AIBMR Life Sciences, Inc.

April 5, 2023

Susan Carlson, PhD
Division Director
Division of Food Ingredients
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

Dear Dr. Carlson:

In accordance with regulation 21 CFR Part 170 Subpart E (Generally Recognized as Safe (GRAS) Notice), on behalf of Microbial Discovery Group (the notifier), the undersigned, Dr. Maureen Dunn, ND, submits, for FDA review, the enclosed notice that *Bacillus subtilis* NRRL 68053 is GRAS under the conditions of its intended use in foods.

Should you have any questions or concerns regarding this notice, please contact me at 253-286-2888 or maureen@aibmr.com.

Sincerely,

Maureen Dunn, ND (agent of the notifier)
Senior Scientific and Regulatory Consultant
AIBMR Life Sciences, Inc. ("AIBMR")

Notice to US Food and Drug Administration of the Conclusion that the Intended Use of *Bacillus subtilis* NRRL 68053 is Generally Recognized as Safe

Submitted by the Notifier:

Microbial Discovery Group
5200 W. Ashland Way
Franklin, WI 53132
Tel: (414) 235-3767
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Prepared by the Agent of the Notifier:

AIBMR Life Sciences, Inc.
1425 Broadway, Suite 458
Seattle WA 98122

April 5, 2023

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Figures and Tables

Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

Microbial Discovery Group (the notifier), hereafter referred to as MDG, is submitting a new GRAS notice in accordance with 21 CFR Part 170, Subpart E, regarding the conclusion that *Bacillus subtilis* NRRL 68053 is Generally Recognized as Safe (GRAS) for its intended use, consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act.

1.2 Name and Address of the Notifier and Agent of the Notifier

Notifier

Jessica Edward
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Microbial Discovery Group
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Agent of the Notifier

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1.3 Name of the Substance

Bacillus subtilis NRRL 68053

Trade name: AmplifiedBiotics™ (AB22).

1.4 Intended Conditions of Use

Bacillus subtilis NRRL 68053 is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. It is not intended to be added to infant formula, or any products that would require additional regulatory review by USDA. The intended addition level to foods is up to 1×10^{10} CFU per serving.

1.5 Statutory Basis for GRAS Conclusion

The conclusion of GRAS status of *B. subtilis* NRRL 68053 for its intended conditions of use, stated in Part 1.4 of this notice, has been made based on scientific procedures.

1.6 Not Subject to Premarket approval

We have concluded that *B. subtilis* NRRL 68053 is GRAS for its intended conditions of use, stated in Part 1.4 of this notice, and, therefore, such use of *B. subtilis* NRRL 68053 is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data and Information Availability Statement

The data and information that serve as the basis for this GRAS conclusion will be available for review and copying during customary business hours at the office of Jessica Edward (Microbial Discovery Group, 5200 W. Ashland Way, Franklin, WI 53132), or will be sent to FDA upon request.

1.8 Exemption from Disclosure under the Freedom of Information Act

None of the data and information in Parts 2 through 7 of this GRAS notice are considered exempt from disclosure under the Freedom of Information Act (FOIA) as trade secret or commercial or financial information that is privileged or confidential.

1.9 Certification of Completion

We hereby certify that, to the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission 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 *B. subtilis* NRRL 68053.

[redacted]

04/05/2023

Jessica Edward
Business Unit Manager
Notifier

Date

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

2.1 Identification

B. subtilis is a gram-positive, rod-shaped, endospore-forming bacterium found in the soil, on plants, in water sources, and in the gastrointestinal tract of humans.[1,2] It has several flagella and is highly motile.[1] While there are members of the *Bacillus* genus that are known to have toxic effects in humans and animals via production of toxins (e.g. *B. anthracis*, *B. cereus*), *B. subtilis* has a long history of safe use for human consumption as will be detailed in Part 6.[3]

A third party, Eurofins, performed gyrB sequencing and the resulting consensus sequence was queried through the NCBI BLASTn database, and *B. subtilis* NRRL 68053 was identified as a member of the *Bacillus subtilis* subsp. *subtilis* group. MDG has completed a hybrid whole genome sequence assembly using the Illumina short reads and Nanopore long reads with the Microbial Genome Sequencing Center for *B. subtilis* NRRL 68053 and a Genome Announcement is pending.

2.2 Taxonomy of *Bacillus subtilis* NRRL 68053

MDG's *B. subtilis* was isolated from mammalian gastrointestinal tract and has been identified according to standard taxonomic guidelines. The taxonomic lineage of the strain is:

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Family: Bacillales

Genus: *Bacillus*

Species: *Bacillus subtilis*

Strain: *Bacillus subtilis* subsp. *subtilis* NRRL 68053

2.3 Manufacturing

2.3.1 Good Manufacturing Practice

All production steps are performed under an approved Hazard Analysis and Critical Control Points (HACCP) plan and are consistent with current Good Manufacturing Practice (cGMP) 117.

2.3.2 Raw Materials

Raw materials used in the production of MDG's *B. subtilis* NRRL 68053 are of appropriate food grade and/or suitable for use in this process. All raw materials used in the manufacture of *B. Subtilis* NRRL 68053 are used in accordance with applicable US regulations, were concluded to be GRAS for their respective uses, or are subjects of effective food contact notifications. MDG's *B. subtilis* NRRL 68053 is not genetically engineered, no growth hormones or other hormones are added to the products, and no solvents are used in the manufacturing process of the microorganism. MDG's *B. subtilis* NRRL 68053 is certified Kosher.

2.3.3 Manufacturing Narrative and Flowchart

Raw materials for the media are received from approved vendors and checked against their purchase order upon arrival. To prepare the commercial media, the raw materials are weighed with clean scoops to the weights indicated on the media make sheet. Clean, potable city water is used as part of the media formula and is added to a tank. The media is sterilized at a minimum temperature of 121°C for 90 minutes and then cooled to the inoculation temperature.

The tank is aseptically inoculated using quality controlled (QC) inoculum stored under frozen conditions. Thereafter, the tank run parameters for media temperature, air flow, and stir speed are set and incubation for biomass growth starts. The vegetative cells will eventually become stressed and start to sporulate, starting the sporulation phase. The spore mass is separated from the growth media by centrifugation. The resulting slurry is collected in clean HDPE drums or totes. There are QC checks for strain identity, spore count, and coliforms. The slurry is placed into freeze dry trays using clean transfer systems and trays of the slurry are frozen after filling. Racks of frozen slurry trays are removed from the freezer and transferred to the Freeze Dryer. Material lots are recorded on a log sheet and the run cycle is started.

Towards the end of the drying cycle, the dryer is opened, a tray is removed, and the dried cake material is emptied into a new low-density polyethylene (LDPE) bag in the collection drum. When all of the trays on a rack have been emptied the bag is closed and the lid is put on the bin. There is a QC check for water activity. The dried

material is milled in a clean pulverizer and collected in new, clean LDPE bags and placed in drums. The *Bacillus* spore powder is moved to the QC HOLD area and stored at ambient temperature. The spore powder is tested to verify it is the strain of *Bacillus* that was grown and free of contaminants and pathogenic bacteria. Powder that meets the specifications for approval is released.

QC passed material is given a 'QC PASS' sticker and removed from the 'QC HOLD' area into inventory.

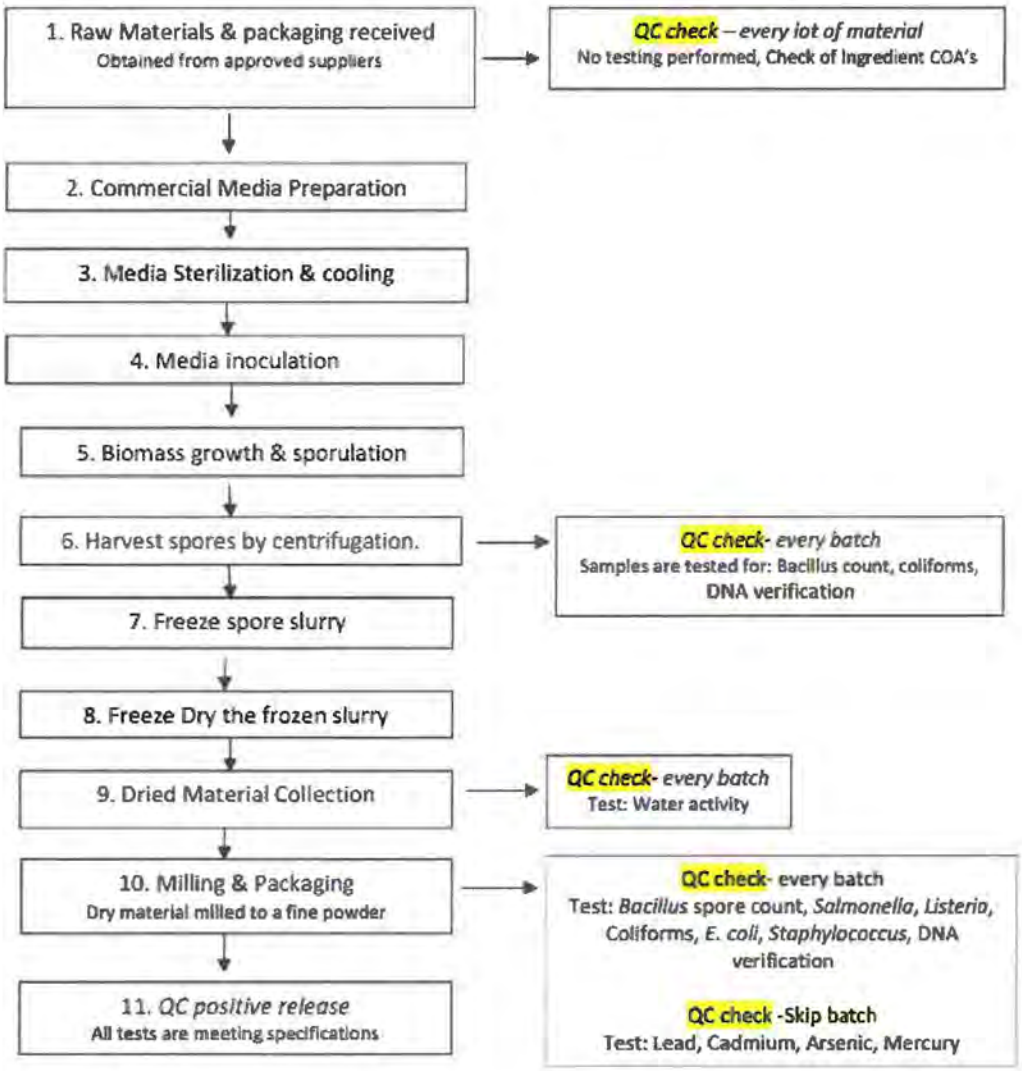

Figure 1. *Bacillus subtilis* NRRL 68053 Manufacturing Flowchart

2.4 Specifications and Batch Analyses

2.4.1 Specifications

The specifications for the food-grade product *B. subtilis* NRRL 68053, along with the specification methods, which have been validated for their stated purpose, are listed in Table 1 below.

