AIBMR Life Sciences, Inc.

July 21, 2020

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OFFICE OF FOOD ADDITIVE SAFETY

Susan Carlson, PhD
Division Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Department of Health and Human Services
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 Danisco USA, Inc. (the notifier), the undersigned, Jessica Gruber, submits, for FDA review, the enclosed notice that *Bacillus subtilis* Bss-19 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 jessica@aibmr.com.

Sincerely,

Jessica Gruber, ND (agent of the notifier)
Scientific and Regulatory Consultant
AIBMR Life Sciences, Inc. ("AIBMR")

September 11, 2020

Dr. Carlson and staff,

To prevent any potential confusion, this submission replaces one that should have arrived at your office on July 29, 2020, but apparently was lost by FedEx.

If you did indeed receive that first shipment, please disregard this duplicate.

Thank you!

Jared

Jared Douglas Brodin
Director of Information Services
AIBMR Life Sciences, Inc.

Notice to US Food and Drug Administration of the Conclusion that the Intended Use of Bss-19 is Generally Recognized as Safe

Submitted by the Notifier:

Danisco USA, Inc.
DuPont Nutrition & Biosciences
Four New Century Parkway
New Century, Kansas 66031

Prepared by the Agent of the Notifier:

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

July 21, 2020

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Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

Danisco USA, Inc. (the notifier), an affiliate of DuPont Nutrition & Biosciences (hereafter referred to as DuPont) is submitting a new GRAS notice in accordance with 21 CFR Part 170, Subpart E, regarding the conclusion that *Bacillus subtilis* Bss-19 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

Elizabeth McCartney
Regulatory Affairs Specialist
Danisco USA, Inc.
DuPont Health & Biosciences
3329 Agriculture Drive
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Agent of the Notifier

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Tel: (253) 286-2888
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1.3 Name of the Substance

The name of the substance is *Bacillus subtilis* Bss-19.

1.4 Intended Conditions of Use

B. subtilis Bss-19 (hereafter referred to as Bss-19) 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 x 10^{10} CFU per serving.

1.5 Statutory Basis for GRAS Conclusion

The conclusion of GRAS status of Bss-19 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 Bss-19 is GRAS for its intended conditions of use, stated in Part 1.4 of this notice, and, therefore, such use of Bss-19 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 Elizabeth McCartney (DuPont Health & Biosciences, 3329 Agriculture Drive Madison, Wisconsin 53716), 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 Bss-19.

(b) (4)

21 July 2020

Elizabeth McCartney
Regulatory Affairs Specialist
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]

Dupont's Bss-19 is also known as *B. subtilis* BS7711. Additionally, DGCC12972 is used as the internal identification within the DuPont Global Culture Collection.

2.1.1 Taxonomy of Bss-19

Dupont's *B. subtilis* inaquosorum strain Bss-19 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: inaquosorum strain Bss-19

2.1.2 Genetic Identification of Bss-19

The whole genome of Bss-19 was sequenced, assembled, and included a single 16S rRNA gene copy. When compared to publicly available sequences, the 16S rRNA copy of Bss-19 was found to be most closely related to the type strain *B. subtilis* subspecies inaquosorum KCTC 13429 (99.93% identical).

Whole genome sequencing and assembly of Bss-19 revealed seven single nucleotide variations between the assemblies when compared to publicly available *B. subtilis* inaquosorum strain DE111 sequence. Four of these changes were confirmed by PCR. Two of the single nucleotide variations resulted in amino acid changes indicating that these strains do not have identical genomes.

Genomic average nucleotide identity (ANI) comparison of the whole draft genome sequence of strain Bss-19 to relevant, closely related strains: DE111, KCTC 13429 (the type strain for the subspecies inaquosorum), and strain 168 (the type strain for

the closely related subspecies *B. subtilis* subsp. *subtilis*) was performed. ANI values above 95% are considered representatives of members of the same species. ANI calculations, shown in Table 1, indicate Bss-19 is of the same species as DE111 and KCTC 13429; but that inaquosorum may be considered a separate species from the subtilis subspecies. DuPont states that ANI is calculated using two factors to produce a third. Overall percent identity of the portions of the sequence that align (ANI) and fraction of the two genomes which do align (coverage) are multiplied to produce the Hadamard product. The Hadamard product is usually a smaller percent identity than the initially reported percent identity unless coverage is complete for both genomes.

