Type Culture Collection (ATCC), USA, under strain designation SD-7280.

The product *Bacillus subtilis* PLSSC (SD-7280) is a spore preparation which contains no viable vegetative cells. Commercial preparations of *Bacillus subtilis* PLSSC (SD-7280) are known as BioSEB BS and SEBtilis.

In this GRAS notice, the *Bacillus subtilis* strain PLSSC is also referred by names such as *'Bacillus subtilis* PLSSC'*; 'B. subtilis* PLSSC*'* or *Bacillus subtilis* SD-7280, *Bacillus subtilis* subspecies *subtilis.*

1.4 Intended Conditions of Use

Based on history of safe use in food and demonstrated safety, *Bacillus subtilis* PLSSC is intended to be used as food ingredient in the following food categories at a level of approximately $1x10^6$ to $6x10^9$ colony forming units (cfu)/serving:

Baked goods and baking mixes, breakfast cereals, beverages and beverage bases, coffee and tea; milk and milk products, dairy product analogs, fruit juices, condiments and relishes, confections and frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, gelatins, jams and jellies, puddings and fillings; grain products and pastas; hard candy, soft candy, chewing gum, extracts, and flavorings, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant protein products, processed fruits, processed vegetables and vegetable juices, snack foods, soups and soup mixes, sugar and sweet sauces, toppings, and syrups.

Based upon the estimated number of servings of food consumed per day in the United States and the highest intended addition level of *Bacillus subtilis* per serving, the estimated daily intake (EDI) of the strain is $1.1x10^{11}$ cfu/day. (This EDI would be reached only if all target foods indeed contained *B. subtilis* at the maximum addition level.). The intended use of *B. subtilis* strain PLSSC is identical to the use of a *Bacillus subtilis* strain previously determined to be GRAS (GRN No. 000831 for *B. subtilis* DE111). It therefore provides an alternate source of the microorganism in the spore preparation added to these foods, but would not result in any change in exposure to the species.

B. subtilis PLSSC is not intended for use in foods that are targeted toward infants, such as infant formulas or foods formulated for infants, nor in meat and poultry products that come under USDA jurisdiction.

1.5 Statutory Basis for GRAS Status

Advanced Enzyme Technologies Ltd., has determined that the intended use of *Bacillus subtilis* PLSSC is GRAS through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

1.6 Premarket Exempt Status

Since Advanced Enzyme Technologies Ltd. has determined that the intended use of *Bacillus subtilis* PLSSC is GRAS, its use as described is exempt from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data Availability

Advanced Enzyme Technologies Ltd. agrees to make the data and information that are the basis for the determination of GRAS status available to FDA upon request. Such data and information may be sent by Advanced Enzyme Technologies Ltd. to FDA either in electronic format or on paper or reviewed during customary business hours at the home office of JHeimbach LLC, located at 923 Water Street, Port Royal VA 22535.

1.8 FOIA Statement

None of the data and information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

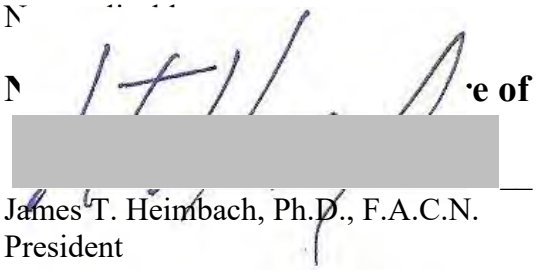
1.9 Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of *Bacillus subtilis* strain PLSSC.

1.10 FSIS Statement

N

1.11 N e of Notifier



James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to Advanced Enzymes Technologies Ltd.

Part 2. Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity/ Identification

The notified microorganism is a spore preparation of *Bacillus subtilis* PLSSC. This strain is a member of the *subtilis* subgroup of *Bacillus subtilis*. The diluents used in the manufacturing of *Bacillus subtilis* PLSSC are approved as either food additives or GRAS substances.

2.1.1 SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES

Name of the food ingredient: *Bacillus subtilis* PLSSC

Synonyms: *Bacillus subtilis* strain PLSSC / *Bacillus subtilis* (strain PLSSC)/ *B. subtilis* PLSSC

Taxonomy:

Kingdom: Bacteria

Phylum: Firmicutes (Gram positive spore forming bacteria)

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: Bacillus

Species: subtilis

Bacillus subtilis was originally named *Vibrio subtilis* by Christian Gottfried Ehrenberg and renamed *Bacillus subtilis* by Ferdinand Cohn in 1872 (Ehrenberg 1835; Cohn 1872). *Bacillus subtilis* has historically been classified as an obligate aerobe, though evidence exists that it is a facultative anaerobe. A strain of *Bacillus subtilis,* formerly known as *Bacillus natto* in Japan and Korea, is used in the commercial production of the Japanese food natto as well as the similar Korean food cheonggukjang.

2.1.2 DESCRIPTION/SOURCE INFORMATION AND GENOTYPIC, PHENOTYPIC CHARACTERIZATION OF THE ORGANISM

B. subtilis PLSSC is a nonpathogenic, non-toxicogenic naturally encapsulated spore-forming bacterium, light brown to brown coloured powder, that was originally isolated from soil. *Bacillus subtilis* is a member of subgroup *subtilis* of *Bacillus subtilis*. *B. subtilis* PLSSC is deposited in the American Type Culture Collection (ATCC), USA, with deposition number SD-7280.

2.1.2.1 Genotypic Characterization

Genotypic characterization of *B. subtilis* PLSSC was carried out using 16S rRNA and genomic sequencing.

a) 16S rRNA

B. subtilis PLSSC was identified using 16S rRNA and *gyrB* genes as phylogenetic markers. The *Bacillus* genus comprises strains which are closely related, and *Bacillus* species can be distinguished from one another using 16S RNA and gyrB gene sequences. Based on these sequence analyses, the strain *B. subtilis* PLSSC is identified as *Bacillus subtilis* subsp. *subtilis*.

b) Genomic Sequencing

Hybrid assembly was performed using Illumina and nanopore reads by MaSuRCA Hybrid Assembler (Zimin et al. 2013). In this case, *B. subtilis* KCTC 3135 strain was used as a reference. The final genome assembly was 4,204,670 bp in size with 43.58% G+C content. Gene prediction was done for the assembled genome using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016). The whole-genome shotgun project was deposited in NCBI/GenBank under the accession number CP031129.

The assembled genome of *B. subtilis* PLSSC was compared with other bacillus genomes present in the RefSeq genome database using NCBI-BLASTN (Altschul et al. 1990). *B. subtilis* (taxid:1423) was chosen as reference database for NCBI-BLASTN. The BLASTN results indicated ~99% sequence homology of the *de-novo* assembled genome with the genome of the reference strain *B. subtilis* KCTC 3135.

c) Determination of mol G+C%

The genomic DNA G+C content, defined as the proportion of guanines and cytosines within the overall number of nucleotides in the genome, is one of the features in taxonomic descriptions of micro-organisms (Meier-Kolthoff et al. 2014). *B. subtilis* G+Cs mol% for the final genome assembly of 4,204,670 bp is 43.58%, which is similar to the G+C mol% of other *Bacillus subtilis* strains, reported as 43.5% to 43.9% (GRN 831).

d) Safety assessment in relation to antibiotic resistance genes

A homology search between the assembled genome of *B. subtilis* PLSSC and antibiotic resistance genes/proteins was performed using the Comprehensive Antibiotic Resistance Database (CARD). In this case, BLASTX was used with criteria: similarity >30%, coverage >70%, and e-value $<1e^{-02}$ for the identification of significant hits. Through the above analysis, 717 putative antibiotic resistance genes were identified which belonged to the following functions: defense mechanism (474); signal transduction mechanism, transcription (136); carbohydrate transport and metabolism, amino acid transport and metabolism, inorganic ion transport and metabolism, general function prediction only (32); cell wall/membrane/envelope biogenesis (10); coenzyme transport and metabolism, energy production and conversion (06); general function prediction only (38); replication, recombination and repair (2); coenzyme transport and metabolism, energy production and conversion (1); tunicamycin (1) (DUT89_01190); aminoglycoside 6-adenylyl-transferase (1) (DUT89_13540). Critically important antimicrobials (CIAs) or highly important antimicrobials as per WHO (2016) and EFSA (2012) were screened in the data, which were analyzed post homology alignment of the assembled genome of strain *Bacillus subtilis* PLSSC and CARD. Full coding genes for resistance to tunicamycin (tmrB gene) and beta-lactamase were found on the genome. These genes are inherent to the species and hence referred to as intrinsic resistance.

The absence of mobile elements in the flanking regions of the above-mentioned antibiotic resistance genes was determined using ISfinder web-based software (Siguier et al. 2006) and using ACLAME database (Leplae et al. 2009). None of the genes coding for or contributing to resistance to antimicrobials has mobile elements in its flanking region, and do not pose any safety concerns.

To confirm the genotype analysis, *B. subtilis* PLSSC was tested as per CLSI guidelines for its

sensitivity/resistance against nine antibiotics: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol. *B. subtilis* PLSSC was sensitive to all the tested antibiotics. The minimum inhibitory concentration (MIC) breakpoint values observed for *B. subtilis* PLSSC were below or equal to the EFSA breakpoints (2012) for all the antibiotics.

e) Analyses of risk associated with virulence factor genes

Virulence factor genes/proteins were downloaded from the Virulence Factor Database (VFDB) (Chen et al. 2004). A homology search between the assembled genome of *B. subtilis* strain PLSSC and virulence factor proteins was performed using BLASTX (criteria: similarity >30%, coverage >70%, and e-value $<1e^{-02}$) to identify significant hits. Multiple putative virulent factor genes were identified through the VFDB, but the majority of them were related to transport mechanisms and so could not be considered as harmful. Also, most of the genes identified were related to extracellular structure, which could be correlated to the adhesion property which is a desirable probiotic trait.