Table 1. *Bacillus subtilis* NRRL 68053 Product Specifications

Tested Parameters	Acceptance Criteria	Methods
Microbiology		
Total *Bacillus* spore count	$\geq 1.50 \times 10^{11}$ CFU/g	MDG LAB 505a
Salmonella species	Not detected (per 25 g)	AOAC RI-121501
Listeria species	Not detected (per 25 g)	FDA BAM Chapter 10
Coliform	< 100 CFU/g	FDA BAM Chapter 4
Escherichia coli	< 10 CFU/g	FDA BAM Chapter 4
Staphylococcus aureus	< 10 CFU/g	FDA BAM Chapter 12
Yeast & mold	< 300 CFU/g	FDA BAM Chapter 18
Enterobacteriaceae	< 100 CFU/g	CMMEF Ch. 9.62
Physical		
Appearance	Light beige to tan powder	Visual check
A_w	< 0.350	MDG LAB 375
Heavy Metals[a]		
Arsenic	≤ 3 mg/kg or ppm	AOAC 2013.06
Cadmium	≤ 1 mg/kg or ppm	AOAC 2013.06
Mercury	≤ 1 mg/kg or ppm	AOAC 2013.06
Lead	≤ 2 mg/kg or ppm	AOAC 2013.06

Abbreviations: AOAC, Association of Official Analytical Chemists; A_w, water activity; BAM, bacteriological analytical manual; CFU, colony forming units; CMMEF, compendium methods microbiological examination foods; ppm, parts per million.

[a] Skip-lot testing, further described in subpart 2.5.

2.4.2 Batch Analyses

Production conformity and consistency of MDG's *B. subtilis* NRRL 68053 are tested in production lots. Batch analyses for the month of August 2021 and skip lot testing for heavy metals during the month of July 2021, are shown below and are reasonably consistent and met the product specifications for microbial analyses and physical composition.

Table 2. *Bacillus subtilis* NRRL 68053 Batch Analyses

Tested Parameters	Acceptance Criteria	Lot No./Date of Manufacture		
		Lot# 2021062201A 8-2021	Lot# 2021070701A 8-2021	Lot# 2021071301A 8-2021
Microbiology				
Total spore count	$\geq 1.5 \times 10^{11}$ CFU/g	7.6×10^{11}	4.9×10^{11}	6.4×10^{11}
Salmonella species	Not detected (per 25 g)	Not detected (per 25 g)	Not detected (per 25 g)	Not detected (per 25 g)
Listeria species	Not detected (per 25 g)	Not detected (per 25 g)	Not detected (per 25 g)	Not detected (per 25 g)
Coliform	< 100 CFU/g	< 10/g	< 10/g	< 10/g
E. coli	< 10 CFU/g	< 10/g	< 10/g	< 10/g
S. aureus	< 10 CFU/g	< 10/g	< 10/g	< 10/g
Yeast & mold	< 300 CFU/g	< 10/g	< 10/g	< 10/g
Enterobacteriaceae	< 100 CFU/g	< 10/g	< 10/g	10/g
Physical Characteristics				
Appearance	Light beige to tan powder	Light beige to tan powder	Light beige to tan powder	Light beige to tan powder
A_w	< 0.350	< 0.350	< 0.350	< 0.350
Heavy Metals[a] (mg/kg)		**Lot# 1 7/2021**	**Lot# 2 7/2021**	**Lot# 3 7/2021**
Arsenic	≤ 3	< 0.037	< 0.037	< 0.037
Cadmium	≤ 1	< 0.03	< 0.03	< 0.03
Mercury	≤ 1	< 0.044	< 0.044	< 0.044
Lead	≤ 2	< 0.029	< 0.029	< 0.029

Abbreviations: A_w, water activity; CFU, colony forming units.
[a] Skip-lot testing, further described in subpart 2.5.

2.5 Heavy Metals

For purposes of evaluation during the GRAS conclusion, heavy metal analysis was performed for each lot of *B. subtilis* NRRL 68053 reported in Table 2 above. However, for normal commercial production, in accordance with the company's Standard Operating Procedures (SOP), heavy metal analysis of a randomly chosen lot grown in each commercial production tank is conducted according to a skip lot procedure. Skip lot testing is done according to a sound statistical sampling plan and is conducted in accordance with the Hazard Analysis and Critical Control Point regulations at 21 CFR 117. According to MDG, skip-lot testing is justified based on the following factors:

- The raw materials used are food grade or USP grade and conform to standards for maximum levels of heavy metals;
- The production equipment is made of stainless-steel IBC; and

- Because of the nature of the spore growth (cultivation of spores in a closed system) heavy metals are not introduced during the process.

2.6 Antibiotic Resistance

Resistance to therapeutic antibiotics by microbial pathogens is currently considered one of the greatest challenges in medicine and public health, as some infectious diseases may become virtually untreatable if they become non-respondent to current therapies. Antibiotic resistance may be classified into two types;

- intrinsic/natural (when resistance is inherent to a bacterial species, and is a trait generally shared by all members of that species), and
- extrinsic/acquired (when a strain of a typically susceptible species is resistant to a given antimicrobial drug).

Extrinsic/acquired resistance can occur either from the gain of exogenous DNA or mutation of indigenous genes.[4, 5] The gain of exogenous DNA occurs through horizontal gene transfer (HGT) via transformation, transduction or conjugation and many of the antibiotic resistance genes are carried on mobility elements such as plasmids, transposons, or phages.[6,7] While intrinsic resistance likely presents a very low risk of dissemination, extrinsic/acquired resistance, especially when the relevant genes are associated with mobile genetic elements such as plasmids and transposons, can be transferred to pathogens or other commensal bacteria.[8] It is generally recommended that resistance to antibiotics be assessed in all probiotic strains prior to marketing.[4, 9-12]

European Food Safety Authority (EFSA) has published guidance documents with regard to antimicrobial susceptibility for bacteria that are intended to be used as feed additives and/or as production organisms.[4, 13] Phenotypic evaluation of antibiotic resistance involves testing the capacity of a microorganism to survive in a medium containing different concentrations of antibiotics. Whereas most microorganisms can survive at low concentrations of many antibiotics, resistance is defined as the capacity to grow at antibiotic concentrations similar to those reached in the human body during therapeutic intervention.

EFSA has provided MIC (minimum inhibitory concentration) values for a select list of clinically relevant antibiotics including chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline, and vancomycin.[13] These MIC values help define a bacterial strain as sensitive/susceptible to an antibiotic when its growth in otherwise appropriate media is inhibited by the antibiotic at a concentration equal to or lower than its established MIC cut-off value. On the other hand, a bacterial strain is defined as resistant when it is able to grow despite the presence of an antibiotic at a concentration greater than or equal to the established MIC cut-off value. The MIC cut-off values are specific

to individual bacterial species and are intended to be a tool to aid in distinguishing strains with intrinsic and/or acquired resistance. MDG performed MIC testing as recommended by EFSA on *B. subtilis* NRRL 68053 the results of which are summarized in Table 3 below with a subsequent discussion.

MDG further evaluated the genetic sequence of its strain for known antibiotic resistance genes. Four genes were identified through a Comprehensive Antibiotic Resistance Database (CARD) BLASTn search, that were characterized as strict matches—the search parameter for sequence identity was set at ≥ 75%. The genetic nature of the antibiotic resistance in these strains was evaluated and shown in Table 4 below, with a discussion below.

Table 3. *Bacillus subtilis* NRRL 68053 Assessment of Antimicrobial Susceptibility

Antimicrobial Agent	Phenotypic MIC		
	***B. subtilis* NRRL 68053 observed (μg/mL or mg/L)**	**EFSA susceptible breakpoints for *Bacillus* spp. (mg/L or μg/mL)[13]**	***B. subtilis* NRRL 68053 EFSA assessment**
Chloramphenicol	3	8	Sensitive
Clindamycin	1	4	Sensitive
Ciprofloxacin	0.94	n.r.	n.r.
Erythromycin	0.064	4	Sensitive
Gentamicin	0.094	4	Sensitive
Kanamycin	0.75	8	Sensitive
Linezolid	1	n.r.	n.r.
Rifampicin	0.25	n.r.	n.r.
Streptomycin	8	8	Sensitive
Tetracycline	12[b]	8	Resistant[a]
Tigecycline	0.094	n.r.	n.r.
Vancomycin	0.5	4	Sensitive

Abbreviations: MIC, Minimum Inhibitory Concentration; n.r., not required; spp. species.
[a]Within two-fold dilution

Table 4. *Bacillus subtilis* NRRL 68053 Assessment of Antibiotic Resistance Genes

Resistance genes identified in *B. subtilis* NRRL 68053 (enzyme/function)	Gene commonly found in *B. subtilis*?	Likely to be Intrinsic?	Associated antibiotic resistance	PR found in *B. subtilis* NRRL 68053?	Gene transfer risk?
aadk (aminoglycoside 6-adenylyltransferase)	Yes[14]	Yes	Streptomycin	No	No PR present & no MGE found, therefore unlikely to be a risk.
mphK (macrolide 2'-phosphotransferase K)	Yes[15] (GenBank NCBI Locus NG_065846)	Yes	Macrolide antibiotics (e.g. erythromycin)	Not in the relevant antibiotics tested.	No PR present & no MGE found, therefore unlikely to be a risk.
ykkC/D (member of the small multidrug resistance family of efflux pumps)	Yes[16, 17]	Yes	Substrates include tetracycline, streptomycin, & chloram-phenicol	PR to tetracycline[a] but not in the other relevant antibiotics tested.	PR present for tetracycline[a] but no MGE found, therefore unlikely to be a risk.
pgsA-A64V (phosphatidylglycerol synthase)	Yes[18]	Yes	Daptomycin	Not tested as EFSA does not recommend MIC testing for daptomycin.[13]	No MGE found, therefore unlikely to be a risk.

Abbreviations: MGE, mobile genetic elements; MIC, minimum inhibitory concentration; NA, not available; PR, phenotypic resistance.

[a]Within two-fold dilution of EFSA MIC

As summarized in Table 3, *B. subtilis* NRRL 68053 was susceptible to all of the antibiotics recommended for testing by EFSA with MIC values at or below the EFSA breakpoints, except for tetracycline. The strain showed intermediary resistance to tetracycline, as the MIC value for this antibiotic was within a two-fold dilution of the EFSA cut-off, which is still generally considered acceptable. This is because the technical variation of the phenotypic method applied to determine antibiotic susceptibility allows for a certain amount of latitude around acceptable results; there is precedence for accepting MIC levels that exceed their cut-off values by a single two-fold dilution due to this normal variation around the mean. For example, EFSA's "Scientific Opinion on the safety and efficacy of Oralin® (*Enterococcus faecium*) as a feed additive for calves for rearing, piglets, chickens for fattening, turkeys for fattening and dogs",[19] in which the Oralin®'s MIC value exceeded the MIC cut-off for kanamycin by a single two-fold dilution, was

considered to be within normal variation and did not raise concerns for safety by EFSA.

With regard to the antibiotic resistance genes, four were identified upon CARD search. One, *pgsA-A64V*, is associated with daptomycin resistance and, therefore, was not phenotypically tested as EFSA does not recommend testing it for this species. Two other antimicrobial resistance genes, aadk and mphK, did not show correlating phenotypic resistance therefore, further analyses was not required. The last of the four genes, *ykkC/D*, conveys resistance to tetracycline, which overlaps with the phenotypic results listed in Table 3 and is discussed below, in terms of its low likelihood of HGT. Importantly, all of the genes have been identified in *B. subtilis* strains in the literature, as shown in Table 4, and thus are further likely to be intrinsic.

In order to complete the HGT risk assessment, particularly concerning the tetracycline resistance gene, evaluation for mobile genetic elements (MGE) was conducted using the Pathogenicity Island Database, which comprises a variety of tools designed to identify pathogenicity genomic islands that encode for a multitude of virulence factors and facilitate horizontal transfer of virulence genes. This evaluation demonstrated that *B. subtilis* NRRL 68053 does not carry any MGE and thus, the possibility of gene transfer of the native tetracycline resistance is low.