Table 1. Genomic Average Nucleotide Identity Comparison of Bss-19 to Other Relevant Strains

	B. subtilis inaquosorum		*B. subtilis* subtilis
	strain DE111	strain KCTC 13429	strain 168
Coverage	99.84%	93.86%	84.52%
Percent identity (ANI)	99.98%	98.83%	93.00%
Hadamard product	99.83%	92.77%	78.61%

DuPont states that whole genome sequences of Bss-19, DE111, and KCTC 13429 aligned using the Mauve Progressive Alignment[4] tool indicate strong conservation between Bss-19 and DE111, but KCTC 13429 appears to contain unique regions ranging in size from 2 to~40 kb.

2.2 Manufacturing

2.2.1 Good Manufacturing Practice

All production steps of Bss-19 are consistent with current Good Manufacturing Practice (cGMP) guidelines in an FDA regulated and inspected facility.

2.2.2 Raw Materials

Raw materials used in the production of DuPont's Bss-19 are of appropriate food grade and are suitable to the application to produce the final food grade product.

2.2.3 Manufacturing Narrative and Flowchart

Master Seed

The source organism used is *Bacillus subtilis* Bss-19.

DuPont takes great care to ensure the quality of bacteria fermentation products. These quality control processes begin with the identification, storage, and handling of the bacteria seed stocks.

A Master Seed repository is maintained for each of the bacterial strains at the DuPont Global Culture Collection (DGCC) in Niebüll, Germany. The repository is a collection of purified, tested, and qualified Master Seed stocks derived from single strain isolates stored at -180°C in liquid nitrogen to maintain long term cell viability. Each seed lot in the culture bank is fully characterized to ensure the identity of the seed strains.

Whole genome sequencing is conducted to establish the identity of each bacteria to the genus, species, and strain level prior to preservation. The microbiological quality of the Master Seeds is determined by testing for microbiological contamination at the DGCC. These identity and purity specifications are absolute acceptance criteria for the Master Seeds. If a Master Seed vial lot fails any of the required tests, the lot is placed on Quality Control (QC) hold to prohibit use and the lot is subsequently destroyed.

Working seed

Working seeds are prepared under controlled conditions from master seed stock maintaining effective acceptance criteria at DGCC. All Working Seeds are prepared under controlled conditions from Master Seed stock meeting established acceptance criteria and each new lot of Working Seeds is held in quarantine pending QC testing (strain identity and purity as described for the Master Seeds) and release. If the Working Seed vial lot fails any of the required tests, the lot is placed on QC hold and destroyed. Qualified, tested Working Seed stocks are stored at -80°C until used in production fermentation.

The use of tandem Master and Working seed inventories reduces the risk of genetic drift over time due to excessive sub-culturing of strains and ensures the integrity of the strain collection. All steps in the preparation of Master and Working seed are documented in a specified database, allowing traceability of every seed preparation down to each single batch of raw material used.

Fermentation process

The fermentation begins by withdrawing one of the working seed vials and scaling-up via a series of fermentations until a commercial size batch is complete.

The fermentation media contains a buffer system to minimize pH shifts. The pH profile for each batch is monitored against a standard to ensure repeatability. The pH in the fermenter is monitored on digital display and on recording charts. By consulting these charts, the growth and sporulation characteristic of a given fermentation can be determined.

After fermentation is complete, an additional aeration step is performed in the fermenter. Airflow is increased and back pressure decreased for a specified amount of time. The fermenter is then normally cooled to stop the fermentation at a specified time. Cooled fermentate is pumped through a continuous flow centrifuge and the bacterial spores are concentrated. The bacterial spore concentrate is pumped to a spray dryer where it is atomized and dried. After spray drying, the bacterial spore concentrate is bulk packaged with lot control and traceability, then stored until QC release against specified criteria.

The fermentation production process is a closed system with no product exposure from seed inoculation to cell harvest. Prior to each fermentation batch, all mixing tanks, lines, fermenter, centrifuge, and spray dryer are cleaned via automated clean-in-place systems. Systems are then either steamed or chemically sanitized prior to product contact.

Packaging

Bulk packaging of the product is carried out in a controlled environment within the DuPont Rochester facility.

HEPA filter is used in the packaging room for high performance as the final filter for particulate removal when clean air is required.

Final packages are heat sealed before passing through a metal detection x-ray system.

Quality Systems

The DuPont Rochester plant has fully implemented HACCP plans, Standard Operating Procedures and Quality Control programs to ensure the quality of each product. A quality control laboratory is maintained on site. Quality control personnel are qualified by training and experience to test products and to release product based on specifications. In addition, a third-party approved laboratory with ISO 17025 certification performs QC testing for DuPont under contract.

The Quality Control unit utilizes an SAP computer quality control system for the specification, quality control data entry and product release. No product can be released for use without acceptance by the Quality Control unit, according to specified acceptance criteria.