To further confirm non-virulence of *Bacillus subtilis* PLSSC, *in vitro* cytotoxicity testing against Vero cells was carried out as recommended by EFSA (2014). The fluorescence values observed for the samples from *B. subtilis* PLSSC were less than 20% of the positive control fluorescence, indicating that the strain did not have any cytotoxic effect. The results show no safety concern in *B. subtilis* PLSSC with regard to virulence factors. (Refer also to section 2.1.4)

f) Identification of biogenic amine producing genes

Protein sequences of short-listed biogenic-amine producing genes (amino acid decarboxylases) were downloaded from the Uniprot database. BLASTX was performed between the assembled genome and biogenic-amine producing proteins. Only one amino acid decarboxylase, arginine decarboxylase (DUT89_07295), was identified with 100% homology against biogenic-amine producing proteins. Phenotypic analysis was carried out to evaluate the functionality of the arginine decarboxylase gene and it was found to be non-functional or not expressed at a level sufficient to produce detectable amounts of biogenic-amine under the tested conditions.

g) Identification of mobile elements in assembled genome

Mobile elements are DNA sequences that can move around the genome, often affecting the activity of nearby genes. These mobile elements include DNA transposable elements, transposons, transposases, plasmids, and bacteriophage elements. Mobile elements were predicted from the assembled genome by using ISfinder web-based software and ACLAME database version 0.4.

Twelve insertion sites (IS element regions) were identified in the assembled genome. In addition, all the nucleotide sequences which include plasmids, viruses, and prophages were downloaded from ACLAME database. A homology search (BLASTN) was performed between the nucleotide sequences downloaded (1,25,190) from the above-mentioned database and the assembled genome. There were 497 regions that had significant hits (coverage ≥50% and e-value $\leq 1e^{-05}$) against the mobile element nucleotide sequences downloaded from ACLAME database (Leplae et al. 2009).

Mobile elements were not found in the vicinity of regions of concern such as antibiotic resistance genes, virulence factor genes, and biogenic-amine producing genes, suggesting stability of the genome and consistent safe use of the strain.

h) Analyses of toxin genes

Gene mining was performed to find genes related to diarrheal enterotoxin bceT, hemolytic enterotoxin operon (*hbl* genes – *hblA, hblC, hblD*), non-hemolytic enterotoxin operon (nhe ABC genes – *nheA, nheB, nheC*), cytotoxin K (*cytK*), enterotoxin FM (*entFM*), and emetic toxin cereulide (*cesB*). None of these toxin producing genes was identified in the genome, indicating that *B. subtilis* PLSSC does not produce these toxins.

i) Identification of CRISPR associated regions in assembled genome

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences were screened in the assembled genome of *B. subtilis* PLSSC using CRISPR Finder (Couvin et al. 2018). CRISPRs are direct repeats found in the DNA of many bacteria (~40% of sequenced bacterial genomes). Two CRISPRs were identified from the assembled genome of *B. subtilis* PLSSC. The presence of CRISPR system indicates an advantage in promoting genome stability by acting as a barrier to entry of foreign DNA elements.

Conclusion

The *de novo* assembled genome of *B. subtilis* PLSSC is generated without gaps, which resulted in a single scaffold. There are no significant mobile elements identified in the vicinity of the loci which have significant homology against antibiotic resistance genes, virulence factor genes, biogenic-amine producing genes, or enterotoxin genes. The presence of a CRISPR sequence in the assembled genome indicates an advantage in promoting genome stability by acting as a barrier to the entry of foreign DNA elements. In conclusion, *B. subtilis* PLSSC does not contain any sequences/genes in the genome that are risk associated, thus confirming the safety of the strain through the genome-based approach.

2.1.2.2 Phenotypic and Biochemical characterization

Bacillus subtilis PLSSC is a motile, rod shape, endospore forming, Gram positive, catalase positive bacterium. Cell size ranges from 0.7 µm to 0.8 µm in width and 2.0 µm to 3.0 µm in length. *B. subtilis* PLSSC colonies are seen with swarming activity on agar surfaces. After 24 hours of incubation on Nutrient Agar at 37°C, colonies are opaque, thick, round to irregular,

wrinkled (rugose), cream colored or brown, with a dull surface. *B. subtilis* PLSSC produces ellipsoidal to cylindrical endospores, located terminally or sub-terminally, and sporangia are not swollen.

Biochemical tests were performed on *B. subtilis* PLSSC following the standard microbiological methods recommended by *Bergey's Manual of Systematics of Archaea and Bacteria. B. subtilis* PLSSC tests positive for catalase, oxidase (variable), gelatinase, protease (casein), and amylase enzymes. The strain shows a negative result for indole production and methyl red but shows positive results on Voges-Proskauer and citrate tests. *B. subtilis* PLSSC shows a negative test result for urease, but positive for the presence of the enzyme nitrate reductase. In the TSI test, the strain shows no gas or hydrogen sulfide production but shows yellow (acidic) butt and slant.

B. subtilis PLSSC ferments D-glucose, sucrose, maltose, starch, dextrin, glycerol, mannitol, xylose, D-fructose, D-galactose, inulin, D-mannose, D-sorbitol, and D-trehalose.

The results of biochemical tests of *B. subtilis* PLSSC are comparable to the reference strain of *Bacillus subtilis* ATCC 6051; the data provided by Logan et al. (2015)--*Bacillus*: *Bergey's Manual of Systematics of Archaea and Bacteria* are provided below in Table 1. The strain was characterized as a member of the genus of *Bacillus* and species *subtilis.*

Table 1. Results of Morphological and Biochemical Tests

Test	Results	
	***Bacillus subtilis* PLSSC**	***Bacillus subtilis* ATCC 6051**
Colony Characteristics	Colonies white to cream colored, circular to irregular, thick, opaque with wrinkles and dull surface	Colonies white to cream colored, circular to irregular, thick, opaque with wrinkles and dull surface
Gram Staining	Gram positive	Gram positive
Cell Morphology	Cells motile, rod shaped	Cells motile, rod shaped
Size	Cells 0.7 µm - 0.8 µm in width and 2.0 µm - 3.0 µm in length	Cells 0.7 µm - 0.8 µm in width and 2.0 µm - 3.0 µm in length
Arrangement	Single cells or pairs or in short chains	Single cells or pairs or in short chains
Catalase Test	Positive	Positive
Oxidase Test	Positive	Positive
Nitrate Reduction Test	Positive	Positive
Endospore Stain	Spores ellipsoidal to cylindrical, located terminally or subterminally, and do not deform the cell	Spores ellipsoidal to cylindrical, located terminally or subterminally, and do not deform the cell
Motility	Motile	Motile
Oxygen Requirement	Aerobic	Aerobic
Fermentation Type	Heterofermentative	Heterofermentative
Indole Test	Negative	Negative
Methyl Red Test	Negative	Negative
Voges-Proskauer Test	Positive	Positive
Citrate Utilization Test	Positive	Positive
Urease Test	Negative	Negative
Triple Sugar Iron (H_2S) Test	No production of hydrogen sulfide, Acidic Slant and Butt	No production of hydrogen sulfide, Acidic Slant and Butt

Test	Results	
	***Bacillus subtilis* PLSSC**	***Bacillus subtilis* ATCC 6051**
Gelatin Hydrolysis Test	Positive	Positive
Casein Hydrolysis Test	Positive	Positive
Starch Hydrolysis Test	Positive	Positive
L(+) Lactic Acid	Positive	Positive
Lecithinase Production	Negative	Negative
Hemolysis	Negative	Negative
Bile degradation	Positive	Positive
Sugar Fermentation Tests		
D-Glucose	Acid produced, No gas produced	Acid produced, No gas produced
Sucrose	Acid produced, No gas produced	Acid produced, No gas produced
Maltose	Acid produced, No gas produced	Acid produced, No gas produced
Starch	Acid produced, No gas produced	Acid produced, No gas produced
Dextrin	Acid produced, No gas produced	Acid produced, No gas produced
Glycerol	Acid produced, No gas produced	Acid produced, No gas produced
Mannitol	Acid produced, No gas produced	Acid produced, No gas produced
Xylose	Acid produced, No gas produced	Acid produced, No gas produced
Rhamnose	No acid produced, No gas produced	No acid produced, No gas produced
D-Fructose	Acid produced, No gas produced	Acid produced, No gas produced
D-Galactose	Acid produced, No gas produced	Acid produced, No gas produced
D-Mannose	Acid produced, No gas produced	Acid produced, No gas produced
L-Arabinose	No acid produced, No gas produced	No acid produced, No gas produced
Inulin	Acid produced, No gas produced	Acid produced, No gas produced
D-Sorbitol	Acid produced, No gas produced	Acid produced, No gas produced
D-Trehalose	Acid produced, No gas produced	Acid produced, No gas produced
Source: Logan et al. (2015)		

As can be seen, *B. subtilis* PLSSC's phenotypic characteristics are the same *as Bacillus subtilis* ATCC 6051, which further confirms identity of the *Bacillus subtilis* PLSSC.

2.1.3 ANTIBIOTIC RESISTANCE (SUSCEPTIBILITY)

Three batches of the *B. subtilis* PLSSC strain were assessed for susceptibility to antibiotics following CLSI (2016) guidelines as recommended by EFSA (2018c).

Broth dilution assay was used to evaluate the antibiotic susceptibility of *B. subtilis* PLSSC against nine antibiotics and to determine the minimum inhibitory concentration (MIC). Antibiotics tested included clindamycin, chloramphenicol, ampicillin, gentamicin, erythromycin, kanamycin, vancomycin, streptomycin, and tetracycline, with the results shown in Table 2.

Table 2. Antibiotic Susceptibility of *B. subtilis* PLSSC

Antibiotic	*Staphylococcus aureus* ATCC 29213			*Bacillus subtilis* PLSSC		
	MIC range[1] (µg/ml)	MIC (µg/ml)	Interpretation	MIC break-point[4] (µg/ml)	MIC (µg/ml)	Interpretation
Clindamycin	0.06 – 0.25	0.25	S[3]	4	0.5	S
Chloramphenicol	2 – 16	8	S	8	2	S
Ampicillin	0.5 – 2	2	S	NR[5]	NR	NR
Gentamicin	0.12 – 1	0.5	S	4	≤0.06	S
Tetracycline	0.12 – 1	0.5	S	8	2	S
Streptomycin	NA[2]	NA	NA	8	8	S
Kanamycin	1 – 4	2	S	8	1	S
Vancomycin	0.5 – 2	2	S	4	0.5	S
Erythromycin	0.25 - 1	1	S	4	4	S

1. Source: CLSI (2016)
2. NA = not available in CLSI (2012)
3. S = susceptible
4. Source: EFSA (2012)
5. NR = not required (EFSA 2012)

Source: Advanced Enzyme Technologies

The minimum inhibitory concentration (µg/ml) for the tested antibiotics against *B. subtilis* strain PLSSC was within the recommended breakpoints specified by EFSA (2018c) for the antibiotics chloramphenicol, erythromycin, gentamicin, kanamycin, vancomycin, clindamycin, streptomycin, and tetracycline.