In summary, this strain did not show phenotypic resistance to any antibiotics tested, except for tetracycline (within an acceptable two-fold dilution of the EFSA MIC). Further, the strain contained one of four antibiotic resistant genes present (*ykkC/D)*, which is known to be associated with resistance to tetracycline, however, there were no MGE found in the genome, thus the possibility of gene transfer to commensal bacteria is low. The other three antimicrobial resistance genes found in the genome of *B. subtilis* NRRL 68053 were not associated with phenotypic resistance, and again, due to lack of MGE located in the strain, concern of gene transfer is low.

2.7 Genomic Analysis for Virulence and Pathogenicity

While *B. subtilis* taxonomic units would not typically be expected to contain toxigenic sequences, some *Bacillus* species have the capacity for toxin production, particularly harmful surfactin-like lipopeptides.[4, 20] In addition to *B. subtilis* strains' not harboring any acquired resistance to clinically relevant antimicrobials, EFSA's Qualified Presumption of Safety (QPS) program, which generically assesses the safety of taxonomic groups or units and provides guidance on additional safety measures for certain species, also requires that *B. subtilis* strains must be absent of toxigenic activity. EFSA QPS is further discussed in subpart 6.2.1.[3] MDG evaluated the potential of *B. subtilis* NRRL 68053 to produce toxins that have been demonstrated to be virulent to hosts, by examining genomic sequence similarities to toxigenic genes with the PIV4 and Virulence Factor Analyzer through the

Virulence Factor Database (VFDB) databases. The *B. subtilis* NRRL 68053 genome was also compared to *B. subtilis* 168—a commonly used reference or type strain.[21, 22]

It should be noted that while virulence genes can help bacteria survive during periods of stress and do not necessarily cause damage to the host, toxin genes produce compounds which can cause damage to the host. Therefore, all toxin genes are considered virulence genes but not all virulence genes are toxigenic.[23] The results of MDG's testing are summarized in the table below.

Table 5. Virulence Factors Sequences Identified in *Bacillus subtilis* NRRL 68053

Closest Virulence Factor (VF) identified**	Related gene	Gene present in *B. subtilis* NRRL 68053	Gene present in *B. subtilis* 168*	Role of VF/reports in literature of gene found in *Bacillus species*	Conclusion/VF likely to be toxigenic?
Polyglutamic acid capsule	*cap*A	orf01726	Yes	Polyglutamic acid (PGA) is produced mainly by gram-positive bacteria in the *Bacillus* genus, such as *B. subtilis*, *B. anthracis*, *B. licheniformis*, & *B. pumilus*. Synthesis of PGA is associated with nutrient starvation/limitation during the stationary growth phase.[24] Genes *cap*BCAD responsible for the formation of PGA are found in *B. anthracis* and are orthologs/homologs of the *pgs*BCAE, genes found in *B. subtilis/licheniformis* strains.[24, 25] *Cap*D is necessary for anchoring polyglutamate to the peptidoglycan and *cap*E is required for polyglutamate synthesis.[26] Importantly, *cap*BCADE located in plasmid pXO2 are required for the capsulation formation.[24, 25, 27]	These genes were also present in the reference strain, and therefore they are likely to be intrinsic and unlikely to show toxigenic activity. PGA has been described as "anionic, biodegradable, water-soluble, non-toxic, and edible."[28] MDG examined the strain and no MGEs (including plasmids) were found in the genome (thus it is unlikely to be acquired or transferrable) and *capE* was not present in the genome, which is required for polyglutamate synthesis.
	*cap*B	orf01724			
	*cap*C	orf01725			
	*cap*D	orf00009			
Hemolysin III	*hlyIII*	orf01273	Yes	Reports in the literature of hemolysin III found in *B. cereus*.[29] A putative hemolysin gene, although Joerling et al., (2020) states that it is unclear if	MDG tested the strain for hemolytic activity, and it displayed alpha hemolysis (negative for beta hemolysis) as is discussed

Closest Virulence Factor (VF) identified**	Related gene	Gene present in *B. subtilis* NRRL 68053	Gene present in *B. subtilis* 168*	Role of VF/reports in literature of gene found in *Bacillus species*	Conclusion/VF likely to be toxigenic?
				hemolysin III truly represents a hemolysin gene and "may play a negligible role in the formation of strong hemolysis."[30] Additionally, Ramarao et al., (2013) states that the role of *hlyIII* "has not been investigated in vivo and remains a matter of speculation."[29]	further in subpart 2.9. They further examined the strain and no MGEs (including plasmids) were found in the genome (thus it is unlikely to be acquired or transferrable). The gene is present in the reference strain, therefore is likely to be intrinsic and unlikely to show toxigenic activity.
Bacillibactin	*dhbA*	orf02137	Yes	These genes are involved in the binding of iron, in response to iron limitation in the environment, and allow iron scavenging.[31] There are reports of this gene being present in *B. subtilis*.[31]	These genes are likely present to help bacteria survive during periods of stress. Additionally, they are reported in the literature to be found in *B. subtilis*.[32] The genes are present in the reference strain, therefore are likely to be intrinsic and unlikely to show toxigenic activity.
	dhbB	orf02140			
	dhbC	orf02138			
	dhbE	orf02139			
	dhbF	orf02141			

Abbreviations: MGE, mobile genetic elements.
* Reference strain

All ten genes identified from the virulence databases, including PGA (*capa*, *capb*, *capc*, and *capd*), hemolysin III (*HylIII*), and bacillibactin (*dhbA*, *dhbB*, *dhbC*, *dhbE*, and *dhbF*) found in *B. subtilis* NRRL 68053 are likely to be intrinsic as they are present in the reference strain, *B. subtilis* 168.

Several authors emphasize that there are limitations to our understanding and knowledge of VFs. For example, Pariza et al., (2015), stated that "our knowledge of how VFs work, and interact with one another, in the promotion of illness versus health, is incomplete."[23, 33] Hill (2012) states that a genuine VF would be defined as a "product, structure, or strategy that helps a microbe gain access to or survive in normally noncolonized body sites or cellular compartments, cause damage to the body, cause dysregulation of the immune system to the extent of creating disease symptoms, or cause a neurological response that again leads to disease symptoms."

[23] Whereas he states that "niche factors would include products or strategies that promote motility, bile tolerance, immune evasion in nonsterile body sites, macro- and micronutrient acquisition, attachment mechanisms, and various other colonization and microbe-host communication strategies."[23] Therefore, he proposed that VFs should be divided into two main categories including 1) "niche factors" or VFs which promote survival and 2) those which cause damage to the host.[23] Hill concludes that many VFs should be categorized as "niche factors" as they are promoting colonization and survival and "are often shared by harmless commensal organisms occupying the same body site."[23, 33] Further, Hill states that "most microbes proposed for use as probiotics will possess many of the colonization and survival strategies used by pathogens, which may well have been established in the literature as bona fide VFs."

In conclusion, the "VF genes" found in *B. subtilis* NRRL 68053 are intrinsic and likely to be categorized in the Hill et al., (2012) "niche" category as they promote colonization and survival and are unlikely to damage the host, and therefore would not be considered toxigenic.

2.8 Cytotoxicity

Finally, because EFSA requires *Bacillus* spp. be free of toxigenic activity, MDG had a third-party laboratory, Emery Pharma, conduct an *in vitro* cytotoxicity test on *B. subtilis* NRRL 68053 supernatant, using Vero (epithelial) cells. Cytotoxicity was measured after a 60 minute incubation of the test article with Vero cells at a concentration of 10%, using a lactate dehydrogenase (LDH) assay and was done in accordance with EFSA's "Guidance on the characterisation of microorganisms used as feed additives or as production organisms"[13] according to Emery Pharma. Absorbance values above 20% of the absorbance obtained from the maximum LDH release control (Promega 10x Lysis Solution) indicates cytotoxicity. The supernatant from *B. subtilis* NRRL 68053 as shown in the tables below, showed no significant cytoxocity against Vero cells under the conditions of the assay.

Table 6. *Bacillus subtilis* NRRL 68053 Supernatant Percent LDH Released by Vero Cells

Test Article	% LDH signal*			
	Replicate #1	Replicate #2	Replicate #3	Average
***B. subtilis* NRRL 68053 supernatant**	5.52%	1.98%	2.02%	3.18%
Untreated	1.87%	0.89%	0.89%	1.22%

Abbreviations: LDH, lactate dehydrogenase.
*% LDH signal determined by the following formula: (sample optical density (OD) – average EC buffer OD) divided by (max LDH release OD – average EC buffer OD)

As a control for endogenous LDH signal in the bacterial medium used to generate the test article, the test article was also tested in EC buffer (in the absence of Vero cells). The results of this assay were utilized in the data shown in Table 6 above, as denoted in the equation below the table.

Table 7. *Bacillus subtilis* NRRL 68053 Percent LDH Signal in EC Buffer and Supernatant Controls

Test Article	% LDH signal			
	Replicate #1	Replicate #2	Replicate #3	Average
***B. subtilis* NRRL 68053 supernatant in EC buffer**	1.58%	1.58%	1.55%	1.62%

Abbreviations: LDH, lactate dehydrogenase.

2.9 Hemolysis

MDG evaluated *B. subtilis* NRRL 68053 for its hemolytic activity (effect on red blood cells (RBCs)) by streaking and incubating it overnight on commercial blood agar plates (BAP; tryptic soy agar enriched with 5% sheep's blood). The purpose of this test is to differentiate the bacteria's ability to fully lyse the RBCs (beta hemolysis), "partially lyse" (alpha hemolysis), or not affect them at all (gamma hemolysis). Hemolytic activity is determined by the color of the agar under or surrounding the bacterial colonies as follows: beta clears the color, alpha results in green-brown color, but gamma doesn't affect a change, so the color remains red.[34] MDG's production strain displayed alpha hemolytic activity confirmed by the green-brown color change in the BAP.

Importantly, rather than a true lysis, alpha hemolysis reduces the red blood cell hemoglobin to methemoglobin, which causes the green-brown color change in the BAP medium. According to Buxton (2005), alpha hemolysis can be equated with "bruising" the cells which is confirmed upon microscopic inspection that shows that the cell membranes remain intact.

2.10 Physical or Technical Effect

MDG's *B. subtilis* NRRL 68053 is not intended to produce any physical or other technical effects that are relevant to the safety of the ingredient.

Part 3: Intended Use and Dietary Exposure

For the purpose of this GRAS notice, MDG's *B. subtilis* NRRL 68053 is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. For example, it maybe be used in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary, snacks, and other foods. It is not intended to be added to infant formula, alcoholic beverages, or any products that would require additional regulatory review by USDA. The intended addition level to foods is up to 1 x 10^{10} CFU per serving (which is similar to levels of fermentation bacteria found in traditionally fermented food products).[35]

While *B. subtilis* NRRL 68053 is considered a novel strain, it's intended uses may be partially substitutive with regard to other GRAS *B. subtilis* strain intended uses (e.g. GRNs 831, 905, 955), or with regard to traditional uses of strains of the *B. subtilis* species.

Several older publications were located that looked at dietary patterns of Americans by analyzing the number of servings of foods consumed in a day. A publication from the USDA's Center for Nutrition Policy and Promotion (October 2000) states that men aged 51 and older consume the largest number of servings of food per day, at 18.2 servings/day.[36] Comparatively, women aged 19–24 consumed the least, at 12.5 servings/day. This data came from detailed 14-day food diaries from 5,752 adults in the 1992–1994 time period. Millen et al., (2005) used 24-hour dietary recall and diet history questionnaire data from the Eating at America's Table study (1997–1998) to analyze the mean number of servings per day consumed of food guide pyramid food groups by adults.[37] There were 497 women and 436 men that completed the study. The results (from the study's Table 1) suggest that the mean intake for men was approximately 27.8 servings per day and for women was 19.5 servings per day.