Each bacteria fermentation product must meet specifications and must have a confirmation of identity through a PCR (polymerase chain reaction) test method based on strain specific primers for release of the product. Microbiological testing is performed by trained QC microbiologists in the Rochester plant laboratory and an approved external laboratory using standard methods.

Cleaning and quality testing of the process rooms and equipment are under the control of Manufacturing and Quality Assurance, following the established SOPs. Room access is controlled by appropriate signage, and additional protective gowning must be worn in processing rooms where product is potentially exposed. Operator sign-off for cleaning, sanitation and testing are required on proper documentation.

Process rooms are segregated from other manufacturing areas with appropriate closures. Room air quality is controlled via HEPA air filtration of incoming air and maintenance of positive pressure in the process rooms relative to adjacent processing areas. HEPA filtration operation is monitored for performance; air quality is monitored monthly by Quality Assurance.

Rooms and equipment used in manufacturing are approved for production only after cleaning, sanitization, and inspection. Prior to qualification of the process room for production, as specified in the appropriate SOP, the Spray Dried room is sprayed from ceiling to floor with 145–160°F water. All clean, out of place equipment having any product contact surfaces is thoroughly scrubbed / foamed with a neutral detergent cleaner, rinsed with cold water, and sanitized with an acid-based sanitizer. The floor is sanitized with acid-based sanitizer.

Batch records are maintained as per Standard Operating Procedures and are provided to Quality Assurance for each lot produced. Quality Assurance is responsible for batch ticket review. The flowchart of the manufacturing process is shown in Figure 1.

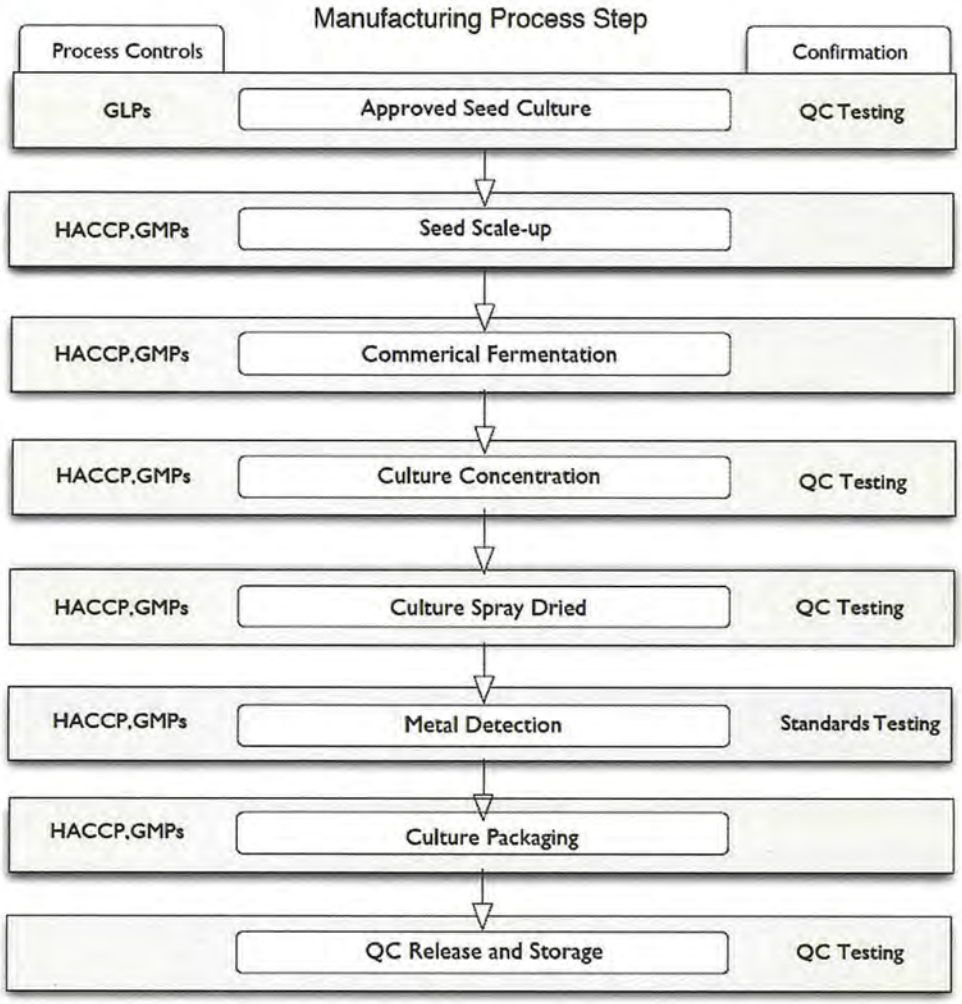

Figure 1. Manufacturing Flowchart

2.3 Specifications

The specifications for the food-grade product Bss-19, along with the specification methods, which have been validated for their stated purpose, are listed in Table 2 below.