The antibiotic sensitivity profile of *B. subtilis* PLSSC was also checked by the disk diffusion method as described by CLSI (2012). The antibiogram profile was compared with the control strain, *Bacillus subtilis* ATCC 6051. Antibiotics tested included amoxycillin–clavulanic acid, cefaclor, cefoxitin, ceftizoxime, ceftriaxone, ceftazidime, amikacin, cefazolin, cefprozil, doxycycline, gentamicin, imipenem, kanamycin, lomefloxacin, nafcillin, nalidixic acid, neomycin, nitrofurantoin, norfloxacin, streptomycin, tobramycin, azithromycin, chloramphenicol, ciprofloxacin, ofloxacin, moxifloxacin, minocycline, meropenem, vancomycin, levofloxacin, cefepime, cefixime, cefotaxime, clindamycin, oxacillin, erythromycin, cefuroxime, and tetracycline.

Both strains were resistant to metronidazole. The reference strain *B. subtilis* ATCC 6051 was resistant to aztreonam but *B. subtilis* PLSSC strain was sensitive to it. The reference strain was sensitive to rifampicin while *B. subtilis* PLSSC was intermediate at the tested concentration.

2.1.4 VIRULENCE ACTIVITY

A test for cytotoxicity using Vero cells was performed to demonstrate that *B. subtilis* PLSSC is free from toxigenic potential (EFSA 2014).

The test is based on the principle that the DNA intercalating agent propidium iodide will stain DNA of cells having leaky cell membranes, thereby enhancing the resulting intracellular fluorescent signal. The DNA of intact cells would not show any uptake of propidium iodide, resulting in basal level, negligible fluorescence. Positive controls contained Triton x 100 treated cells with leaky cell membranes (100% fluorescence). Cytotoxicity of *B. subtilis* PLSSC at three different concentrations was measured in triplicate. A compound is considered to be active if the fluorescence unit (FU) values of the test sample are 20% or above of the values obtained from the positive controls. The study showed that *B. subtilis* PLSSC did not elicit cytotoxicity on Vero cells (Table 3).

Table 3. Test for Detection of Cytotoxicity Using Vero cells

Test Article	Fluorescence Units in Live Cells	Percent Fluorescence
Background	2.38	1.88
Positive control	126.68	100.00
Negative control	5.75	4.54
B. subtilis PLSSC – 10 µl	14.20	11.21
B. subtilis PLSSC – 50 µl	13.44	10.61
B. subtilis PLSSC – 100 µl	16.69	13.71
Source: Advanced Enzyme Technologies		

Percent fluorescence values for *B. subtilis* PLSSC samples were less than 20% of the positive control fluorescence, indicating that the sample did not have any cytotoxic effect *in vitro* at 10-100 µl sample volume for the 2-hour incubation period.

2.1.5 ANTIMICROBIAL ACTIVITY

B. subtilis PLSSC was evaluated for its antimicrobial activity following CLSI (2012) guidelines as recommended by EFSA (2018) against five selected microorganisms (*Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Enterococcus faecalis* ATCC 29212); the United States Pharmacopoeia (USP 2008) against two microorganisms (*Escherichia coli* ATCC 8739 and *Staphylococcus epidermis* ATCC 12228); and the Food and Agriculture Organization (FAO 2006) against *Staphylococcus aureus* ATCC 6538. *B. subtilis* PLSSC showed an absence of antimicrobial activity against all of the selected test microorganisms.

2.1.6 ACID AND BILE SALT TOLERANCE

The spore preparation of *B. subtilis* PLSSC was tested by Advanced Enzyme Technologies for its ability to survive under different simulated gastrointestinal conditions through an *in vitro* study.

After 24 hours of exposure, *B. subtilis* PLSSC was stable in simulated saliva (95%), simulated intestinal fluid (100%), simulated colonic fluid (100%), fasting-state simulated gastric juice

(91%), and fed-state simulated gastric juice (96%) for up to 24 hours. The *in vitro* study concluded that *B. subtilis* PLSSC was stable and maintained its survivability under different simulated gastrointestinal conditions.

2.1.7 ENTEROTOXINS

B. subtilis PLSSC was found negative for enterotoxins when tested by ELISA and immunechromatography (LFD).

Further, *B. subtilis* PLSSC was also examined for the presence of enterotoxins (hemolysin, hbl; nonhemolytic, nhe; cytotoxin, cytK) and emetic toxin (cereulide, ces) producing genes using a molecular approach. The absence of PCR products for the toxin-related genes in *B. subtilis* PLSSC confirms the absence of the above-mentioned toxins.

B. subtilis PLSSC was concluded to be negative for non-hemolytic enterotoxins and emetic toxin.

Conclusion

B. subtilis PLSSC strain has been analyzed for risk-associated factors following genome-based analyses and phenotypic/biochemical studies. Various studies and analyses carried out on this strain showed no safety concerns.

2.2 Manufacturing Process

2.2.1 OVERVIEW

B. subtilis PLSSC is produced as spores by fed-batch type fermentation in accordance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing facility is ISO 9001:2015, ISO 22000, and GMP certified. Fermentation is a well-known process that occurs in food and has been used for the cultivation of microorganisms for decades, if not centuries. Liquid-state or submerged fermentation is used to produce *B. subtilis* PLSSC. The typical fermentation batch size ranges from 100 L to 50,000 L, preferably 20,000 L.

The key steps for production of *B. subtilis* PLSSC are fermentation, recovery, formulation, and packaging. The process is illustrated in Figure 1.

2.2.2 FERMENTATION

2.2.2.1 Raw materials

The following food-grade approved materials are used in the fermentation process (inoculum, seed, and main fermentation). There are no ingredients based on milk, soy, or any of the eight most widely recognized allergens.

- Potable water
- A carbon source
- A nitrogen source
- Salts
- Vitamins (as a part of complex fermentation materials)
- pH adjustment agents
- Foam control agent (at ≤0.1%)

2.2.2.2 Inoculum (Seed)

A suspension of a pure culture of *B. subtilis* PLSSC is aseptically transferred to an inoculum flask containing fermentation medium.

The culture is grown in the flask under optimum conditions in order to obtain a sufficient amount of biomass, which is subsequently be used as inoculum for the seed fermentation.

2.2.2.3 Seed Fermentation

The inoculum is aseptically transferred to the seed fermenter containing seed fermentation medium. When a sufficient amount of biomass has developed (typically up to 17 hours), the content of the seed fermenter is used for inoculation of the main fermentation.

2.2.2.4 Main fermentation

During the main fermentation, the growth (cell-mass) of *B. subtilis* PLSSC takes place and the vegetative cells are later converted to spores during late growth/stationary phase.

The fermentation in the main fermenter is operated as a batch and fed-batch fermentation. First, the content of the seed fermenter is aseptically transferred to the main fermenter containing fermentation medium. The fermentation process is continued for a predetermined time or until laboratory test data show that the desired biomass production has been obtained or that the rate

of biomass production has decreased below a predetermined production rate. When the desired spore count is reached, the fermentation is complete.

2.2.3 RECOVERY

The purpose of the recovery process is to separate the *B. subtilis* PLSSC spores from the fermentation media, concentrate the spores, and prepare dried powdered biomass.

The vegetative cells of *B. subtilis* PLSSC are converted to spores at the end of fermentation and are suspended in the fermentation media. During recovery, spores are separated from fermentation medium.

The steps of recovery include:

- Primary separation of spores (biomass) from the soluble media components
- Washing of concentrated spores (biomass)
- Spray drying

2.2.3.1 Primary Separation

The fermentation broth is passed through a high-speed centrifuge to separate the spores (biomass) from the soluble media components along with water. The spore biomass is collected as a thick slurry and subjected to further processing. Temperature and pH are controlled during this step.

2.2.3.2 Washing

Sterilized and demineralized water is added to the collected biomass slurry. Slurry is again passed through high-speed centrifuge and the washed biomass is collected. Temperature and pH are controlled during this step. The heat treatment assures that no viable vegetative cells remain in the preparation.

2.2.3.3 Spray Drying

The concentrated biomass suspension is spray-dried in presence of approved food-grade stabilizers (e.g., maltodextrin) to obtain the unformulated concentrate.

2.2.4 FORMULATION AND PACKAGING

B. subtilis PLSSC is sold as a powder preparation of different spore counts, depending on the final intended application.

For the manufacturing of the dry spore preparation, the spray-dried unformulated concentrate (not less than 150 billion/g) is further formulated with approved food-grade formulating agents such as maltodextrin and adjusted to a declared spore count.

The *B. subtilis* PLSSC preparation is tested by Quality Control for all quality related aspects and released by Quality Assurance. The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for final preparations.