Using a most conservative estimation of consumption, if 100% of food servings contained *B. subtilis* NRRL 68053 at the maximum concentration of 1 x 10^{10} CFU per serving, highest consumers (men) would be exposed to approximately 1.82–2.78 x 10^{11} CFU/day. Using 70 kg as a standard body weight, this is equivalent to 2.6–4.0 x 10^{9} CFU/kg bw/day). This estimation is considered extremely conservative, as realistically, most foods will not contain *B. subtilis* NRRL 68053 due to the standards of identity of many foods, the fact that it will not be added to foods requiring additional USDA regulatory review, market share limitations, limited food matrix viability, and the fact that the ingredient will likely be "invisible" to many consumers, who may realize they are consuming a food containing a "probiotic" but likely will not be aware of the specific strain that they are consuming, reducing the likelihood that only food products containing this strain will be chosen and consumed. If a more realistic (but still highly conservative) estimate is used that 50% of food servings will contain the maximum intended use

level of *B. subtilis* NRRL 68053, highest consumers (men) would be exposed to approximately 9.1 x 10^{10}–1.4 x 10^{11} CFU/day (using 70 kg as a standard body weight, this is equivalent to 1.3–2.0 x 10^{9} CFU/kg bw/day). For the purpose of this GRAS conclusion, this latter calculation is considered the estimated exposure for the strain.

Part 4: Self-limiting Levels of Use

There are no known inherent self-limiting levels of use.

Part 5: Experience Based on Common Use in Food Prior to 1958

The safety assessment for MDG's *B. subtilis* NRRL 68053 is based on scientific procedures, and thus, experience based on common use in food prior to 1958 is not considered pivotal information. Nevertheless, the historical use of food fermented with *B. subtilis* is extensively discussed in Part 6.

Part 6: Narrative

6.1 History of Consumption

B. subtilis has a long history of human consumption, especially in fermented foods in Asia and Africa. Hong, et al., (2005) describes at least nine probiotics on the market containing *B. subtilis* that are intended for human consumption, many of which have been on the market for decades without safety concerns.[38] Typical levels ranged from 1 x 10^6 to 1 x 10^9 CFU/serving.[38]

B. subtilis is well known for its use in the traditional Japanese fermented soybean food called natto, which has a bacterial concentration reported as approximately 1 x 10^8 CFU/g.[1, 38] Consumption of a 100 g serving of natto containing this concentration of bacteria is equivalent to consumption of approximately 1 x 10^{10} CFU/serving. *B. subtilis* natto is recognized as FOSHU (Food For Specified Health Use) by the Japanese Ministry of Health, Labour, and Welfare.[39]

Further, *B. subtilis* has a long history of being commonly used in non-soybean based fermented foods, including locust bean as "dawadawa" and "kinda" in Africa, fish such as "karati", "bordia", and "lashim" in India and black-gram products such as "maseura" and "wari" in India.[40]

B. subtilis is also listed in the inventory published by the International Dairy Federation (originally a collaboration with the European Food and Feed Culture's Association) documenting microbial species with technological beneficial roles in fermented food products, specifically as relates to use in soy (natto), emphasizing the species' long history of use in human food.[41, 42]

6.2 Regulatory Opinions

B. subtilis NRRL 68053 has never been released on the market and therefore it does not have regulatory status. However, there is international regulatory status for *B. subtilis*, some of which is summarized below.

6.2.1 Europe

6.2.1.1 EFSA QPS

EFSA has developed an approach to safety assessments of microorganisms called Qualified Presumption of Safety (QPS). QPS generically assesses the safety of taxonomic groups or units (e.g., a bacterial species) independent of any particular pre-market authorization process. Any strain of microorganism, the identity of which can be unambiguously established and assigned to a QPS group, does not need to undergo further safety assessment by EFSA other than to satisfy any qualifications specified in the QPS assessment. QPS is generally not based on a particular intended use unless stated in a specific qualification. Microorganisms not

considered suitable for QPS remain subject to full safety assessments. The first QPS list was established in 2007.[3] A full evaluation of the QPS list is undertaken every three years and results are published as Scientific Opinions, while the list of microorganisms is maintained and re-evaluated approximately every six months to include new notifications to EFSA, and published as Panel Statements.[43, 44] *B. subtilis* was granted QPS status in the first EFSA QPS publication in 2007, based on the substantial body of knowledge available on the species. However because some species within the *Bacillus* genus possess toxigenic traits, a QPS qualification for this species is the absence of toxigenic activity.[3] The other qualification is that the individual strains should not harbor any acquired antimicrobial resistance genes to clinically relevant antimicrobials. *B. subtilis* remains on the most recent EFSA QPS lists.[43-47]

6.2.1.2 EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)

EFSA FEEDAP concluded that several strains of *B. subtilis* are potentially efficacious as feed additives. They stated that *B. subtilis* DSM 28343 has the potential to be efficacious in pigs for fattening and is considered safe at 2 x 10^8 CFU/kg, provided that the identity of the strain is conclusively established, that there is evidence that the strain is not toxigenic, and that it does not show resistance to antibiotics of human and veterinary importance.[48] Additionally, the FEEDAP concluded that *B. subtilis* PB6 shows "some potential to be efficacious in laying hens.[49] This conclusion on efficacy for laying hens can be extended to minor poultry species raised for laying when the additive is used at the same minimum dose."[49]

6.2.2 United States

6.2.2.1 FDA GRAS

In the US, companies can notify FDA of their conclusion of GRAS status for a particular bacterial species/strain or ingredient on an individual basis, and for specific intended uses. It was estimated in 2009 that approximately 40% of food enzymes marketed in Europe were produced by bacterial/fungal recombinant strains, and vitamins, amino acids, and polysaccharides are also obtained from recombinant strains.[50] There are 25 GRAS notices related to *B. subtilis* strains (mainly as recombinant strains utilized to isolate enzymes) listed in FDA's GRN inventory. Of these, 20 have received the no questions ("NQ") letter from FDA, one was ceased to be evaluated at the notifier's request (this was actually a notice for *B. subtilis* itself, and the reason for requesting that FDA cease to evaluate is unknown), five notices (GRNs 831, 905, 955, 956, and 969) received the no questions letter for *B. subtilis* strains to be used in foods, and are thus most relevant to the current safety assessment, and four notices are pending approval. It is worth noting that GRNs

such as 969, 956, and 905 that have received FDA no questions letters have reported minor adverse events including gastrointestinal events associated with consumption of the ingredient used in clinical trials and it was concluded that these do not present a safety concern. A brief summary of these notices is shown in Table 8.

Table 8. FDA GRAS Notices That Include *Bacillus subtilis* Strains

FDA GRN Number	Strain Description	Date of Closure	Status	Maximum Intended Use	Exposure Estimates by the Notifiers
20	Pullulanase derived from *B. subtilis* carrying a gene encoding pullulanase from *B. naganoensis*	September 1999	NQ	0.0225–0.8/gram dry substance of starch in starch hydrolysis and alcoholic beverages and at 0.0225–10/g dry substance of flour in bakery products	"Worst case" human exposure to the residues of enzyme—0.02 mg TOS/kg bw/day
114	Pectate lyase enzyme preparation from *B. subtilis*	January 2003	NQ	0.5–1.0% of weight	N/A
205	Pullulanase enzyme preparation from *B. subtilis* expressing the pullulanase gene from *B. acidopullulyticus*	December 2006	NQ	25 liters per ton of starch dry substance	N/A
274	Branching glycosyltransferase enzyme preparation from *B. subtilis* expressing a branching glycosyltransferase gene from *Rhodothermus obamensis*	June 2009	NQ	0.4–40 kg/ton of starch dry substance	Maximum daily intake—1.8 mg TOS/kg bw/day
406	1,4-α-glucan branching enzyme preparation from *B. subtilis* strain 168 expressing the glucan branching enzyme gene from *Aquifex aeolicus* strain VF	September 2012	NQ	500 U/g substrate, which is equivalent to 0.07 mg TOS/g of substrate in the production of cyclic dextrin	Exposure at 90th percentile—0.46 mg TOS/kg bw/day
476	Asparaginase enzyme preparation produced by genetically modified *B. subtilis*	February 2014	NQ	20 mg TOS/kg	Maximum daily intake—0.27 mg TOS/kg bw/day
562	*B. subtilis*	April 2015	With-drawn		

FDA GRN Number	Strain Description	Date of Closure	Status	Maximum Intended Use	Exposure Estimates by the Notifiers
579	Lactase from *Bifidobacterium bifidum* produced in *B. subtilis*	November 2015	NQ	Milk—1.1 mg TOS/g Infant formula—1.3 mg TOS/g of GOS	Dairy products—1.4 mg TOS/kg bw/day Infant formula—1.9 mg/kg bw/day
592	β-glucanase from *B. subtilis*	October 2015	NQ	37 mg TOS/kg of grist	156 µg TOS/kg bw/day
649	β-galactosidase enzyme preparation from *B. circulans* produced in *B. subtilis*	November 2016	NQ	GOS at levels up to 0.3% of the lactose starting material	Dietary exposure to the enzyme preparation from its intended use is not expected to occur
714	Subtilisin from *B. amyloliquefaciens* produced in *B. subtilis*	February 2018	NQ	Up to 369 mg TOS/kg substrate	4.15 mg TOS/kg bw/day
746	Maltogenic amylase from *Geobacillus stearothermophilus* produced in *B. subtilis*	June 2018	NQ	Maximum of 20 mg TOS/kg flour	0.18 mg TOS/kg bw/day
751	Maltogenic alpha-amylase from *B. stearothermophilus* produced in *B. subtilis*	July 2018	NQ	Up to 49.5 mg TOS/kg starch raw material	0.32 mg TOS/kg bw/day
831	*B. subtilis* DE111	October 2019	NQ	Infant formula for term infants—maximum level of 2×10^8 CFU/100 mL Various foods for adults—10^{6-10} CFU/serving	Infant formula 90th percentile—213.4 mL/kg bw/d and exposure 4.27×10^8 CFU/kg bw/day EDI—1.3×10^{11} CFU/day
861	Pullulanase from *Bacillus deramificans* produced in *Bacillus subtilis*	July 2020	NQ	Maximum use of 186 mg TOS/kg	0.5 mg TOS/kg bw/day
905	*Bacillus subtilis* SG188	June 2020	NQ	10^9 CFU/serving in conventional foods	Maximum of 5×10^9 viable spores/day
955	*Bacillus subtilis* strain BS-MB40 PTA-122264 spore preparation	May 2021	NQ	2×10^9 CFU/serving in various foods	Maximum exposure 3.64×10^{10} CFU/day
956	*Bacillus subtilis* ATCC SD-7280	August 2021	NQ	6×10^9 spores/serving in various foods	Maximum exposure 1.1×10^{10} spores/day

FDA GRN Number	Strain Description	Date of Closure	Status	Maximum Intended Use	Exposure Estimates by the Notifiers
969	*Bacillus subtilis* "Bss-19" spore preparation	October 2021	NQ	1×10^{10} CFU/serving in foods	Highest exposure 2.78×10^{11} CFU/day
974	Maltogenic alpha-amylase enzyme preparation produced by *Bacillus subtilis*	February 2022	NQ	22 mg TOS/kg cereal and baking flours raw material	Maximum exposure 0.195 TOS/kg bw/day
989	*Bacillus subtilis* expressing a modified gene encoding a variant of the wild-type subtilisin from *B. clausii*		Pending		
991	Chitonase enzyme preparation produced by *Bacillus subtilis*		Pending		
1007	*Bacillus subtilis* strain R0179		Pending		
1011	Alpha-amylase enzyme preparation produced by *Bacillus subtilis* strain AR-651 expressing the gene encoding alpha-amylase from *Thermoactinomyces vulgaris*	July 2022	NQ	Maximum level of 100 mg TOS/kg four in baked foods	Maximum exposure 0.89 mg TOS/kg bw/day
1042	Menaquinone-7 derived from a specific strain of *Bacillus subtilis* natto		Pending		

Abbreviations: CFU, colony forming units; EDI, estimated daily intake; GOS, galacto-oligosaccharides; N/A, data not available; NQ, FDA filed the GRAS without questions ("no questions" letter); TMDI, Total Theoretical Maximum Daily Intake; TOS, Total Organic Solids

6.2.2.2 FDA New Dietary Ingredient Notifications

The Federal Food, Drug, and Cosmetic ACT (FD&C Act) in section 21 U.S.C. 350b(a) requires that all manufacturers and distributers of a New Dietary Ingredient (NDI) not yet present in the food supply be submitted to the FDA a minimum of 75 days prior to its introduction. There was one NDI notification relevant to this safety assessment that was filed by FDA summarized in the table below. Note that NDI notifications that were objected to by FDA were excluded from the table. This is because objections by FDA were most often related to incomplete notifications and/or the notifier's inability to establish the identity of the dietary ingredient (and not necessarily due to a safety issue), and thus those notifications are not considered to be useful to this safety assessment.