Table 2. Bss-19 Specifications

Tested Parameters	Specification	Method
Viable cell count (CFU/g)	$\geq 2.25 \times 10^{11}$	In-house method
Color[1]	White to cream	Visual Inspection
Form[1]	Freeze-dried powder	Visual Inspection

Heavy Metals[2]		
Arsenic (ppm)	< 1.0 ppm	AOAC 984.27
Cadmium (ppm)	< 0.2 ppm	AOAC 984.27
Mercury (ppm)	< 0.05 ppm	EPA 7471
Lead (ppm)	< 0.5 ppm	AOAC 984.27
Microbiological Tests		
Coliform (CFU/g)	< 10	AOAC 991.14
Escherichia coli (CFU/g)	< 10	AOAC 991.14
Salmonella	Negative in 50 g	AOAC RI-121501
Listeria	Negative in 25 g	FDA BAM Chapter 10
Staphylococcus coag+ (CFU/g)	< 10	FDA BAM Chapter 12
B. cereus (CFU/g)	< 10	AOAC 980.31
Molds and Yeast[3]	< 100 CFU/g	USP 41-NF 36

Abbreviations: CFU, colony forming unit; AOAC, Association of Official Analytical Chemists; EPA, Environmental Protection Agency; FDA BAM, Food and Drug Administration's Bacteriological Analytical Manual; ppm, parts per million

[1] Internal Specification, not reported on CoA

[2] Based on annual surveillance testing, not generally reported on CoA

[3] Tested on bulk intermediate powder, not generally reported on CoA

2.3.1 Batch Analysis

Production conformity and consistency of DuPont's Bss-19 are tested in production lots. Batch analyses of three non-consecutive lots are shown below and are reasonably consistent and met the product specifications for marker compounds, microbial analyses, and heavy metals (see Table 3).

Table 3. Bss-19 Batch Analyses

Tested Parameters	**Specification**	**Lot No./Date of Manufacture**		
		1493413902 03/29/2019	**1493441559 04/11/2019**	**1493438028 04/19/2019**
Viable cell count (CFU/g)	$\geq 2.25 \times 10^{11}$	4.03×10^{11}	3.63×10^{11}	3.73×10^{11}
Microbiological Tests				
Coliforms (CFU/g)	< 10	< 10	< 10	< 10
Escherichia coli (CFU/g)	< 10	< 10	< 10	< 10
Salmonella spp.	ND in 50 g	Negative/50 g	Negative/50 g	Negative/50g
Listeria spp.	ND in 25 g	Negative/25 g	Negative/25g	Negative/25g
Staphylococcus aureus (CFU/g)	< 10	< 10	< 10	< 10
B. cereus (CFU/g)	< 10	< 10	< 10	< 10
Heavy Metals				
Arsenic (ppm)	< 1.0	0.05	0.05	0.06
Cadmium (ppm)	< 0.2	0.03	0.02	0.02
Mercury (ppm)	< 0.05	< 0.02	< 0.02	< 0.02
Lead (ppm)	< 0.5	< 0.010	< 0.010	< 0.010

Abbreviations: CFU, colony forming units; ND, not detected; ppm, parts per million

2.3.2 Residual Pesticide Analysis

In accordance with standard operating procedures, DuPont is committed to annual surveillance testing of Bss-19 for pesticide residues. All lots in Table 3 of Bss-19 were analyzed using AOAC 2007.01 for the presence of hundreds of residual pesticides by an independent laboratory. All lots were free of all pesticides tested and complied with the product specifications.

2.3.3 Shelf–Life Stability

A long-term stability study for Bss-19 is currently underway.

2.4 Antibiotic Resistance

Resistance to therapeutic antibiotics in 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 their 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.[5, 6] 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.[7,8] 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, can be transferred to pathogens or other commensal bacteria.[7, 9]

It is generally recommended that resistance to antibiotics be assessed in all probiotic strains prior to marketing.[5, 10-13] Antibiotic resistance is a complex phenomenon, in which microbial genetics and environmental stimuli both play an important role. Assessing resistance both phenotypically and genotypically is generally recommended. As detailed below, antibiotic susceptibility of Bss-19 was evaluated using both approaches.

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.