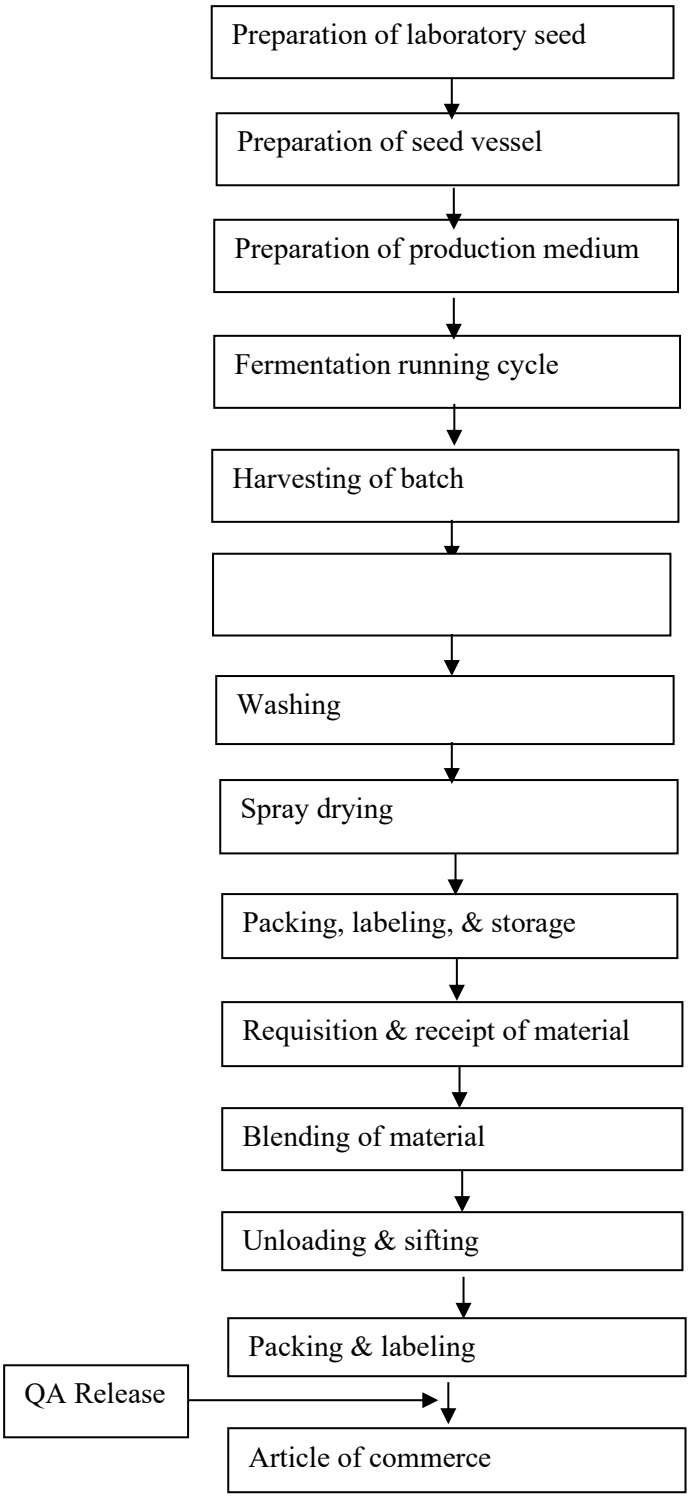


Figure 1. Manufacturing Process for *B. subtilis* PLSSC

2.3 Product Specifications and Compliance with Specifications

2.3.1 PRODUCT SPECIFICATIONS

Specifications for *B. subtilis* PLSSC preparation have been established by Advanced Enzyme Technologies Ltd. and are summarized in Table 4. All analytical methods have been validated for this purpose.

Table 4. Product Specifications for *B. subtilis* PLSSC

Product specification	Advanced Enzyme Technologies Ltd.	
	Limits	Reference Method
Total viable count/Assay (cfu/g)	Not less than 150 billion viable spore counts/g	Internal method
Appearance/Description	Light brown to brown colored powder	Visual
Microscopy/ Identity	Rod-shaped cells containing round or ellipsoidal spores located either centrally or subterminally	Internal method
Moisture/Loss on Drying	Not more than 7.0%	AOAC 926.08
Sieve test	100% through 40 mesh	Internal method
Arsenic	Not more than 2.0 ppm	AOAC 984.27
Cadmium	Not more than 1.0 ppm	AOAC 984.27
Lead	Not more than 3.0 ppm	AOAC 984.27
Mercury	Not more than 0.5 ppm	EPA 7471
Total yeast & mold count	Not more than 100 cfu/g	Harmonized method (IP, BP, EP and USP)
Total coliforms	Not more than 100 cfu/g	FDA Bacteriological Analytical Manual
E. coli	Absent in 10 g	Harmonized Pharmacopoeial method (EP, BP, USP, and IP)
Salmonella spp.	Absent in 10 g	Harmonized Pharmacopoeial method (BP, USP and IP)
P. aeruginosa	Absent in 1 g	Harmonized method (IP, BP, EP and USP)
Staphylococci spp.	Absent in 1 g	Harmonized method (IP, BP, EP and USP)
Listeria monocytogenes	Absent in 25 g	Internal method
Source: Advanced Enzyme Technologies		

2.3.2 COMPLIANCE WITH SPECIFICATIONS

Three batches of *B. subtilis* PLSSC were analyzed and the results compared with food-grade specifications. As shown in Table 5, all tested batches were in compliance, demonstrating that the production process is in control.

Table 5. Batch Analysis of Compositional Variability of *B. subtilis* PLSSC

Parameter	Specification	Batch		
		101833	101834	101835
B. subtilis viable spore count	Not less than 150 billion viable spore counts/g	162 billion viable spore count/g	171 billion viable spore count/g	168 billion viable spore count/g
Description	Light brown to brown colored powder	Light brown colored powder	Light brown colored powder	Light brown colored powder
Microscopy/ Identity	Rod shaped cells containing round or ellipsoidal spores located either centrally or subterminally	Complies	Complies	Complies
Sieve test	100% pass through 40 mesh	Complies	Complies	Complies
Moisture/Loss on drying (%)	Not more than 7.0%	6.38%	6.45%	6.29%
Heavy Metal Analysis				
Arsenic	Not more than 2.0 ppm	Complies	Complies	Complies
Cadmium	Not more than 1.0 ppm	Complies	Complies	Complies
Lead	Not more than 3.0 ppm	Complies	Complies	Complies
Mercury	Not more than 0.5 ppm	Complies	Complies	Complies
Microbial Analysis				
Total yeast & mold count	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
Total Coliform	Not more than 100 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g	Less than 10 cfu/g
E. coli	Absent in 10g	Complies	Complies	Complies
Salmonella spp.	Absent in 10g	Complies	Complies	Complies
P. aeruginosa	Absent in 1g	Complies	Complies	Complies
Staphylococci spp.	Absent in 1g	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25g	Complies	Complies	Complies
Source: Advanced Enzyme Technologies				

2.4 Shelf-Life Stability

Stability testing was performed on *B. subtilis* PLSSC to assess its shelf-life stability. In a real-time stability study, the samples were stored in an environmental chamber at 25±2°C and 60±5% relative humidity for 24 months. In an accelerated stability study, samples were stored in an environmental chamber at accelerated storage conditions (40±2°C and 75±5% relative humidity) for a period of six months.

The real-time stability studies showed less than 10% loss of viable count in 12 months. In the accelerated stability study, the activity drop of *B. subtilis* PLSSC was less than 15%. Ongoing real time data show little change and variability over time.

Based on above observation and as per ICH guideline Q1E, the proposed shelf life of *B. subtilis* PLSSC is 2 years under real-time storage conditions, when stored in simulated market packing [e.g. double polybag bag in HDPE drum (powder)].

The shelf-life storage stability results obtained in the present studies corroborate the results presented in another GRAS notice for *B. subtilis* [GRN831 (2019)]

Part 3: Intended Use and Dietary Exposure

B. subtilis PLSSC is intended for addition at a level of $1x10^6$ to $6x10^9$ cfu/serving to a wide variety of conventional foods. The food categories as defined in 21 CFR §170.3(n) to which *B. subtilis* PLSSC is to be added are listed below:

(1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours and mixes, requiring preparation before serving.

(2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix.

(3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks, drinking water, sport 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.

(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.

(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.

(12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils.

(16) Fresh fruit juices, including only raw fruits, citrus, melons, and berries, and home prepared "ades" and punches made therefrom.

(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.

(26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors.

(28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads.

(30) Milk, whole and skim, including only whole, low-fat, and skim fluid milks.

(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products.

The intended use level of *B. subtilis* PLSSC and the food categories to which it will be added are the same as those for *Bacillus subtilis* DE111 described in GRN 000831. Thus, the intended use of *B. subtilis* PLSSC merely represents an alternative strain with no increase in consumer exposure to the species.

The NOAEL for *B. subtilis* PLSSC, based on a 90-day oral toxicity study (described in Section 6.3) is 1000 mg/kg bw/day, corresponding to $1.62x10^{11}$ cfu/kg bw/day. The Acceptable Daily Intake (ADI) determined from the NOAEL (ADI = NOAEL x 70/100, where the body weight of a healthy individual is considered to be 70 kg and a safety factor of 100 is employed) is $1.13x10^{11}$ cfu/person/day.

According to USDA *Nutrition Insights*, a publication of the USDA Center for Nutrition Policy and Promotion (2000), males aged 51 or older consume the greatest number of servings of food per day, about 18.2 servings from the following categories: grains, fruits, vegetables, milk, meat and others (fats, oils, sweets). Based upon the maximum number of servings of food consumed per day in the US and the highest intended addition level of *B. subtilis* PLSSC, the maximum potential Estimated Daily Intake (EDI) is $1.1x10^{11}$ cfu/day, which is lower than the ADI.

As explained, this EDI assumes that all foods consumed contain the strain at the maximum intended level. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Consequently, there are no safety concerns with the intended use level of *B. subtilis* PLSSC.

Part 4: Self-Limiting Levels of Use

There are no self-limiting levels of use of *Bacillus subtilis* spores from *B. subtilis* PLSSC in food applications.

Part 5: Experience Based on Common Use in Food before 1958

The statutory basis for our conclusion of GRAS status in the notice is scientific procedures rather than on common use in food prior to 1958.

Part 6: Narrative

6.1. History of Consumption of *Bacillus subtilis*

There is a long history of consumption of *Bacillus subtilis* in human food and use of *Bacillus subtilis* as a food ingredient in commercial food preparations is described in GRN 831. Use of other *Bacillus* strains, such as *Bacillus coagulans,* as food ingredients in commercial food preparations has been described in several GRNs, including GRN 399, 526, 597, 601, 660, 691.

B. subtilis strains have been used as starter cultures for fermentation of various foods, fodders, and feed additives for centuries (Wang & Fung 1996; Harwood et al. 2018). They are being used in the preparation of health-promoting traditional fermented foods such as natto (Southeast Asia) and ogiri (Africa), *dawadwa* (West Africa), *Axone and Piak* (India), *shuǐdòuchǐ càixīn* (China), *Thuanao* (Thailand), and *Cheonggukjang* (Korea) according to Lefevre et al. (2015), Cutting (2011, Shieh (2009, and Shurtleff et al. (2012). *B. subtilis* is a dominant microorganism in the fermented maize product pozol (Wacher et al. 1993) and in alkaline fermentation of soya products (Inatsu et al. 2006). The bacterium is also used in conventional food products like natto sushi and miso soup. *B. subtilis* based fermented products are widely used in various countries which have long traditions of consuming fermented foods. The occurrence of *Bacillus* species (*B. subtilis, B. licheniformis*, etc.) as components of fermented soy or locust beans was demonstrated by Keitarou et al. (2019) using random amplified polymorphic DNA analysis and other molecular analyses.