Table 9. FDA NDI Notification for *Bacillus subtilis*

Species	NDI Notice	Intended Use
Bacillus subtilis	RPT1167 (NC) *Bacillus subtilis* ANA3	3×10^9 CFU/5 mL/day

Abbreviations: CFU, colony forming units; NC, no comments; NDI, New Dietary Ingredient.

6.2.2.3 Code of Federal Regulations

A thorough search for the current regulatory status of *Bacillus subtilis*, relevant to its use in food in the United States, was conducted with the following searched entities: "*Bacillus subtilis*" and "*B. subtilis*". There are three regulations in the 21 CFR for enzyme preparations allowed in foods, derived from nonpathogenic/nontoxigenic *B. subtilis* strains, as follows:

- 21 CFR 173.115 Alpha-acetolactate decarboxylase enzyme from recombinant *B. subtilis;*
- 21 CFR 184.1148 Carbohydrase enzyme from *B. subtilis*; and
- 21 CFR 184.1150 Protease enzyme from *B. subtilis.*

Of note, a number of *B. subtilis* strains are registered with the Environmental Protection Agency (EPA) as microbial pesticides for various uses.[51] EPA describes *B. subtilis* as "a ubiquitous bacteria commonly found in various ecological niches including soil, water, and air which does not have a history of pathogenicity from contact in the environment".[51] Further, the EPA concluded that it is "a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low."[52]

6.2.3 Health Canada

All natural health products (NHPs) sold in Canada are subject to the *Natural Health Products Regulations*, which came into force on January 1, 2004. To be legally sold in Canada, all natural health products must have a product license. To get a product license, proper safety and efficacy evidence must be provided. Once Health Canada has assessed a product and decided it is safe, effective, and of high quality, it issues a product license along with an eight-digit Natural Product Number (NPN), which must appear on the label. This number indicates that the product has been reviewed and approved by Health Canada.

The safety and efficacy of NHPs and their health claims must be supported by proper evidence. Evidence may include clinical trial data or references to published studies, journals, pharmacopoeias, and traditional resources. The type and amount of supporting evidence required depends on the proposed health claim of the product and its overall risks.

B. subtilis is classified as an NHP under Schedule 1 (substances including plant, plant material, algae, bacterium, fungus, or non-human animal material) of the Natural Health Product Regulations. Additionally, *B. subtilis* strain DE111 and d-ribose derived from *B. subtilis* strain ATCC 21951 for use at 5 g/serving up to 15 g/day are both considered non-novel food ingredients.[53] There are 74 products

containing *B. subtilis* that are approved to be marketed under the NHPs Regulations of Health Canada. Further, there are ten enzymes (α-acetolactate decarboxylase, amylase, asparaginase, glucanase, hemicellulose, lactase, pentosanase, protease, pullulanase, and xylanase) derived from *B. subtilis* that are permitted in a variety of foods.[54] Unfortunately, the data that Health Canada relied upon to make their determination is not available to the public.

6.2.4 Food Standards Australia New Zealand (FSANZ)

The Food Standards Code Standard 1.5.1, part of the FSANZ, came into force in December 2000. To be legally sold in Australia or New Zealand all novel food or ingredients, or 'non-traditional' ingredients, must be approved by FSANZ. To be approved, these novel foods and/or ingredients are subject to a pre-market safety assessment and FSANZ evaluates the public health and safety of the ingredient, taking into consideration the toxicological and nutritional factors. Once FSANZ has assessed the novel ingredient or food it is approved and added to The Food Standards Code Standard 1.5.1 list and permitted to be sold.

Novel Foods of the Australia New Zealand Food Standards Code Standard 1.5.1 lists four *B. subtilis* strains; CU1, R0179, BS-GA28, and DE111.[55] There were no safety concerns identified at intended levels of use for *B. subtilis* strain DE111 up to 1×10^{11} CFU/serving, strain R0179 up to 1×10^{9} CFU/serving, and strain CU1 up to 6.5×10^{9} CFU/day (there was not an intended use level provided for BS-GA28).[55, 56]

6.3 Safety Information

Toxicological studies have been published on various strains of *B. subtilis* and are summarized in subpart 6.3.1. Additionally, human studies on *B. subtilis* strains are discussed in subpart 6.3.2. There were no human or toxicological studies located for *B. subtilis* NRRL 68053 specifically, as it is a novel strain. The studies reviewed do not suggest any concerns related to the safety of the strain. A comprehensive literature search was conducted on *B. subtilis* for the safety assessment described in Part 6 of this GRAS notice through January 24, 2023.

6.3.1 Toxicological Studies on *Bacillus subtilis* strains

Spears et al., (2021) studied *B. subtilis* MB40 in a 14-day repeated dose oral toxicity study.[57] Groups of 10 Sprague-Dawley [Crl:CD(SD)] rats/sex/group each were administered the test article at doses of 500, 1000, and 2000 mg/kg/bw/day (equivalent to 9.25×10^{10}, 1.85×10^{11}, or 3.7×10^{11} CFU/kg bw/day) by gavage (control group rats were given deionized water). The animals were weighed, observed for clinical signs, and evaluated for mortality and moribundity for each of the 14 days. Clinical pathology and gross pathological examinations were performed on day 15 and selected organs were weighed. There were no mortalities and no reported test article-related effects, with regard to clinical observations or body weights. Macroscopic findings were considered incidental and unrelated to administration of the test article. Higher mean adrenal weights were observed in the low and high dose treated male groups (500 and 2000 mg/kg bw/day), higher mean testes weights were found in the low dose group (500 mg/kg bw/day) and there were some statistically significant differences in hematology coagulation parameters. However, all of these findings were considered unrelated to the administration of the test article as they were also not dose-dependent and were within the laboratory's historical range. The NOAEL for *B. subtilis* MB40 was 2000 mg/kg bw/day, equivalent to 3.7×10^{11} CFU/kg bw/day, which was the highest dose tested. The study was performed using guidelines from FDA Redbook 2000 Testing Guideline IV.C.3.a, Short-Term Toxicity Studies with Rodents.[57]

Zhang et al., (2013) studied *B. subtilis* strain Tpb55 in an acute gavage toxicity study and a maximum tolerable dose study in mice and rats, respectively, in which animals were observed for 14 days after treatment.[58] The LD_{50} was determined to be greater than 5000 mg/kg in both the mice and rats, as no deaths occurred and in addition no "symptoms of poisoning", or abnormal anatomic structures were observed. There was no increase in the incidence of micronuclei or chromosomal aberrations in *in vivo* mouse assays up to the highest dose tested (2500 mg/kg bw/day administered (by gavage) for two days or five days in a bone marrow polychromatic erythrocyte micronucleus study and a primary spermatocyte chromosomal aberration study, respectively). The spore content was 3×10^{10} CFU/g/day (7.5×10^{10} CFU/kg bw/day).

Two toxicity studies published in Korean and Chinese, respectively, were identified and the translated abstracts are described below. Both studies were also described in GRN 831 on *B. subtilis* DE111, which received the FDA no questions letter. Kyoung-Hoon et al., (2015) administered a single oral dose of *B. subtilis* JNS to mice at 2000 mg/kg bw followed by an observation period of 14 days.[59] The authors reported that no significant change in the general condition of the animals, no clinical signs or mortality was observed. Nor were there any gross lesions observed at necropsy. Nakamura et al., (1999) performed a 90-day subchronic toxicity study using *B. subtilis* gum (gum and strain not specified) in both sexes of F344 rats by feeding of CRF-1 pellet diet containing 0%, 0.18%, 0.55%, 1.66% and 5% of the test article (the abstract did not state the amount of *B. subtilis* used in the study).[60] Five groups consisted of 10 males and 10 females each and the rats were randomly allocated to the five groups. No animals died during the administration period and there were no differences in body weights or food intakes among groups of either sex. Kidney weight was significantly increased in both sexes in groups given concentrations of 1.66% or more *B. subtilis,* but the increases were slight and no correlating serum biochemistry changes or kidney histopathology was observed. The authors concluded that the treatment of *B. subtilis* gum in the diet for 90 days does not exert any treatment related effects at the highest dose tested.

Sorokulova et al., (2008) described a number of studies on *B. subtilis* VKPM B2335 (BS3) in mice and rabbits.[61] In one study, groups of 10 BALB/c male mice were each administered the test article intravenously and intraperitoneally at 5×10^7, 5×10^8, and 5×10^9 CFU/mouse and orally at doses of 5×10^7, 5×10^8, and 5×10^{11} CFU/mouse (authors state that the control group mice (three total) were given "sterile PBS" by the appropriate route of administration). Animals were observed for seven days, and on days two and seven, five animals from each group were euthanized and internal organs were observed macroscopically. For the groups treated orally, tissues were collected for histopathological examination (liver, kidneys, lungs, spleen, intestine, mesenteric lymph nodes, brain, thymus, and tissues around the throat). There were no treatment related deaths, even in groups given the strain intravenously. There were no adverse effects observed related to animal activity or weight. All animals were reported to be clinically healthy. There were no differences in visceral organ appearance or histopathological examinations between treated and control groups. The authors also described a 10-day repeated dose study using oral administration (method of oral administration not provided) in groups of ten mice (1×10^6 CFU/day), rabbits (1×10^9 CFU/day), and piglets (1×10^9 CFU/day), as well as a 30-day repeated dose study using groups of ten rabbits (1×10^9 CFU/day). There were no clinical effects or effects on body weight, changes in hematology values or effects observed upon gross or histopathological examination in treated groups compared to controls.

Hong et al., (2008) performed a repeated-dose gavage study of *B. subtilis* natto in 6 male New Zealand White rabbits as compared to an equal number of controls.[62] A dose of 1 x 10^9 spores was given to the treated animals daily for 30 days. Blood samples were taken on the last day and the liver, kidneys, spleen, small intestines, and mesenteric lymph nodes were collected for histopathological examination. There were no clinical changes or changes in feed intake, and no changes in hematology or visceral organs or tissues were observed compared to controls. The authors additionally studied the effect of a single dose (1 x 10^{12} CFU) via gavage of *B. subtilis* natto in guinea pigs, as briefly described in the same publication. There were no findings related to changes in appetite, behavior, feces, weight gain, or histopathology 17 days after administration in feed.