Following EFSA recommendations and guidelines, DuPont assessed the phenotypic susceptibility of Bss-19 to the antibiotics detailed in the guidelines for *Bacillus* species, namely gentamycin, kanamycin, erythromycin, clindamycin, tetracycline, streptomycin, vancomycin, and chloramphenicol.[14] One additional antibiotic, Ampicillin, was also tested although it is not a requirement per the EFSA guidelines for *Bacillus* species. The EFSA guidelines define a bacterial strain as sensitive or susceptible to an antibiotic when it is inhibited at a concentration of a specific antimicrobial equal or lower than the established cut-off value for that particular compound.

The assays on Bss-19 were performed using the ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that include all antibiotics recommended by EFSA. All MIC values were below or equal to the microbial break points defined for *Bacillus* species except for chloramphenicol. Results are shown in Table 4 and indicate that Bss-19 is phenotypically sensitive to all antibiotics included in EFSA's guidelines for the *B. subtilis* species except for chloramphenicol.

Table 4. Bss-19 Phenotypic Resistance to Antibiotics Results

Antibiotic	MIC (mg/L)		Assessment
	Bss-19 (observed)	*B. subtilis* Breakpoints[5]	
Ampicillin	0.06	n.r.	Sensitive
Clindamycin	2	4	Sensitive
Chloramphenicol	16	8	Resistant
Erythromycin	0.5	4	Sensitive
Streptomycin	4	8	Sensitive
Gentamycin	1	4	Sensitive
Kanamycin	4	8	Sensitive
Tetracycline	0.25	8	Sensitive
Vancomycin	1	4	Sensitive

MIC = Minimum Inhibitory Concentration; n.r. = not required (according to EFSA guidelines for *B. subtilis)* [14]

Genotypic evaluation of antibiotic resistance is a procedure in which the whole bacterial genome (chromosome and peripheral genetic elements, if any) is screened for putative genes of antibiotic resistance, as described in genetic databases. It is therefore a complementary procedure to the phenotypic assessment, in which the main objective is to discard the potential of transferring putative genes of antibiotic resistance to other microbes.

Comparison of all annotated Bss-19 protein sequences to the Comprehensive Antibiotic Resistance Database (CARD)[15] revealed a putative homolog to a chloramphenicol acetyltransferase resistance protein (53% identity over 100% of query length). This gene was also found in both the *B. subtilis* inaquosorum DE111 and *B. subtilis* inaquosorum KCTC13429 genomes. Three types of HGT were

evaluated by DuPont and included: conjugative plasmids, transposases, and prophage/bacteriophage elements. A search of the Bss-19 draft genome sequence versus the ISFINDER database[16] of known mobility element sequences revealed no mobility loci within 15 kb of the putative chloramphenicol resistance gene. Additionally, no intact or potentially active phage or prophage genomes were identified in the assembled Bss-19 genome sequence.

The lack of the presence of known mobility elements near the chloramphenicol resistance gene combined with the absence of active phage or prophage genomes suggests that acquired antibiotic resistance for the strain is not a concern. Additionally, while the observed MIC for Bss-19 was just above the cut-off value established by EFSA, one two-fold dilution above the EFSA cut-off value is still generally considered acceptable. This is because of the technical variation of the phenotypic method applied to determine antibiotic susceptibility. There is a certain amount of technical variability in all phenotypic antibiotic-resistance testing. Guidelines for this testing allow for this normal (technical) variation around the mean. The phenotypic test performed is generally based on two-fold broth dilutions. There is precedent for accepting levels that exceed the MIC cut-off by a single two-fold dilution due to 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*",[17] 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.

2.5 Genomic Analysis for Virulence and Pathogenicity

The genome of Bss-19 was screened using the Database of Bacterial ExoToxins for Human Health (DBETH)[18] which revealed a single hit (70% identity over 90% of query length) to a hemolysin protein (HLY3_BACCE). However, the Bss-19 gene encoding this protein is highly conserved across the genus including many strains known to not be pathogens. Additionally, as discussed in Part 2.7, Bss-19 showed no hemolysis when analyzed in vitro.

A comparison of all Bss-19 protein sequences to the virulence factor database (VFDB)[19] revealed no significant matches, indicating that Bss-19 does not contain any detected virulence factors.

2.6 Resistance to Gastric Acidity and Bile Salts

In many cases, probiotic microorganisms should be viable upon reaching the gastrointestinal tract. Some important traits that are believed to be relevant for surviving the passage through the gastrointestinal tract are tolerance to the acidic environment of the stomach and tolerance to the concentration of bile salts found in the small intestine. The extremely acidic environment of the stomach (pH of

approximately 2.0–3.0) kills the majority of potentially pathogenic bacteria, preventing infection.

For assessment of Bss-19, a modified gastric juice was utilized to simulate contact with a moderately acidic stomach fluid environment. Bile tolerance was estimated by determining the % recovery on bile containing agar medium compared to a non-bile containing control medium.