B. subtilis fermented corn soyabean meal and acidified foliage silage are commonly used animal feeds in China (Shi et al. 2017) and Europe (Lara et al. 2016).

B. subtilis fermented foods have been reported to produce various health effects as fermented dietary fibers are softer and food products have very low sodium content compared to the quantity of sodium in traditionally preserved products. *B. subtilis* is used for its hydrolytic capabilities to produce a precursor-rich environment which subsequently enhances the flavor profile of various fermented food products (Beaumont 2002). The long history of use of *Bacillus subtilis* in human food confirms its safety for human consumption.

6.2 Regulatory History of *Bacillus subtilis*

B. subtilis strains have long been known to be safely consumed by the general human population.

The American Type Culture Collection (ATCC 2020) has classified different strains of *B. subtilis* as Bio-safety Level 1, indicating that it is a well-characterized agent which does not cause disease in healthy humans

The European Food Safety Authority granted *Bacillus subtilis* Qualified Presumption of Safety (QPS) status in 2008 (EFSA 2007) and has renewed its status annually since then. Further, *B. subtilis* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC, as it is globally regarded as a safe microorganism.

B. subtilis R0179 was self-determined to be GRAS in February 2012, by Institut Rosell-Lallemand for application as a heat-stable microorganism in baked goods, juices, and drinks. (Nutrition Insight 2012).

US FDA had no questions regarding the GRAS notice (GRN 831) for *B. subtilis* DE111 and its use in infant formula.

B. subtilis R0179 is included in Health Canada's Natural Health Product Ingredient Database.

B. subtilis DE111 is considered by Health Canada as not novel and phylogenetically equivalent to *B. subtilis* strain R0179.

Food Standards Australia New Zealand (FSANZ) identified no safety concerns associated with *B. subtilis*.

B. subtilis subsp. natto is approved in Japan as FOSHU (Food for Specific Health Use)

B. subtilis subsp. *inaquosorum* is recognized by Japan's Ministry Health, Labor, and Welfare.

The Australian Advisory Committee on Novel Foods (ACNF) granted "non-traditional food" status to *B. subtilis* DE111 as not a novel food. The Committee deemed it not necessary to perform further public health and safety assessment following their hazard identification process.

Several GRAS notices mention use of *B. subtilis* for production of food enzymes.

6.3 Safety of *Bacillus subtilis*—Oral Toxicity and Genotoxicity Studies

The safety of *Bacillus subtilis* PLSSC and other strains has been evaluated in animal research, including acute, subacute, subchronic, chronic studies of oral toxicity and genetic toxicity assays.

6.3.1. STUDIES OF BACILLUS SUBTILIS PLSSC

B. subtilis PLSSC, the notified strain, has been investigated in a series of toxicity studies complying with OECD guidelines and conducted in accordance with the principles of Good Laboratory Practice (GLP) as published by the OECD (ENV/MC/CHEM (98)17).

Acute oral toxicity test (OECD Test No. 423, 2001): Using the step-wise method, 2 groups of n=3 female Wistar rats aged 8-9 weeks and weighing 202-212 g were dosed via gavage with 2000 mg/kg bw spore preparation ($3.24x10^{11}$ spores/kg bw) and observed for 14 days. No indications of toxicity were reported. Based on the results, the estimated LD_{50} for *B. subtilis* PLSSC in female Wistar rats was >2000 mg/kg bw.

Repeated-dose 90-day oral toxicity test (OECD Test No. 408, 2018): Four groups of 10 male and 10 female Wistar rats, 7-8 weeks old and weighing 189-229 g (males, mean = 208.94 g) and 162-192 g (females, mean = 175.44 g) were assigned to receive daily oral gavage of 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. Rats were examined daily for signs of toxicity, morbidity, and mortality. They were subjected to detailed clinical examinations at day 0 and weekly thereafter during the treatment and recovery period. Ophthalmic examinations were performed on the control and high-dose rats at the beginning and end of dosing. At week 13, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were taken at the end of dosing and after recovery. All animals were subjected to necropsy and weights of kidneys, liver, adrenals, testes, epididymis, uterus, thymus, spleen, brain, ovaries, and heart were recorded. Histological evaluations were performed on all tissues from control and high-dose rats.

There was no mortality and no clinical abnormalities in rats treated at any dose. Ophthalmological examination revealed no abnormalities, nor did the neurotoxic assessment. There was no effect on feed intake or body weight gain, hematological or biochemical parameters, or absolute or

relative organ weights and no histopathology. The no observed adverse effect level (NOAEL) of *B. subtilis* PLSSC spore preparation in the Wistar rat, following oral administration for 90 days, was the highest dose tested, 1000 mg/kg bw/day providing 1.62 x 10^{11} spores/kg bw/day.

Bacterial reverse mutation test—Ames assay (OECD Test No. 471, 1997): The test was conducted using *Salmonella typhimurium* tester strains TA97a, TA98, TA100, TA102, and TA1535 in the presence and absence of S9 metabolic activation. The test was conducted in triplicate at concentrations of 0, 50, 150, 500, 1500, and 5000 µg/plate. No significant increase in the number of histidine revertant colonies was reported, and it is concluded that, under the conditions of this study, *B. subtilis* PLSSC spore preparation is non-mutagenic.

***In vitro* mammalian chromosomal aberration test in human lymphocytes (OECD Test No. 473, 2016):** Cultures of human peripheral blood lymphocytes were exposed to *B. subtilis* PLSSC spore preparation at concentrations of 0, 156.25, 312.50, and 625 µg/ml in the presence and absence of metabolic activation for 3 or 24 hours. No significant concentration-related increase was reported in the incidence of structural chromosome aberrations at any tested concentration, and it was concluded that *B. subtilis* PLSSC is non-clastogenic in the presence and absence of microsomal enzymes.

***In vivo* micronucleus test in mice (OECD Test No. 474, 2016):** Four groups of 5 male Swiss albino mice were gavaged with *B. subtilis* PLSSC spore preparation at doses of 2000 mg/kg bw on two consecutive days, after which bone marrow was aspirated and examined microscopically. A total of 20000 polychromatic erythrocytes per mouse were examined for the presence of micronucleated cells. No evidence of toxicity was seen in treated mice or in their bone marrow with no increase in the incidence of micronucleated polychromatic erythrocytes. Based on the results obtained, it was concluded that *B. subtilis* PLSSC is non-mutagenic under the conditions tested.

6.3.2. STUDIES OF OTHER STRAINS OF BACILLUS SUBTILIS

Safety assessments of other strains of *B. subtilis* have been reported in numerous toxicity studies. A few representative studies are described below in Table 6.

Table 6. Safety Studies of Other Strains of *Bacillus subtilis*				
Reference	**Type of Study**	**Animal Model**	***Bacillus subtilis* Strain & Dose**	**Study Outcome**
Hong et al. (2008)	Repeated dose 28-day oral toxicity study	New Zealand White rabbits	*B. subtilis* HU36 /Natto $1x10^9$ spores/ml	No adverse effects on feed intake or the general health status of the animals. No changes in selected visceral organs and tissues. No significant differences in hematological indexes.
	Acute oral toxicity	Harley Dunkin Guinea pigs	*B. subtilis* HU36/Natto $1x10^{12}$ spores/pig	No noticeable effect on feed intake. Significant weight gain at day 7. No signs of inflammation or pathological changes. No differences in hematological indexes.
Zhang et al. (2013)	Acute oral toxicity	Rabbits	*B. subtilis* Tpb55 $15x10^{10}$ cfu/kg bw	LD50 > 5,000 mg/kg bw ($1.5x10^{11}$ cfu/kg bw)
Harrington et al. (1998)	Acute oral toxicity	Rats	*B. subtilis* QST 713 >$1.13x10^8$ cfu/rat	No toxic or clinical effects after oral administration LD50 >$1.13x10^8$ cfu/kg bw NOAEL = $1.13x10^8$ cfu/kg bw/day
	Repeated dose 28-day oral toxicity study	Rats	*B. subtilis* QST 713 >$1.13x10^8$ cfu/rat/ day	No adverse effects
Kim et al. (2015)	Acute oral toxicity	ICR mice	*B. subtilis* JNS 2,000 mg/kg	No significant change in general conditions, mortality, body weight, clinical signs, autopsy findings, or presence of gross lesions. Up to 2,000 mg/kg bw of *B. subtilis* JNS had no adverse effect on ICR mice.
Sorokulova et al. (2008)	Acute oral toxicity	BALB/c mice	*B. subtilis* VKPM B2335 (BS3) $5x10^7$, $5x10^8$, $2x10^{11}$ cfu/mouse	No adverse effect on mouse activity and weight. No signs of inflammation or any other pathological changes in analyzed organs and tissues. No treatment-related deaths.
	Repeated dose 28-day oral toxicity study	BALB/c mice, rabbits, piglets	*B. subtilis* VKPM B2335 (BS3) Mice: $1x10^6$ cfu/ day; rabbits and piglets: $1x10^9$ cfu/ day	No adverse effects on the general health. No changes in the organs and tissues. No differences in hematological indexes.
Tompkins et al. (2008)	Repeated dose 28-day oral toxicity study	Sprague-Dawley albino rats	*B. subtilis* R0179 $2x10^9$ cfu/kg bw/ day	No adverse effects on general health, no changes in organs and tissues, no differences in the hematological indexes.

The above studies show that *B. subtilis* strains in general have been found safe in animal studies.

6.4 Safety of *Bacillus subtilis* —Human Studies

Several researchers carried out studies with different *B. subtilis* strains on human subjects and evaluated the safety aspects. Many such studies are summarized in Table 7.