Tompkins et al., (2008) performed a 28-day repeated dose study in groups of ten Sprague-Dawley albino rats. *B. subtilis* R0179 or *E. Faecium* R0026 was administered by gavage at dose of 2 x 10^9 CFU/kg bw/day *B. subtilis* R0179. The control group was administered the vehicle (authors did not state what the vehicle was) via gavage.[63] Animals were monitored daily for potential signs of toxicity and groups were compared for mortality, morbidity, behavior, body mass, food consumption, gross pathology, intestinal colonization, and infection. Any changes in skin, fur, eyes, mucous membranes, secretions/excretion, autonomic activity, gait, posture, handling response, sensory reactivity, and movement were noted. At the end of treatment, the liver, kidneys, spleen, heart, and lungs were subjected to histopathology and microbiological exams. No findings, other than a lower heart mass (10%) in female rats, were noted. The heart to body weight ratio was not affected by the treatment in these animals, and no histopathological findings in the heart were mentioned. The *B. subtilis* strain was not observed microbiologically except in the intestinal content of treated animals.

Lastly, cell-free supernatants of *B. subtilis* KATMIRA were evaluated by a bacterial reversal mutation assay (Ames Salmonella assay).[64] The cell-free supernatants were negative for inducing mutations in this assay.

6.3.2 Human Studies

The safety of *B. subtilis* NRRL 68053 has not been formally investigated in healthy adult subjects. Additionally, many human clinical studies have been and continue to be published on other *B. subtilis* strains.[65-68] EFSA's recent mandated Scientific Opinion published in December 2022, includes a thorough search and review of literature published since the previous EFSA QPS (June 2022) as well as a full evaluation of the QPS list and it continues to maintain QPS status. There were no published human clinical studies since the last QPS Opinion with trials utilizing other *B. subtilis* strains.

6.3.3 Opportunistic Infections

With the exception of the members of the *Bacillus cereus* group (e.g., *B. cereus*, *B. anthracis*, *B. thuringiensis*), the virulence of members of the *Bacillus* genus may be considered very low. Identified risk factors for *Bacillus* bacteremia include drug addiction, hemodialysis, and leukemia (all of which may contribute to immunosuppression).[69] Based on two retrospective studies investigating *Bacillus* bacteremia, the presence of central venous catheters may increase risk of *Bacillus* bacteremia in immunocompromised patients.[69, 70]

Rare infections caused by *B. subtilis* have been described in the literature. For example, a 73 year old male with chronic lymphocytic leukemia had a positively identified recurrent septicemia caused by *B. subtilis*.[71] Another case report in the literature involved a patient with an esophageal perforation who had bacteremia and mediastinitis due to co-infection with *B. subtilis* and *B. licheniformis*.[72] Overall, infections with *B. subtilis* occur at very low rates, and generally occur in hospital settings in immunocompromised patients and/or during medical procedures.[71-73]

6.4 Allergenicity

B. subtilis NRRL 68053 does not contain or have added any of the nine major allergens (milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanuts, sesame, and soybeans) identified, and required to be disclosed in labeling, in the Food Allergen Labeling and Consumer Protection Act (FALCPA). No reports of allergic reactions to *B. subtilis* were found in our investigations.

6.5 Reported Adverse Events

B. subtilis NRRL 68053 has never been released on the market and therefore has no past sales or past reported adverse events to account for. However, rare adverse events associated with *B. subtilis* have been reported, as summarized below. All databases were accessed on January 24, 2023.

As described above, *B. subtilis* has a long history of safe consumption by humans and animals.[74] Today, *B. subtilis* is available in supplements from numerous companies. According to a search of the National Institutes of Health's Dietary Supplement Label Database, which contains information taken from the labels of dietary supplement products available in the U.S. marketplace, the search term "*Bacillus subtilis*" returned 1806 products that contain this species as an ingredient.

FDA

B. subtilis NRRL 68053 is a novel product and thus no FDA letters regarding concern for safety to companies that market products containing *B. subtilis* NRRL 68053 were located. A search of MedWatch and FDA's Recalls, Market

Withdrawals, & Safety Alerts search engine had no mention of *B. subtilis*. No FDA letters regarding concern for safety to companies that market products containing *B. subtilis* were located.

FAERS

FDA's Center for Food Safety and Applied Nutrition Adverse Event Reporting System (FAERS AE) revealed five reported cases of adverse events which included one death. These were found under the category "*Bacillus subtilis.*" These events occurred in 2001 (one case; case ID 3642080), 2014 (three cases; case IDs 10156610, 10214359, and 10220659) and 2019 (one case; case ID 16155058); one case involved a subject between 3–11 years old, two cases occurred in subjects between 65–85 years old, and in two cases the ages were not specified. Reported symptoms ranged significantly, including chest discomfort, multiple organ dysfunction syndrome, gastrointestinal disorders; urinary tract infection, and respiratory failure.

Adverse event reports are only associations and reported products may not be causally related to the adverse events. The FAERS website include the following caveats regarding their AERs as seen below.

"...while FAERS contains reports on a particular drug or biologic, this does not mean that the drug or biologic caused the adverse event. Importantly, the FAERS data by themselves are not an indicator of the safety profile of the drug or biologic. Some additional limitations to note include:

***Duplicate and incomplete reports are in the system**: There are many instances of duplicative reports and some reports do not contain all the necessary information.*

***Existence of a report does not establish causation**: For any given report, there is no certainty that a suspected drug caused the reaction. While consumers and healthcare professionals are encouraged to report adverse events, the reaction may have been related to the underlying disease being treated, or caused by some other drug being taken concurrently, or occurred for other reasons. The information in these reports reflects only the reporter's observations and opinions.*

***Information in reports has not been verified**: Submission of a report does not mean that the information included in it has been medically confirmed nor it is an admission from the reporter that the drug caused or contributed the event.*

***Rates of occurrence cannot be established with reports**: The information in these reports cannot be used to estimate the incidence (occurrence rates) of the reactions reported."*

6.6 Basis for the GRAS Conclusion

MDG's *B. subtilis* NRRL 68053 has been the subject of a thorough safety assessment as described in this GRAS Panel Report. The totality of evidence supporting safety is comprised of data and information that establish the safety of *B. subtilis* NRRL 68053 under the conditions of its intended use and data and information that is corroborative of safety.

6.6.1 Data and Information that Establish Safety

The scientific data, information, and methods forming the basis of this conclusion are:

- The establishment of identity via:
  - Complete genome sequencing and analysis with the Microbial Genome Sequencing Center services;
  - gyrB sequencing and the resulting consensus sequence was queried through the NCBI BLASTn database, demonstrating unequivocally that NRRL 68053 is a strain of the *B. subtilis* subspecies *subtilis*;
- The method of manufacture of *B. subtilis* NRRL 68053, which is described. In brief, non-genetically engineered *B. subtilis* NRRL 68053 cells are incubated and stressed, causing them to sporulate and grow. They are centrifuged, frozen, and then freeze dried. *B. subtilis* NRRL 68053 is produced using food grade materials and consistent with current good manufacturing practices;
- The specifications, as well as batch analyses, show that all specifications are met for each batch, and demonstrate safe production methods and robust quality control standards for *B. subtilis* NRRL 68053;
- The analyses and resulting data show that the strain is sensitive to the clinically relevant antimicrobials per European Food Safety Authority (EFSA) minimum inhibitory concentration (MIC) cut-offs and guidelines and in cases of resistance, further investigation by MDG showed that the resistance is not expected to be transferable;
- An analysis demonstrated that there are ten genes related to virulence factors (VF) present in the genome of *B. subtilis* NRRL 68053 (via comparison of genomic sequences to known virulence sequences in the Pathogenicity Island Database and the Virulence Factor Database). However, the genes present are considered intrinsic to this species and are correlated with promoting survival and are otherwise unlikely to show toxigenic activity; and

- *B. subtilis* has EFSA QPS status for use in food or feed, at any reasonable dose/intended use, suggesting no further regulatory review prior to introduction of new *B. subtilis* strains into the European food supply, other than the qualifications that it must be verified to not possess toxigenic traits or harbor acquired antimicrobial resistance genes.
- Previous GRAS notices for *B. subtilis* strains (GRNs 831, 905, 955, 956, and 969) received no questions letters from FDA for use in foods. GRNs 831 and 969 both listed similar intended use levels at addition levels of up to 1×10^{10} CFU per serving. GRN 831 listed an estimated exposure of 1.3×10^{11} CFU/day by assuming consumption at the maximum intended use addition level in 50% of food servings daily. GRN 969 listed the highest exposure of 2.78×10^{11} CFU/day. GRNs 905, 955, and 956 had slightly lower intended use levels and estimated exposure levels.

6.6.2 Data and Information that are Corroborative of Safety

- Published toxicology studies and animal feed studies on various *B. subtilis* strains, showing no indication of safety issues;
- The documented long history of safe human consumption of *B. subtilis* as a common bacterial species in fermented foods,[41] such as in natto (with concentrations of approximately 1×10^8 CFU/gram, equivalent to approximately 1×10^{10} CFU/100 g serving) over decades without known concerns for safety;[62, 63] and
- Agreement in the literature that it is highly unlikely that a microorganism maintained in pure culture, with a history of safe use, would become unsafe as a result of mutation (genetic drift), production changes, or delivery format changes.[33, 75, 76]

6.6.3 General Recognition

The scientific data, information, and methods herein reported, that provide the basis of this GRAS conclusion by scientific procedures are published and available in the public domain. Part 7 of this GRAS notice contains the citations for the published studies. These publicly available data and information fulfill the requirement of the GRAS standard for general availability of the scientific data, information, and methods relied on to establish the safety of *B. subtilis* NRRL 68053 for its intended conditions of use. The peer-review of the published studies and lack of Letters to the Editor or other dissenting opinions provide ample evidence of general recognition among qualified experts that there is reasonable certainty that consumption of *B. subtilis* NRRL 68053 for its intended use is not harmful. The general availability and acceptance of these scientific data, information, and

methods satisfy the criterion of the GRAS standard that general recognition of safety requires common knowledge throughout the 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.

6.6.4 Data and Information that are Inconsistent with the GRAS Conclusion

We have reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with a conclusion that MDG's *B. subtilis* NRRL 68053 is reasonably certain to be safe under the conditions of its intended use.

6.6.5 Information that is Exempt from Disclosure under FOIA

There are no data or information in this report that are considered trade secret or commercial or financial information that is privileged or confidential.

Part 7: Supporting Data and Information

Literature searches for the safety assessment described in Part 6 of this GRAS notice were conducted through January 24, 2023.

7.1 Data and Information that are *not* Generally Available

All of the information described in this report is generally available.

7.2 References that *are* Generally Available

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November 6, 2023

Re: Responses to FDA's GRN 1143 Questions

Dear Dr. Deng,

Please find responses to FDA's questions concerning *B. subtilis* NRRL 68053 (GRN 1143) below. FDA's questions are in BLACK, while the notifier responses are in BLUE:

1. Please clarify whether *B. subtilis* NRRL 68053 has been deposited in a culture collection organization and please provide a depository number if it is available.

   Response: Yes, *B. subtilis* NRRL 68053 has been deposited at the Agriculture Research Service Culture Collection (NRRL) and the depository number is B-68053.

2. On page 9, the notifier states that a genome announcement for *B. subtilis* NRRL 68053 is pending. Please clarify whether the genome data will be available in a public domain, e.g., a NCBI accession number.

   Response: The genome announcement will be published in a Microbiology Resource Announcements® (ASM Journals), which will be available in the public domain.