Culture was obtained from seed vials, inoculated into strain specific medium, and grown overnight. An aliquot of the overnight broth culture was pelletized, washed, and resuspended. An aliquot of the resuspended pellet was mixed with tempered gastric juice (hydrochloric acid and pepsin (0.32%) and pH 3.5). An aliquot was immediately diluted and plated in TSA agar with and without 0.3% ox-gall bile salt for a T0 control and a bile test result. The balance of sample in gastric juice was incubated for one hour at which time the final aliquot was taken for T1 plating using the same TSA media. Plates were allowed to solidify and incubated at 32°C under aerobic conditions for 48 hours. The results are shown in Table 5.

Table 5. Acid Tolerance and Bile Tolerance of Bss-19 in its Vegetative State and Spore Form

	Internal Identification	Acid tolerance		Bile tolerance		Date Tested
Vegetative state	DGCC12972*	<*	0%	<*	0%	5/13/2019
Spore form	DGCC12972*	****	100%	***	88.4%	5/30/2019

*DGCC12972 (DuPont Global Culture Collection) is used as the Internal Identification for Bss-19

Acid Tolerance rating
**** Excellent (>90% survival in hydrochloric acid and pepsin, 0.32% (wt/v) at pH 3.5)
*** Very Good (80–90% survival)
** Good (70–79% survival)
* Fair (<69% survival)

Bile Tolerance Rating
**** Excellent (>90% survival in 0.3% bile salt containing medium)
*** Very Good (80–89% survival)
** Good (70–79% survival)
* Fair (<69% survival)

Bss-19 in the vegetative state exhibited 0% survival following exposure to a low pH solution and 0% survival in bile salt solutions. Bss-19 in its spore form exhibits >90% survival in a low pH solution and >80% survival in a bile salt solution. These results suggest the spore form is necessary for survival through the gastrointestinal tract.

2.7 Hemolysis

Cultures of the strain were grown overnight in strain specific medium and temperature. An aliquot of the overnight culture was streaked onto prepared blood

agar plates and incubated at a strain specific temperature for 18 and 24 hours. Results showed that Bss-19 did not promote hemolysis when cultured on Sheep's blood agar plates.

As discussed in Part 2.2, genomic analysis of Bss-19 revealed a single hit to a hemolysin protein (HLY3_BACCE). However, the Bss-19 gene encoding this protein is highly conserved across the genus including many strains that do not show pathogenic activity. This is supported by the absence of hemolysis observed when the strain is grown on blood agar.

2.8 Biogenic Amine Formation

Some species and/or strains of lactic acid bacteria are able to produce biogenic amines (organic, basic, nitrogenous compounds formed mainly by the decarboxylation of amino acids), likely for use as metabolic energy and/or to increase acid resistance.[20] These amines are present in a wide range of foods (e.g., fermented food products), and although they are involved in many natural physiological processes, consuming large quantities of these amines can have undesirable consequences in some individuals. For example, if they are not properly biotransformed in the body, they can cause release of adrenaline/noradrenaline, cause gastric acid secretion, increased cardiac output, heart rate, and blood pressure, migraines, and increased blood sugar.[20] Biogenic amine formation in fermented foods has been reviewed by EFSA (2011)[21] and Spano (2010).[20] Histamine and tyramine are considered the most concerning with regard to food safety.[21]

Generally, detection of strains possessing amino acid decarboxylase deaminase activity is helpful to aid in mitigating the accumulation of these amines in food products. Per assessment, Bss-19 did not contain histidine decarboxylase-encoding genes or tyrosine decarboxylase-encoding genes in its genome. Hence, it is unlikely that this strain can produce histamine or tyramine.

2.9 D(-)/L(+)-Lactic Acid Production

Bss-19 produces lactic acid (lactate) from the fermentation of carbohydrates. Lactate exists in two forms, a dextrorotary enantiomer (D-lactate) and a levorotary enantiomer (L-lactate). In humans, over 99% of lactate found in the blood is L-lactate. Testing D-lactate production by food microorganisms has been historically recommended likely because until relatively recently, it was believed that humans had a poor capacity for metabolizing D-lactate.[11] Some lactic acid bacteria as well as several other members of the intestinal microflora produce a mixture of L- and D-lactate.[22] More recent studies have shown that much of the human gut microbiota produces D-lactate with no evidence of D-lactic acidosis, and in fact, humans are able to metabolize this isoform.[23-29] D-lactate accumulation may only occur in cases

of impaired D-lactate metabolism and/or in subjects with a disturbed gastrointestinal function following bowel resection or Short Bowel Syndrome (SBS).[25, 29-32]

Cultures of the strain were grown overnight in strain specific medium and temperature. An aliquot of overnight broth culture was pelletized, and the supernatant was retained. The supernatant was inactivated at 80°C for 15 minutes and diluted to achieve the desired total lactic acid concentration range. D(-)/L(+)-lactic acid detection was performed using a calorimetric measurement of lactate dehydrogenase activity for the respective isomers. Total lactic acid and isomer specific measurements were determined relative to control samples. Bss-19 produced an average of 100% of the L(+)-lactic acid isomer and 0% of the D(-)-lactic acid isomer.