Table 7. Human Studies of *Bacillus subtilis*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Hanifi et al. (2015)	Randomized, double-blind, placebo-controlled trial	81 apparently healthy adults aged 18-50 years	*B. subtilis* R0179 at 0, 0.1, 1.0 or $10x10^9$ cfu/day	4 Weeks	*B. subtilis* R0179 survives passage through the human GI tract and is well tolerated by healthy adults at intakes from 0.1 to 10×10^9 cfu/day.
Cuentas et al. (2017)	Randomized, double-blind placebo-controlled study	50 adults aged 18-65 years, suffering from occasional constipation and/or diarrhea but otherwise apparently healthy	*B. subtilis* DE111 at 0 or 10^9 cfu/day	90 days.	The study provided evidence that DE111 is safe.
Penet et. al. (2019)	Randomized, double-blind, placebo-controlled, multi-center trial	100 apparently healthy participants aged 18 to 75 years with bloating, abdominal discomfort, and gas	*B. subtilis* MB40 at 0 or $5x10^9$ cfu/day	4 weeks	MB40 supplementation at a dose of $5x10^9$ cfu daily for 4-weeks was safe and well-tolerated as all biometric, vital, and hematological measures remained within normal laboratory range.
McFarlin et al. (2017)	Randomized, double-blind placebo-controlled study	28 responders to a screen for post-prandial dietary endotoxemia	$4x10^9$ spores of *B. subtilis HU58, B. indicus HU36, B. coagulans, B. licheniformis,* and *B. clausii*/day	30 days	The authors reported that the supplementation reduced symptoms indicative of “leaky gut” syndrome with no reported adverse effects.
Dound et al. (2017)	Open-label study	18 apparently healthy participants, 9 M, 9 F	*B. subtilis* HU58 $2x10^9$ cfu/day	8 weeks	*Bacillus subtilis* HU58 was well tolerated clinically and found to be safe as per the organ function tests in all the subjects. No serious adverse events were reported during the period of therapy.

Table 7. Human Studies of *Bacillus subtilis*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Hatanaka et al. (2018)	Randomized, double-blind, placebo-controlled trial	80 apparently healthy participants aged 20 - 80 years suffering from loose stools	*B. subtilis* spores at 0 or $2.2x10^9$ cfu/day	8 weeks	A physician asked about any signs of headache, abdominal pain, nausea, and digestive problems; no adverse effects association with the intervention were reported.
Lee et al. (2010)	Randomized, double-blind, placebo-controlled trial	51 patients with constipation and 53 healthy adults receiving bowel cleaning pre-colonoscopy	$3x10^9$ cfu *B. subtilis* and $2.7x10^{10}$ cfu *Streptococcus faecium*/day	2 weeks	AEs were assessed. The degree of discomfort associated with bloating, abdominal cramps, nausea or vomiting was recorded. AEs were less frequent in the patients receiving probiotics than in the control group.
Lefevre et al. (2015)	Randomized, double-blind, placebo-controlled study	100 apparently healthy men & women aged 60 - 74 years	*B. subtilis* CU1 at 0 or 2.10^9 spores/day	4 consumption periods of 10 days each with 18-day washouts.	The authors reported that, "There were no abnormal values of biological parameters at the end of the study, and no clinically significant variation was observed during the study, on renal and hepatic functions," and concluded that the use of *B. subtilis* in elderly humans is safe.
Hatanaka et al. (2019)	Randomized, placebo-controlled, double-blind, parallel-group study	44 individuals (18 M, 26 F) aged 46.1±13.8 years	*B. subtilis* C-3102 at 0 or $4.8x10^{10}$ cfu/day	4 weeks	The results revealed no medical-related problems in both the C-3102 and placebo groups. This study proved the safety of 4-weeks continuous consumption of *B. subtilis* C-3102 tablets providing $4.8x10^{10}$ cfu/day
Pushkarev et al. (2007)	Randomized, placebo-controlled, double-blind, parallel-group study	71 patients with infravesical obstructions	*B. subtilis* 3H at 0 or $5x10^9$ cfu	Single administration	No adverse effects of the intervention were reported.

Table 7. Human Studies of *Bacillus subtilis*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Townsend et al. (2018)	Randomized, double blind, placebo-controlled study	25 male baseball athletes aged 20.1±1.5 years	*B. subtilis* DE111 at 0 or 10^9 cfu/day	12 weeks	These data indicate that probiotic supplementation had no effect on body composition, performance, hormonal status, or gut permeability, while it may attenuate circulating TNF-α in athletes. The authors stated that "Both DE111 and placebo supplements were well tolerated and no adverse events were reported."
Takimoto et al. (2018)	Randomized, double-blind, placebo-controlled study	76 postmenopausal women aged 57.7±5.0 years	*B. subtilis* C-3102 at 0 or $3.4x10^9$ cfu/day.	24 weeks	Safety was monitored by assessing the hematology, clinical chemistry, and 134 vital signs of each participant, in addition to physical examinations at each clinic visit and recording adverse events. The authors stated that "No adverse effects were reported during the study period."
Vukovic (2001)	Randomized, double-blind, placebo-controlled, multi-center study	63 males and females with acute non-typhoid *Salmonella* gastroenteritis	*B. subtilis* strain IP5832 at 0 or $6x10^9$ cfu/day	7 days	No strain-related adverse effects were reported.
Maher et.al. (2019)	Randomized, double-blind, placebo-controlled study of the tolerance and safety of *B. subtilis* DE111	41 apparently healthy subjects (18 M, 23 F) aged 19-42 years	*B. subtilis* DE111 at 0 or $5x10^9$ cfu/day	20 days	Tolerance was assessed through analysis of blood biomarkers and C-reactive protein and through a pre- and post-capsule consumption GI symptom questionnaire. The authors reported that B. subtilis was well tolerated and that daily consumption of *B. subtilis* can be recognized as safe.
Toohey et al. (2018)	Randomized, double-blind, placebo-controlled study	23 apparently healthy female athletes aged 19.6 ± 1.0 years	*B. subtilis* DE111 at 0 or:$5x10^9$ cfu/day	10 weeks	The authors stated that "both DE111 and placebo supplements were well tolerated, and no adverse events were reported."

6.5 Decision Tree

The safety of *B. subtilis* PLSSC has also been established using the decision tree for determining safety of microbial cultures to be consumed by humans or animals (Pariza et al. 2015):

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? **YES**

2. Has the strain genome been sequenced? **YES**

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? **YES**

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? **YES**

5. Does the strain produce antimicrobial substances? **NO**

6. Has the strain been genetically modified using rDNA techniques? **NO**

7. 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—it was isolated from soil.**

8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? **NO**

Conclusion: The strain is “deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption” (Pariza et al. 2015).

6.6 Safety Assessment and GRAS Determination

This section presents an assessment that demonstrates that the intended use of *B. subtilis* PLSSC spore preparation is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of *B. subtilis* PLSSC is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of consumers to *B. subtilis* PLSSC under its intended conditions of use is not harmful. In the second step, the intended use of *B. subtilis* PLSSC is determined to be GRAS by demonstrating that the safety of this spore preparation under its intended conditions of use is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether the intended use of a substance (or microorganism) is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1. Data and information relied upon to establish the scientific element of safety must be generally available; and
2. There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the intended use of *B. subtilis* PLSSC spore preparation is safe and is GRAS.

6.6.1 EVIDENCE OF SAFETY

Genomic analysis of *B. subtilis* PLSSC established that it harbors no antibiotic resistance genes flanked by mobile elements, no confirmed virulence genes and none flanked by mobile elements, and no genes encoding toxin production. Phenotypic analysis shows an absence of antibiotic resistance and no production of biogenic amines. No evidence of pathogenicity has been reported, and the species is generally regarded as non-pathogenic as well as non-toxigenic. No indications of toxicity were found in acute and repeated-dose studies of oral toxicity or in genotoxicity assays in strain PLSSC or other strains of *B. subtilis*, and no adverse effects were reported when *B. subtilis* spores are administered to humans. All of these findings support the conclusion that the intended use of *B. subtilis* PLSSC spore preparation is safe.

6.6.2 CONCLUSION OF THE GRAS PANEL

The intended use of *B. subtilis* PLSSC spore preparation has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by genomic analysis of the strain, a record of safe ingestion of numerous strains of *B. subtilis*, toxicity studies of *B. subtilis* PLSSC and other strains, and research in humans with numerous strains of *B. subtilis*, concluding that the expected exposure to *B. subtilis* PLSSC spore preparation is without significant risk of harm. Finally, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the intended use of *B. subtilis* PLSSC spore preparation has been made through the deliberations of a GRAS Panel consisting of Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., with James T. Heimbach, Ph.D., as Advisor to the Panel, who reviewed a monograph prepared by Advanced Enzyme Technologies, as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They independently critically reviewed and evaluated the publicly available information and the potential human exposure to *B. subtilis* PLSSC spore preparation anticipated to result from its intended use, and individually and collectively determined that no evidence exists in the available information on *B. subtilis* PLSSC that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of *B. subtilis* PLSSC spore preparation.

It is the GRAS Panel's opinion that other qualified scientists reviewing the same publicly available data would reach a similar conclusion regarding the safety of *B. subtilis* PLSSC under its intended conditions of use. Therefore, the intended use of *B. subtilis* PLSSC spore preparation is GRAS by scientific procedures.

6.7. Statement Regarding Information Inconsistent with GRAS

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with our conclusion of the GRAS status of the intended use of *Bacillus subtilis* PLSSC (ATCC SD 7280).

6.8. Statement of the GRAS Panel

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of microorganisms intended for addition to foods. We have individually and collectively critically evaluated the publicly available information on *Bacillus subtilis* PLSSC summarized in a monograph, *GRAS NOTIFICATION Bacillus subtilis PLSSC (ATCC SD 7280)*, prepared by Advanced Enzyme Technologies, and other materials deemed appropriate or necessary.

We have individually and collectively determined that no evidence exists in the available information on *B. subtilis* PLSSC or other strains of *B. subtilis* that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of *B. subtilis* PLSSC.