3. The notifier compares the *B. subtilis* NRRL 68053 genome to that of *B. subtilis* 168 – "a commonly used reference or type strain" (page 18). We note that *B. subtilis* 168 is a whole-genome sequenced strain that has been used in industrial biotechnology. To support the safety conclusion of this GRAS notice, please provide the following information:

   - genome similarity between *B. subtilis* NRRL 68053 and the *B. subtilis* strains that have been used in food, such as *B. subtilis* strains of the five GRAS notices (GRNs 000831, 000905, 000955 and 000956 and 000969) listed on pages 29-30 which have received "no questions" letters from the FDA.

     Response: Direct sequence comparisons of the *B. subtilis* NRRL 68053 genome to those of the strains that are the subjects of the

above noted GRAS notices have not been performed. However, some gross/phenotypic similarities can be stated in that none of the strains harbor transferable antibiotic resistance genes and all of the strains exhibit similar minimum inhibitory concentration test results to clinically important antibiotics; none of the stains harbor putative virulence factors that result in toxigenicity of the strains; and none of the stains produce hemolysins. Therefore, the strains are considered non-pathogenic and non-toxigenic.

It is, also, important to note that there are reports in the literature such as the EPA (1997) state that *B. subtilis* is considered a "benign organism as it does not possess traits that cause disease,[1]" implying that the species has low concern with regard to safety. Gu et al. (2019) state that the "majority of *Bacillus* are non-pathogenic," and only a few species of *Bacillus* (such as *B. anthracis* and *B. cereus*) are known to cause disease in animal and humans.[2] Additionally, there are numerous publications regarding safety of microbial species and strains, such as Pariza et al. (2015) and more recently, Roe et al. (2022).[3, 4]

Roe et al. (2022) states that "if sufficient history of safe use is known for oral consumption of a specific bacterial species, and the strain of interest has been properly identified to the strain level, its genome properly sequenced, annotated and shown to not contain genes of concern, and intended use of the ingredient falls within an exposure considered to be safe, phase 1 clinical study safety studies are likely not needed for use by generally healthy humans. If there are limitations on the history of safe use of the strain and/or the species exists on the EFSA QPS list for example, then some limited testing may be necessary. This should include a search of phylogenomic databases using the whole genome sequencing to determine presence of various antibiotic resistance, virulence and toxin genes, and phenotypic testing for antibiotic resistance should be conducted according to standard to antibiotic screens."[3]

As stated in the GRAS notice and summarized in Subparts 6.1 and 6.2 on pages 26–27 of 45, the *B. subtilis* species has a long history of safe use, remains on the most recent QPS list, and *B. subtilis* NRRL 68053 has been properly identified to the strain level and its genome has been properly sequenced and annotated, demonstrating that it does not contain genes of concern. Further, a complete genome sequencing was completed, demonstrating that there are no antibiotic resistance, virulence or toxin genes of concern.

- comparison of identified virulence factors in *B. subtilis* NRRL 68053 and those in the *B. subtilis* strains that have been used in food.

Response: The polyglutamic acid (PGA) proteins found in *B. subtilis* NRRL 68053 have been identified widely across *B. subtilis* species and contribute to the functional properties in fermented soybean based foods.[5] PGA produced by *Bacillus* species can be found in many Asian fermented soybean products such as "natto" and "kinema", and give the food a sticky texture. A key criterion for high quality kinema is its' exceptional stickiness, which Chettri et al. (2016) attribute to the production of PGA by *Bacillus* species.[5] Given the international use and consumption of these foods, this virulence factor (VF) is unlikely to be toxigenic.

Regarding the hemolysin gene (*hylIII*), there is a similar report of a hemolysin protein in *B. subtilis* Bss-19 in GRN 969. The authors noted that this strain contains hemolysin protein (HLY3_BACCE) but did not have any safety concerns as this "protein is highly conserved across the genus including many strains known to not be pathogens." Further, *B. subtilis* NRRL 68053 was negative for beta-hemolysis, indicating that the presence of this protein is unlikely to show toxigenic activity.

In regard to bacillibactin, GRN 955 demonstrated that their strain (*B. subtilis* MB40) also contains this VF. Harwood et al. (2018) state that "many members of the genus *Bacillus* synthesize and secrete bacillibactin."[6] Furthermore, the authors state that there are no direct reports of toxicity nor are there any well-documented reports of human or animal toxicity associated with this compound. Additional information on bacillibactin can be found below in the response to question 3(c).

In summary, *Bacillus* species containing these VF are commonly found in food and not associated with pathogenicity. Thus, there are minimal to no known safety concerns with their presence in *B. subtilis* NRRL 68053.

- clarification whether *B. subtilis* NRRL 68053 produces any secondary metabolites.

  Response: *Bacillus subtilis* NRRL 68053 whole genome sequence was queried through the antiSMASH pipeline which permits the rapid genome-wide identification, annotation, and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genomes. It integrates and cross-links with a large number of in silico secondary metabolite open-source analysis tools. The inputs for the pipeline were set for relaxed detection strictness with extra features including KnownCultureBlast, TFBS analysis, ActiveSiteFinder, SubCLusterBlast, and RPEFinder.

The output data yielded 100% sequence similarity to the secondary metabolites listed below in the table. Additionally, there were no hits for sequence similarities in 90–100% range.

Secondary Metabolite	**Description**	**Metabolite Present in Other *B. subtilis* GRNs That Received a "No Questions" Letter from FDA**
Bacillaene	A polyketide synthase (PKS) gene cluster, which belongs to a large family of secondary metabolites that includes many bioactive compounds with antibacterial and physiologically relevant bioactivities.[6, 7] The *pks1* biosynthesis gene cluster is orthologous to *B. subtilis* strain 168's PKS gene cluster for similar bioactive molecules. This metabolite has been characterized in both *B. subtilis* and *B. amyloliquefaciens* species.[7] In a study performed by Harwood et al. (2018), it was reported that 77% (55 of 68 strains) of the examined *B. subtilis* strains contained this metabolite. They state that there "are no direct reports of toxicity" to organisms higher than prokaryotes.[6]	GRN 955
Bacillibactin	An endogenous non-ribosomally peptide synthetase (NRPS) siderophore which chelates iron as a survival mechanism.[6] This metabolite has been characterized in both *B. subtilis* and *B. amyloliquefaciens* species.[8, 9] In a study performed by Harwood et al. (2018), it was reported that 99% (67 of 68 strains) of the examined *B. subtilis* strains contained this metabolite. They state that there "are no direct reports of toxicity associated with bacillibatin."[6]	GRN 955
Bacilysin	NRPS surfactant-like dipeptide, induces lysis of the microbial cell wall by inhibiting glycosamine-6-phosphate synthase.[10] It is responsible for impairing the formation of microbial cell wall development rather than direct antimicrobial activity. Additionally, it acts as a signaling molecule either directly or indirectly and affects various cellular functions, as well as spore quality.[11] It has been identified in *B. amyloliquefaciens*, *B. velezensis*, *B. licheniformis, B. pumilus* and *B. subtilis*.[10] In a study performed by Harwood et al. (2018) , it was reported that 93% (63 of 68 strains) of the tested *B. subtilis* strains contained this metabolite.[6] There	GRNs 905 & 955

Secondary Metabolite	Description	Metabolite Present in Other *B. subtilis* GRNs That Received a "No Questions" Letter from FDA
	were no specific toxicological concerns addressed by the authors.[6]	
Pulcherriminic Acid	A cyclic dipeptide synthesized by *Bacillus* and yeast that accumulates iron ions in a severely iron-deficient environment. The microorganism is able to remain competitive when other microorganisms are unable to obtain iron and other necessary inorganic salts for growth. In this way, pulcherriminic acid (PA) can be considered to have antibacterial activity through its ability to out compete other microorganisms in these low-iron environments. "PA is a very effective and safe bioactive antibacterial compound in industry."[12]	N/A
Subtilomycin	A new lantibiotic characterized from *B. subtilis* which produces broad spectrum antimicrobial activity against gram-positive and gram-negative pathogens, as well as several pathogenic *Candida* species. It is proposed that this metabolite facilitates the survival of the microbe in competitive ecological niches. This metabolite is widespread amongst *B. subtilis* strains.[13]	N/A
Subtilosin A	A macrocyclic bacteriocin (antibacterial) metabolite produced by *B. subtilis* from the subtilosin gene (*sbo*) sequences. The biosynthesis sequence exhibits high level of homology to the sequence of the *sbo-alb* gene locus of *B. subtilis* strain 168.[14]	GRN 905 (*B. subtilis* SG188)

Abbreviations: N/A: not available

- a statement that *B. subtilis* NRRL 68053 is nonpathogenic and non-toxigenic.

  Response: *B. subtilis* NRRL 68053 is a non-pathogenic and non-toxigenic organism.

4. For the administrative record, please briefly specify how the purity of the *B. subtilis* NRRL 68053 inoculum for the manufacturing process is ensured.

   Response: To ensure the purity of the *B. subtilis* NRRL 68053 inoculum for the manufacturing process, prior to placing the biomass under cryopreservation, the identity of the strain is confirmed via DNA fingerprinting methods and spore counts and checks for contaminants and pathogenic species are performed according to the ingredient specifications. Additionally, as noted in Subpart 2.3.3 (Manufacturing Narrative and Flowchart), pages 10–11, these checks are repeated during the manufacturing process.

5. Please provide a statement that the manufacturer continuously monitors the fermentation process for contaminants.

   Response: The fermentation process is monitored per every lot and every inoculation process, for contaminants including culture condition, culture

temperature, pH, type of bacteria and presence of contaminants by culture medium sampling.

6. In Table 1 on page 12, we note that the FDA BAM Chapter 4 is a conventional method to enumerate coliform and *E. coli* using the most probable number (MPN) with a sampling size of 50 g. Please clarify if this is the method used in testing for *E. coli* with the specification of 10 CFU/g.

   Response: No, MDG did not use the conventional method to enumerate *E. coli* using the most probable number as described in FDA BAM Chapter 4 sections I.C, I.E, and I.F. Rather, they used the conventional solid medium plating method described in FDA BAM Chapter 4, section I.G. FDA BAM Chapter 4, introductory text states, "Also, there is a solid medium plating method for coliforms that uses Violet Red Bile Agar [VRBA] … This chapter also includes variations of above tests that use fluorogenic substrates to detect *E. coli*." *E. coli* (as well as total coliform) enumeration is performed by 3rd party ISO 17025 certified laboratories—Eurofins or Diebel.

   As required under section I.G, MDG followed the sample preparation steps as described in Section I.C using a 50 g sample size. However, as described in section I.G, results are calculated as follows, "Determine the number of coliforms **per gram** by multiplying the number of suspect colonies by percent confirmed in BGLB by dilution factor … *E. coli* colonies can be distinguished among the coliform colonies on VRBA by adding 100µg of 4-methyl-umbelliferyl-β-D-glucuronide (MUG) per mL in the VRBA overlay. After incubation, observe for bluish fluorescence around colonies under longwave UV light." Thus, despite the use of a 50 g sample size, the reporting units of the specification are CFU/g.

7. The notifier states that they intend to use *B. subtilis* NRRL 68053 as an ingredient in foods, but then provides examples of foods in which the ingredient will be used. Please clarify if the use of the ingredient is in all conventional foods, except for alcoholic beverages, infant formula, products under the jurisdiction of the United States Department of Agriculture, and in foods where standards of identity preclude its use.

   Response: We apologize for the confusion. The ingredient is intended for use in all conventional foods amenable to addition of such ingredients, except for alcoholic beverages, infant formula, products under the jurisdiction of the United States Department of Agriculture, and in foods where standards of identity preclude its use.