2.10 Carbohydrate Analysis

Bss-19 capability to use and ferment different sugars as carbon sources was assessed following the API 50 CH system (Biomeréux, France). Results are shown in Table 6.

Table 6. Sugar Fermentation Capacity (API 50 CH) of Bss-19

Substrate	Growth	Substrate	Growth	Substrate	Growth
Control	-	Inositol	+	Melezitose	-
Glycerol	+	Mannitol	+	Raffinose	+
Erythritol	-	Sorbitol	+	Starch	+
D-Arabinose	-	a-methyl-D-Mannoside	-	Glycogen	+
L-Arabinose	+	a-methyl-D-Glucoside	-	Xylitol	-
D-Ribose	+	N-Acetylglucosamine	-	Gentiobiose	-
D-Xylose	-	Amygdaline	+	D-Turanose	+
L-Xylose	-	Arbutine	+	D-Lyxose	-
Adonidol	-	Esculin	+	D-Tagatose	-
Beta Methyl-D-Xyloside	-	Alicin	+	D-Fucose	-
Galactose	-	Celiobiose	+	L-Fucose	-
Glucose	+	Maltose	+	D-Arabitol	-
Fructose	+	Lactose	-	L-Arabitol	-
Mannose	+	Melibiose	+	Gluconate	-
Sorbose	-	Sucrose	+	2-Keto-gluconate	-
Rhamnose	+	Trehalose	+	5-Keto-gluconate	-
Dulcitol	-	Inulin	+		

2.11 Physical or Technical Effect

Bss-19 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, Dupont's Bss-19 manufactured in accordance with current GMP, 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, 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 lactic acid bacteria found in traditionally fermented food products).[33]

The addition of Bss-19 to some foods may be substitutive with regard to other GRAS *B. subtilis* strains' intended uses (e.g. GRN 831 and 905), or with regard to traditional uses of *B. subtilis*. However, uses in other foods may be considered more novel and additive with regard to exposure. 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.[34] 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.[35] 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 Bss-19 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 Bss-19 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 fermented food 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 Bss-19, highest consumers (men) would be exposed to approximately 9.1 x 10^{10} to 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).

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 GRAS conclusion for Bss-19 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 foods fermented with *B. subtilis* is extensively discussed in Section 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.[36] Typical levels ranged from 1 x 10^6 to 1 x 10^9 CFU/serving.[36]

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, 36] Consumption of a 100g 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.[37]

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.[38, 39]

6.2 Regulatory Opinions

6.2.1 Europe

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 satisfying 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 3 years and results are published as Scientific Opinions (the next mandated Opinion was published in December 2019), while the list of microorganisms is maintained and re-evaluated approximately every 6 months to include new notifications to EFSA, and published as Panel Statements (the most recent Panel Statement was published in June of 2020 and includes research published through December 2019.[40] *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.[40, 41]

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.[42] Fifteen GRAS notices related to *B. subtilis* strains (mainly as recombinant strains utilized to isolate enzymes) are listed in FDA's GRN inventory. Of these, 14 have received the no questions 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). A brief summary of these notifications is shown in Table 7.

Table 7. FDA GRAS Notifications that Include *B. subtilis* Strains

FDA GRN Number	Strain Description	Date of Closure	Comments
20	Pullulanase derived from *B. subtilis* carrying a gene encoding pullulanase from *B. naganoensis*	September 1999	FDA had no questions.
114	Pectate lyase enzyme preparation from *B. subtilis*	January 2003	FDA had no questions.
205	Pullulanase enzyme preparation from *B. subtilis* expressing the pullulanase gene from *B. acidopullulyticus*	December 2006	FDA had no questions.
274	Branching glycosyltransferase enzyme preparation from *B. subtilis* expressing a branching glycosyltransferase gene from *Rhodothermus obamensis*	June 2009	FDA had no questions.
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	FDA had no questions.
476	Asparaginase enzyme preparation produced by genetically modified *B. subtilis*	February 2014	FDA had no questions.
562	*B. subtilis*	April 2015	At the notifier's request, FDA ceased to evaluate the notice.