We unanimously conclude that the intended addition to conventional foods at the levels specified in this monograph of *B. subtilis* PLSSC, produced consistent with current good manufacturing practice (cGMP) and meeting the food-grade specifications presented in the monograph, is safe and is GRAS by scientific procedures.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin
Signature: _____ Date: _____

John A. Thomas, Ph.D.
Adjunct Professor
Indiana University School of Medicine
Indianapolis, Indiana
Signature: _____ Date: _____

James T. Heimbach, Ph.D. (Advisor to the GRAS Panel)
President
JHeimbach LLC
Port Royal, Virginia
Signature: _____  _____ Date: June 2, 2020

6.8. Statement of the GRAS Panel

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of microorganisms intended for addition to foods. We have individually and collectively critically evaluated the publicly available information on *Bacillus subtilis* PLSSC summarized in a monograph, *GRAS NOTIFICATION Bacillus subtilis PLSSC (ATCC SD 7280)*, prepared by Advanced Enzyme Technologies, and other materials deemed appropriate or necessary.

We have individually and collectively determined that no evidence exists in the available information on *B. subtilis* PLSSC or other strains of *B. subtilis* that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of *B. subtilis* PLSSC.

We unanimously conclude that the intended addition to conventional foods at the levels specified in this monograph of *B. subtilis* PLSSC, produced consistent with current good manufacturing practice (cGMP) and meeting the food-grade specifications presented in the monograph, is safe and is GRAS by scientific procedures.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin
Signature: _____ Date: June 3, 2020

John A. Thomas, Ph.D.
Adjunct Professor
Indiana University School of Medicine
Indianapolis, Indiana
Signature: _____ Date: _____

James T. Heimbach, Ph.D. (Advisor to the GRAS Panel)
President
JHeimbach LLC
Port Royal, Virginia
Signature: _____ Date: _____

6.8. Statement of the GRAS Panel

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of microorganisms intended for addition to foods. We have individually and collectively critically evaluated the publicly available information on *Bacillus subtilis* PLSSC summarized in a monograph, *GRAS NOTIFICATION Bacillus subtilis PLSSC (ATCC SD 7280)*, prepared by Advanced Enzyme Technologies, and other materials deemed appropriate or necessary.

We have individually and collectively determined that no evidence exists in the available information on *B. subtilis* PLSSC or other strains of *B. subtilis* that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of *B. subtilis* PLSSC.

We unanimously conclude that the intended addition to conventional foods at the levels specified in this monograph of *B. subtilis* PLSSC, produced consistent with current good manufacturing practice (cGMP) and meeting the food-grade specifications presented in the monograph, is safe and is GRAS by scientific procedures.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin
Signature: _____ Date: _____

John A. Thomas, Ph.D.
Adjunct Professor
Indiana University School of Medicine
Indianapolis, Indiana
Signature: [redacted] Date: 6/2/20

James T. Heimbach, Ph.D. (Advisor to the GRAS Panel)
President
JHeimbach LLC
Port Royal, Virginia
Signature: _____ Date: _____

Part 7: References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403-410.

American Type Culture Collection (ATCC): *Biosafety levels*.

Beaumont, M. 2002. Flavouring composition prepared by fermentation with Bacillus spp. *Int J Food Microbiol* 75:189–196

CFSAN. 2012. *Agency Response Letter GRAS Notice No. GRN 000399*, FDA.

CFSAN. 2015. *Agency Response Letter GRAS Notice No. GRN 000526*, FDA.

CFSAN. 2016. *Agency Response Letter GRAS Notice No. GRN 000597*, FDA.

CFSAN. 2017. *Agency Response Letter GRAS Notice No. GRN 000691*, FDA.

Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jim Q. 2004. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res* 33:D325-328.

CLSI. 2012. *Performance standards for antimicrobial disc susceptibility tests; Approved standards*, 11th Ed. CLSI Document M02-A11. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI. 2015. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standards*, 10th edition. CLSI document M07-A10. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI. 2016. *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria*. 3rd ed. CLSI guideline M45 Wayne, PA: Clinical and Laboratory Standards Institute.

Cohn F. 1872. Untersuchungen über bakterien. *Beitr Biol Pflanzen* 1:127-224.

Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Neron B, Rocha EPC, Vergnaud G, Gautheret D, Pourcel C. 2018. CRISPRCasFinder, an update of CRISRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Res* 46:W246-W251.

Cuentas AM, Deaton J, Davidson J, Ardita C. 2017. The effect of *Bacillus subtilis* DE111 on the daily bowel movement profile for people with occasional gastrointestinal irregularity. *J Prob Health* 5:4.

Cutting SM. 2011. Bacillus probiotics. *Food Microbiol* 28:214-220.

Dound YA, Jadhav SS, Devale M, Bayne T, Krishnan K, Mehta DS. 2017. The effect of probiotic *Bacillus subtilis* HU58 on immune function in healthy human. *Indian Practitioner* 70: September.

EFSA. 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *EFSA J* 587:1-16.

EFSA. 2012. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J* 10:2740.

EFSA. 2014. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. *EFSA J* 12:3665.

EFSA. 2018a. *Scientific opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed* (2018 update).

EFSA. 2018b. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 8: suitability of taxonomic units notified to EFSA until March 2018. *EFSA J* 16.

EFSA. 2018c. EFSA Panel on Additives and Products or Substances used in Animal Feed: Guidance on the characterization of microorganisms used as feed additives or as production organisms. *EFSA J* 16:5206.

Ehrenberg CG. 1835. *Dritter Beitrag zur Erkenntniss grosser Organisation in der Richtung des kleinsten Raumes. Physikalische Abhandlungen der Koeniglichen Akademie der Wissenschaften zu Berlin aus den Jahren 1833–1835*. Pp 145–336.

European Parliament Directive 2000/54/EC of the European Parliament and of the Council.

FAO/JECFA. 2006. *Joint FAO/WHO Expert Committee on Food Additives Monographs: Combined compendium of food additive specifications 4*. Pp 122-123.

Hanifi A, Culpepper T, Mai V, Anand A, Ford AL, Ukhanova M, Christman M, Tompkins TA, Dahl WJ. 2015. Evaluation of *Bacillus subtilis* R0179 on gastrointestinal viability and general wellness: A randomized, double-blind, placebo-controlled trial in healthy adults. *Benef Microbes* 6:19-27.

Harrington K. 1998. *Toxicity/Pathogenicity Testing of QST 713 Following Acute Oral Challenge in Rats: Lab Project Number: L08726SN4*. Unpublished study prepared by IIT Research Institute. 126 p. {OPPTS 885.3050} Cited in a Biopesticide Registration Action Document: *Bacillus subtilis* Strain QST 713 (PC Code 006479)

Harwood CR, Mouillon JM, Pohl S, Arnau J. 2018. Secondary metabolite production and the safety of industrially important members of the *B. subtilis* group. *FEMS Microbiol Rev* 42:721-738.

Hatanaka M, Kanzato H, Tsuda R, Nadaoka I, Yasue M, Hoshino T, Iio SI, Takara T. 2019. Safety evaluation of the excessive intake of *Bacillus subtilis* C-3102 in healthy Japanese adults: A randomized, placebo-controlled, double-blind, parallel-group, comparison trial. *Toxicol Rep* 7:46-58.

Hatanaka M, Yamamoto K, Suzuki N, Iio S, Takara T, Morita H, Takimoto T, Nakamura T. 2018. Effect of *Bacillus subtilis* C-3102 on loose stools in healthy volunteers. *Benef Microbes* 9:357-365.

Hong HA, Huang JM, Khaneja R, Hiep, LV, Urdaci MC, Cutting SM. 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *FEMS Microbiol Rev* 29:813-835.

International Council for Harmonization (ICH). 2003. *Q1E Guideline for Evaluation of Stability Data*. Version 4, February.

Inatsu Y, Nakamura N, Yuriko Y, Fushimi T, Watanasirtum L, Kawamoto S. 2006. Characterization of *Bacillus subtilis* strains in Thua nao, a traditional fermented soybean food in Northern Thailand. *Lett Appl Microbiol* 43:237-242

Keitarou K, Satoshi Y. 2019. Trends in the application of *Bacillus* in fermented foods. *Curr Opin Biotechnol* 56:36-42.

Kim K-H, Jeong C, Joo S-J, Park J-H, Moon J-Y, Cho EJ, Lee H-T, Kwon H-J, Kim B-W, Eom S-H, Lee E-W. 2015. Single dose oral toxicity of *Bacillus subtilis* JNS in ICR mice. *J Korean Soc Food Sci Nutr* 44:24-28.

Lara EC, Basso FC, Assis FBD, Souza FA, Berchielli TT, Reis RA. 2016. Changes in the nutritive value and aerobic stability of corn silages inoculated with *B. subtilis* alone or combined with *Lactobacillus plantarum*. *Anim Product Sci* 56:1867.

Lee H, Kim YH, Kim JH, Chang DK, Kim JY, Son HJ, Rhee PL, Kim JJ, Rhee JC. 2010. A feasibility study of probiotics pretreatment as a bowel preparation for colonoscopy in constipated patients. *Dig Dis Sci* 55:4344-4351.

Lefevre M, Racedo SM, Ripert G, Housez B, Cazaubiel M, Maudet C, Jüsten P, Marteau P, Urdaci MC. 2015. Probiotic strain *Bacillus subtilis* CU1 stimulates immune system of elderly during common infectious disease period: a randomized, double-blind placebo-controlled study. *Immun Ageing* 12:24.

Lefevre M, Racedo SM, Denayrolles M, Ripert G, Desfougères T, Lobach AR, Simon R, Pélerin F, Jüsten P, Urdaci MC. 2017. Safety assessment of *B. subtilis* CU1 for use as a probiotic in humans. *Regul Toxicol Pharmacol* 83:54-65.

Leplae R, Lima-Mendez G, Toussaint A. 2009. ACLAME: A classification of mobile genetic elements, update 2010. *Nucleic Acids Res* 38:D57-D61.

Logan N, Vos P. 2015. Bacillus; Firmicutes / "Bacilli" / Bacillales / Bacillaceae / Bacillus; Cohn 1872, 174AL; *Bergey's Manual of Systematics of Archaea and Bacteria*.

Maher M, Labellarte G. 2019. Tolerance and effect of a probiotic supplement delivered in capsule form. *Food Nutr Sci* 10:626-634.

McFarlin BK, Henning AL, Bowman EM, Gary MA, Carbajal KM. 2017. Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers. *World J Gastroenterol Pathophysiol* 8:117-126.