8. In Table 2 on page 13, the notifier lists the specifications for *B. subtilis* NRRL 68053 and provides results from the analyses of three non-consecutive batches. We note that the batch analyses for lead, cadmium, mercury, and arsenic are significantly lower than the corresponding specifications for each heavy metal. In addition, we note FDA's Closer to Zero initiative that focuses on reducing the dietary exposure to heavy metals. Please lower the heavy metal specifications to reflect the results of the batch analyses and to be as low as possible.

Response: The notifier has lowered their specification limits for lead, cadmium, mercury, and arsenic to better reflect the results of the batch analyses and can be seen in the table below.

Heavy Metals		
	New Specifications	Previous Specifications
Lead	≤ 0.2 mg/kg	≤ 2 mg/kg
Cadmium	≤ 0.2 mg/kg	≤ 1 mg/kg
Mercury	≤ 0.2 mg/kg	≤ 1 mg/kg
Arsenic	≤ 0.2 mg/kg	≤ 3 mg/kg

9. In Part 3, the notifier provides the maximum number of servings consumed per day by men (~ 27.8 servings/day) and women (~19.5 servings/day) based on the data published in Millen et al. (2006). We note the following:
    - The maximum number of servings/day provided in the GRAS notice are higher than expected based on the data in Millen et al. (2006). Please note that the number of ounces/day reported for "Red meat, poultry, fish" accounts for all "Lean meat".

        Response: We acknowledge your point and agree that our exposure estimations were higher than would be expected based on the Millen et al. (2006) data. Also, we note, from a practical perspective, unprocessed "lean meats" are not a suitable format of food for addition of *B. subtilis* NRRL 68053, nor are other whole food products such as vegetables and fruits, making our exposures estimates all the more conservative.
    - To estimate dietary exposure based on the number of servings, we typically use 20 servings/day (the average number of servings for men and women from both the 24-hours dietary recall and the diet history questionnaire). Please revise your dietary exposure accordingly.

        Response: Utilizing FDA's recommendations of consumption of an average number of 20 serving/day of food for men and women and assuming the ingredient will be present at the maximum addition level of 1 x 10^{10} CFU *B. subtilis* NRRL 68053 per serving, the maximum estimated dietary exposure from the intended use of *B. subtilis* NRRL 68053 is 2 x 10^{11} CFU/day. Using 70 kg as an average body weight, this exposure is equivalent to 2.86 x 10^{9} CFU/kg bw/day. This estimate is highly conservative as it assumes the ingredient will be present at the maximum addition level in all foods.
10. For supporting the safety conclusion, the notifier lists five successful GRAS notices (GRNs 000831, 000905, 000955 and 000956 and 000969) which subjects are *B. subtilis* strains. As each GRAS notice stands on its own, for the administrative record, please provide a brief paragraph summarizing the information pertaining to safety for each of these GRAS notices.
    - A GRAS notice to FDA (GRN 831) for *B. subtilis* DE111 received a no questions letter from FDA for use as an ingredient in milk-based and soy-

based infant formula for term infants at a level of 2 x 10^8 CFU/100 mL of formula powder. In FDA's no questions letter dated on August 13, 2019, they summarized the GRN safety narrative for the ingredient, including the following: The notifier "discusses the history of use of the *B. subtilis* strain and published literature demonstrating that *B. subtilis* is found in soil and has been isolated from the healthy human gastrointestinal tract and human breast milk. The notifier states that *B. subtilis* is non-pathogenic and non-toxicogenic. They discuss the results of unpublished studies demonstrating that *B. subtilis* DE111 spore preparation is susceptible to antibiotics and lacks antibiotic resistance genes. Additionally, they state that *B. subtilis* DE111 spore preparation does not possess hemolytic activity. They note the safety assessment of other authoritative bodies as support for the safe use of *B. subtilis* DE111 spore preparation. They include the statement of a panel of individuals. Based on its review, the GRAS panel concluded that *B. subtilis* DE111 spore preparation is safe under the conditions of its intended use. Based on the totality of evidence, they conclude that *B. subtilis* DE111 spore preparation is GRAS for its intended use."

- A GRAS notice to FDA (GRN 905) for *B. subtilis* DSM 32444 received a no questions letter from FDA for use as an ingredient in conventional foods (excluding infant formula and foods under the jurisdiction of the United States Department of Agriculture) at a level of 10^9 CFU/serving. In FDA's no questions letter dated on June 8, 2020, they summarized the GRN safety narrative for the ingredient, including the following: the notifier "states that members of the genera *Bacillus* are commensals within the digestive tract of humans and animals. They describe the history of safe use of *B. subtilis* in fermented foods. They rely on published literature that supports the safety of consumption of *B. subtilis* DSM 32444 spore preparation. The notifier states that numerous strains of *B. subtilis* with close homology to *B. subtilis* DSM 32444 are nonpathogenic and non-toxigenic. Additionally, they describe published human tolerance studies in which adults were fed *B. subtilis* and state that no significant adverse effects were noted in any of these studies. The notifier includes the statement of a panel of individuals. Based on its review, the GRAS panel concluded that *B. subtilis* DSM 32444 spore preparation is safe under the conditions of its intended use."
- A GRAS notice to FDA (GRN 955) for *B. subtilis* strain BS-MB40 PTA-122264 (*B. subtilis* BS-MB40 PTA-122264) received a no questions letter from FDA for use as an ingredient in various conventional foods at a level of up to 2 x 10^9 CFU/serving. In FDA's no questions letter dated on February 8, 2021, they summarized the GRN safety narrative for the ingredient, including the following: the notifier "describes the history of safe use of *B. subtilis* in human food, specifically in fermented food products, and explains that *B. subtilis* has been isolated from water, soil, air, and decomposing plant matter. The notifier performed a literature search through January 2021 and summarizes published literature and governmental reviews that support the safety of consumption of *B. subtilis* BS-MB40 PTA-122264 spore

preparation, including a published 14-day oral toxicity study and two published clinical studies, with no reports of toxicity or treatment-related effects noted. The notifier explains that infection linked to *B. subtilis* is rare. Based on its review, the notifier's GRAS panel concluded that *B. subtilis* BS-MB40 PTA-122264 spore preparation is safe under the conditions of its intended use. Based on the totality of evidence, they conclude that *B. subtilis* BS-MB40 PTA122264 spore preparation is GRAS for its intended use."

- A GRAS notice to FDA (GRN 956) for *B. subtilis* ATCC SD-7280 received a no questions letter from FDA for use as an ingredient in various foods at a level of 6×10^9 spores/serving. In FDA's no questions letter dated on August 18, 2021, they summarized the GRN safety narrative for the ingredient, including the following: the notifier "states that there is a history of safe use of *B. subtilis* in the manufacture of fermented foods, including fermented sausages, fermented vegetables, cereal products, and dairy products. They rely on published literature to support the safety of oral consumption of *B. subtilis* spore preparation. The notifier states that there have been no human infections related to ingesting food products containing *B. subtilis* spores. They include the report of a panel of individuals. Based on its review, the notifier's GRAS panel concluded that *B. subtilis* ATCC SD-7280 spore preparation is safe under the conditions of its intended use."
- A GRAS notice to FDA (GRN 969) for *B. subtilis* ATCC SD-7280 received a no questions letter from FDA for use as an ingredient in various foods at a level of 1×10^{10} CFU/serving. In FDA's no questions letter dated on October 6, 2021, they summarized the GRN safety narrative for the ingredient, including the following: the notifier "discusses the history of consumption of *B. subtilis* and published literature demonstrating that it is found in soil and has been isolated from the human gastrointestinal tract. They state that *B. subtilis* is non-pathogenic and non-toxicogenic. The notifier discusses the results of published human and toxicological studies demonstrating that *B. subtilis* strains are well tolerated and do not induce toxicity, as well as an unpublished acute oral toxicity study in rats demonstrating that *B. subtilis* ATCC SD-7780 does not induce toxicity. They state that *B. subtilis* ATCC SD-7780 is non-pathogenic and non-toxicogenic. The notifier discusses the results of unpublished studies demonstrating that *B. subtilis* ATCC SD-7780 spore preparation is susceptible to antibiotics and lacks antibiotic resistance genes. They discuss published reports of opportunistic infections in immunocompromised individuals and states that these do not present a safety concern for *B. subtilis* ATCC SD-7780 spore preparation. Additionally, they note that the European Food Safety Authority concluded that *B. subtilis* met Qualified Presumption of Safety status in 2007 and has maintained this status. Based on the totality of evidence, they conclude that *B. subtilis* ATCC SD-7780 spore preparation is GRAS for its intended use."

11. Please provide updated information on the literature search(es) performed to prepare the notice. This includes the date(s) (e.g., month and year) of the

search(es), the resource database(s) used (e.g., PubMed), the principal search terms used, and the time period that the search spanned (e.g., 1/2022 to 10/2023).

Response: A comprehensive literature search was performed related to the safety of the ingredient. Literature searches for the safety assessment described in Part 6 were conducted through January 24, 2023. The search engines utilized included PubMed and Medline. The search terms included "*Bacillus subtilis*" and "*B. subtilis*."

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December 18, 2023

Re: Response to FDA's December 1, 2023 Second Set of Questions regarding GRN 1143

Dear Dr. Deng,

Please find response to FDA's query concerning *B. subtilis* NRRL 68053 (GRN 1143) below. FDA's query is in BLACK, while the notifier response us in BLUE:

> In the amendment dated November 6, 2023, Microbial Discovery Group (MGD) lowered the specifications for arsenic, cadmium, lead, and mercury to 0.2 mg/kg each. Based on the results of the batch analyses and what we have seen in other fermentation derived ingredients produced under good manufacturing practices, it may be possible to further lower these specifications. Please consider lowering the specifications for heavy metals to 0.1 mg/kg each or lower in accordance with FDA's closer to zero initiative to reduce dietary exposure to heavy metals.

Response:

Following analysis of historical batch data, the Notifier (MDG) amends their specification limits for cadmium, mercury, and lead to not more than (NMT) 0.1 ppm.

While analysis of all lots of NRRL 68053 produced to date have shown arsenic results of < 0.037 ppm, historical lot data across all bacterial strains produced by MDG have shown arsenic levels may vary from not detected to 0.16 ppm. Therefore, the MDG has amended the NRRL 68053 specification limit for arsenic to NMT 0.175 ppm. The amendment history is shown in the table below:

Heavy Metal Specification Limits			
	Revised 12/19/2023	Revised 10/17/2023	Original
Lead	≤ 0.1 mg/kg	≤ 0.2 mg/kg	≤ 2 mg/kg
Cadmium	≤ 0.1 mg/kg	≤ 0.2 mg/kg	≤ 1 mg/kg
Mercury	≤ 0.1 mg/kg	≤ 0.2 mg/kg	≤ 1 mg/kg
Arsenic	≤ 0.175 mg/kg	≤ 0.2 mg/kg	≤ 3 mg/kg

MDG operates in compliance with the HACCP certification as well as the US requirement under 21 CFR 117 subpart G 117.405-117.475 supply-chain program. According to 21 CFR subpart C, 117.126 (food safety plan) and 117.130 (hazard analysis) there is an established food safety plan that includes the risk assessment of ingredients. Accordingly, limits are set, and analyses performed, for any impurity in a raw material or other ingredient that could present a hazard in the finished product. Given the materials in use, MDG has not assessed that arsenic is a foreseeable concern for the ingredients used in the culture media broth. Nonetheless, at this time, the cause of the variation in arsenic levels (compared to the other three heavy metals that are consistently low) is unknown, and MDG is taking steps to identify and mitigate, if possible, the cause.