Meier-Kolthoff JP, Hans-Peter K, Markus G. 2014. Taxonomic use of DNA G+C content and DNA–DNA hybridization in the genomic age. *Int J Syst Evol Microbiol* 64:352-356. doi: 10.1099/ijs.0.056994-0

National Institutes of Health (NIH). 2012. Dietary supplementary label database. *Nutr Insight* Feb.

Nutrition Insight. 2012. *Heat stable probiotic receives self-affirmed GRAS status*. Available on-line at https://www.nutritioninsight.com/news/heat-stable-probiotic-receives-self-affirmed-gras-status.html.

OECD. 1997. *Test No. 471: Bacterial Reverse Mutation Test*. Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-11

OECD. 1998. *Principles on Good Lab Practice.* Series on Principles of Good Laboratory Practice and Compliance Monitoring; 1-41.

OECD. 2001. *Test No. 423: Acute Oral toxicity - Acute Toxic Class Method*. Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-14.

OECD. 2016a. *Test No. 473:* In Vitro *Mammalian Chromosomal Aberration Test*. Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-22.

OECD. 2016b. *Test No. 474:* In Vivo *Mammalian Erythrocyte Micronucleus Test*. Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-21.

OECD. 2018. *Test No. 408: Repeated dose 90-day oral toxicity study in rodents*. Guidelines for the Testing of Chemicals, Section 4, OECD Publishing; 1-16

Pariza MW, Gillies KO, Kraak-Ripple SF, Leyer G, Smith AB. 2015. Determining the safety of microbial cultures for consumption by humans and animals. *Regul Toxicol Pharmacol* 73:164-171.

Penet C, Kramer R, Little R, Spears JL, Parker J, Iyer JK, Guthrie N, Evans M. 2019. A randomized, double-blind, placebo-controlled, parallel study evaluating the efficacy of *Bacillus subtilis* MB40 to reduce abdominal discomfort, gas, and bloating. *Altern Ther Health Med* Nov 8:AT6199.

Pushkarev AM, Tuĭgunova VG, Zaĭnullin RR, Kuznetsova TN, Gabidullin IuZ. 2007. Use of antagonistic *Bacillus subtilis* bacteria for treatment of nosocomial urinary tract infections. *Zh Mikrobiol Epidemiol Immunobiol* 2:90-93.

Shi C, Zhang Y, Lu Z, Wang Y. 2017. Solid-state fermentation of corn-soybean meal mixed feed with *B. subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. *J Anim Sci Biotechnol* 8:50.

Shieh C-J, Thi L-AP, Shih I-L. 2009. Milk-clotting enzymes produced by culture of *B. subtilis* natto. *Biochem Engineer J* 43:85-91.

Shurtleff W, Aoyagi A. (Eds.) 2012. History of natto and its relatives (1405–2012): Extensively annotated bibliography and sourcebook, Soyinfo Centre, Lafayette, CA, Pp 5-8.

Siguier P, Pérochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res* 34:D32-D36.

Sorokulova IB. 2013. Modern status and perspectives of *Bacillus* bacteria as probiotics. *J Prob Health* 1:1-5.

Sorokulova IB, Pinchuk IV, Denayrolles M, Osipova IG, Huang JM, Cutting SM, Urdaci MC. 2008. The safety of two *Bacillus* probiotic strains for human use. *Dig Dis Sci* 53:954-63.

Takimoto T, Hatanaka M, Hoshino T, Takara T, Tanaka K, Shimizu A, Morita H, Nakamura T. 2018. Effect of *Bacillus subtilis* C-3102 on bone mineral density in healthy postmenopausal Japanese women: A randomized, placebo-controlled, double-blind clinical trial. *Biosci Microbiota Food Health* 37:87-96.

Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J. 2016. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Res* 44:6614-6624.

Tompkins TA, Hagen KE, Wallace TD, Fillion-Forté V. 2008. Safety evaluation of two bacterial strains used in Asian probiotic products. *Canadian J Microbiol* 54:391-400.

Toohey JC, Townsend JR, Johnson SB, Toy AM, Vantrease WC, Bender D, Crimi CC, Stowers KL, Ruiz MD, VanDusseldorp TA, Feito Y, Mangine GT. 2018. Effects of probiotic (*Bacillus subtilis*) supplementation during offseason resistance training in female division I athletes. *J Strength Cond Res* Jun 26:1-9.

Townsend JR, Bender D, Vantrease WC, Sapp PA, Toy AM, Woods CA, Johnson KD. 2018. Effects of probiotic (*Bacillus subtilis* DE111) supplementation on immune function, hormonal status, and physical performance in division I baseball players. *Sports* 6:70.

US FDA. 2011. GRN 399. Notice to US Food and Drug Administration that *Bacillus coagulans* GBl-30, 6086, a Novel Probiotic, is Generally Recognized as Safe for use in Foods. Ganeden.

US FDA. 2014. GRN 526 GRAS Notification for *Bacillus coagulans* Unique 1S2 Unique Biotech Ltd.

US FDA. 2015. GRN 597 GRAS Notification for *Bacillus coagulans* SNZ1969 spore preparation. Sanzyme.

US FDA. 2017. GRN 691 GRAS Conclusion for the Use of *Bacillus coagulans* SANK 70258 Spores Preparation (LACRIS-S) in Select Foods. Mitsubishi.

US FDA. 2018. GRN 831 GRAS Notification for *Bacillus subtilis* DE111in foods. Deerland Probiotics and Enzymes USA.

US Pharmacopeia 2008. *Biological Tests and Assays: Antibiotics–Microbial Assay*, pp 91-96. Rockville, MD, USA.

USDA. 2000. *Consumption of Food Group Servings: People's Perceptions vs. Reality*. Center for Nutrition Policy and Promotion, October 2000.

Vukovic M. 2001. *Bacillus subtilis* IP 5832 (Flonivin BS) shortens the duration of the carrier state in patients with acute non-typhoid salmonella enteritis. *Med Pregl* 54:62-68

Wacher C, Canas A, Cook PE, Barzana E, Owens JD. 1993. Sources of microorganisms in pozol, a traditional Mexican fermented maize dough. *World J Microbiol Biotechnol* 9:269-274.

Wang J, Fung DYC. 1996. Alkaline-fermented foods: A review with emphasis on pidan fermentation. *Crit Rev Microbiol* 22:101-138.

WHO 2016. *Clinically important antimicrobials for human medicine*. 5th Revision.

Zhang CW, Zhang YQ, Gao JM. 2013. *Toxicological and Safety Evaluation of Bacillus subtilis Strain Tpb55*. Advanced Materials Research.

Zimin AV, Marcais G, Puiu D, Roberts M, Salzberg SL, Yorke JA. 2013. The MaSuRCA genome assembler. *Bioinformatics* 29:2669-2677.

JHeimbach LLC

June 17, 2021

Lane A. Highbarger, Ph.D.
Microbiology and Regulatory Review
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients
Food and Drug Administration

Dear Dr. Highbarger:

The letter responds to questions asked by FDA reviewers of GRN956 and relayed to m in an e-mail from you on June 8.

1. The notifier provides specifications for arsenic, cadmium, lead, and mercury and the analyses from three batches. The results provided indicate that the ingredient complies with the specifications but does not provide the actual numerical values. Please provide the actual values for the three batch analyses. We note that heavy metals should be as low as possible in the final ingredient and the specifications should be representative of the results of the batch analyses. Please review the specifications and revise them accordingly to ensure that heavy metals are as low as possible in the final ingredient and consistent with the results of the batch analyses.

The notifier has revised the heavy-metal specifications to tighten them considerably, to 0.3 mg/kg for As and Pb and to 0.15 mg/kg for Cd and Hg. The notifier also provides the results of analyses of three batches of product; all results were lower than the limit of quantitation (LoQ) and so are shown in the following table simply as less than the LoQ:

Heavy Metal	Limit of Quantitation LOQ (mg/kg)	Heavy Metal Concentration in *Bacillus subtilis* Batches			Revised Specification (mg/kg)	Current Specification (mg/kg)
		Batch No. 101834	Batch No. 101835	Batch No. 101833		
Arsenic	0.25	<0.25	<0.25	<0.25	0.3	2
Lead	0.25	<0.25	<0.25	<0.25	0.3	3
Cadmium	0.1	<0.1	<0.1	<0.1	0.15	1
Mercury	0.1	<0.1	<0.1	<0.1	0.15	0.5

923 Water Street #66, Port Royal Virginia 22535, USA
tel. (+1) 804-742-5548 cell (+1) 202-320-3063 jh@jheimbach.com

2. Please describe the process by which vegetative cells are converted to spores at the conclusion of the large-scale fermentation.

Bacillus subtilis grows as vegetative cells in the early growth phase and follows a unique growth kinetic that leads to the conversion of vegetative cells into spores at the later stages of growth. The sporulation process is regulated by multiple parameters including nutrient availability, aeration, pH, temperature, etc. Nutrient stresses developed due to limitation of available carbons and micronutrients, elevation of pH, and metabolic changes contribute to the conversion of vegetative cells into forespores (spore precursors). The external regulation of batch aeration and temperature supports and completes the spore formation process.

3. Please describe the tests used to assure that there are no viable vegetative cells present.

The test described below is carried out to assure the absence of viable vegetative cells in the *Bacillus subtilis* spore preparation.

Briefly, a reconstituted *Bacillus subtilis* spore preparation is subjected to total viable cells (TVC) enumeration using a two-step analysis:

1. The reconstituted spore preparation is heat-treated at 70ºC for 15 minutes and serially diluted; then, TVC analysis is carried out aseptically following standard pour plate technique. The TVC obtained in the first step represents only the *Bacillus subtilis* spore count. Viable vegetative cells, if present in the preparation, are killed due to the heat treatment and do not contribute in the cell count.

2. The reconstituted spore preparation is serially diluted without heat treatment (i.e., no holding at 70ºC for 15 minutes), and aseptically analyzed for TVC following standard pour plate technique, the same as step 1. The TVC obtained in the second step of analysis represents both spores and vegetative cells.

The test results are interpreted as follows:

- A TVC in step 1 higher than or equal to that in step 2 confirms that no viable vegetative cells are present in the spore preparation.
- A TVC in step 1 less than that in step 2 indicates the presence of viable vegetative cells in the spore preparation.

I am confident that these responses adequately address FDA's concerns.

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
President