579	Lactase from *Bifidobacterium bifidum* produced in *B. subtilis*	November 2015	FDA had no questions.
592	β-glucanase from *B. subtilis*	October 2015	FDA had no questions.
649	β-galactosidase enzyme preparation from *B. circulans* produced in *B. subtilis*	November 2016	FDA had no questions.
714	Subtilisin from *B. amyloliquefaciens* produced in *B. subtilis*	February 2018	FDA had no questions.
746	Maltogenic amylase from *Geobacillus stearothermophilus* produced in *B. subtilis*	June 2018	FDA had no questions.
751	Maltogenic alpha-amylase from *B. stearothermophilus* produced in *B. subtilis*	July 2018	FDA had no question
831	*B. subtilis* DE111	October 2019	FDA had no questions.
905	*B. subtilis* DSM 32444	June 2020	FDA had no questions.

6.2.2.2 Code of Federal Regulations

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;*
- 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.[43] 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".[43]

6.2.3 Canada

A number of products containing *B. subtilis* are approved to be marketed under the Natural Health Products Regulations of Health Canada, including products containing *B. subtilis* strain DE111 (NPNs 80077102 & 80080178) and *B. subtilis* strain R0179 (NPNs 80045131, 80051112, 80054028, etc.).

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 the strain of closest similarity, *B. subtilis* DE111 and other *B. subtilis* strains are discussed in subpart 6.3.3. While no published human or toxicological studies were located for *B. subtilis*

Bss-19 specifically, an unpublished acute oral toxicity study was performed on the strain and is summarized in subpart 6.3.2. The studies reviewed do not suggest any concerns related to the safety of the strain.

6.3.1 Toxicological Studies on *B. subtilis* strains

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.[44] 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. Similarly, no increase in the incidence of micronuclei or chromosomal aberrations occurred in in vivo mouse assays up to the highest dose tested (2500 mg/kg bw/day 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 (7.5×10^{10} CFU/kg bw/day) in all of the studies.

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 observation for 14 days.[45] The authors reported that no significant change in general conditions, mortalities, body weight changes, clinical signs, autopsy findings, or presence of gross lesions were observed. Nakamura et *al.* (1999) performed a 90-day subchronic toxicity study in both sexes of F344 rats by feeding of CRF-1 pellet diet containing 0%, 0.18%, 0.55%, 1.66% and 5% *B. subtilis* gum (strain and CFUs of *B. subtilis* were not specified).[46] Five groups consisted of 10 males and 10 females each whereby rats were randomly allocated. 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 these increases were slight and serum biochemistry and histopathology did not show any toxicological effects. The authors concluded that these findings indicated that the treatment of *B. subtilis* gum in the diet for 90 days does not exert toxicity in rats even at the highest dose tested.

Sorokulova et *al.* (2008) described a number of studies on *B. subtilis* VKPM B2335 (BS3).[47] Groups of 10 BALB/c male mice were each administered the test article at doses of 5×10^7, 5×10^8, and 5×10^9 CFU/mouse both intravenously and intraperitoneally, and orally at doses of 5×10^7, 5×10^8, and 2×10^{11} CFU/mouse (control group mice were given sterile PBS). 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 (AEs) observed related to activity and 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 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. There were no AEs noted or changes in hematology values, or gross or histopathological findings 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.[48] A dose of 1×10^9 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 AEs in health status or feed intake, and no changes in hematology or visceral organs or tissues were observed as compared to controls. The authors additionally studied a single dose (1×10^{12} CFU) of *B. subtilis* natto in guinea pigs, as briefly described in the same publication. There were no findings related to 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 three groups of ten Sprague-Dawley albino rats using 2×10^9 CFU/kg bw/day *B. subtilis* R0179 or *E. faecium* R0026 or control administered by gavage.[49] 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 were mentioned. The *B. subtilis* strain was not observed microbiologically except in the intestinal content of treated animals.

Additionally, Cell-Free Supernatants (CFSs) of *B. subtilis* KATMIRA were evaluated by a bacterial reverse mutation assay (Ames Salmonella assay) and showed no mutagenicity.[50]

6.3.2. Unpublished Acute Toxicity Study on *B. subtilis* Bss-19

An initial limit dose of 5000 mg/kg *B. subtilis* Bss-19 was administered to one healthy female Sprague-Dawley rat by oral gavage. Due to the absence of mortality in this animal, two additional female rats received the same dose. All animals were observed for mortality, signs of gross toxicity, and behavioral changes at least once daily for 14 days after dosing. Body weights were recorded prior to administration

and again on days 7 and 14. Necropsies were performed on all animals at terminal sacrifice. All animals survived test substance administration, gained body weight, and appeared active and healthy during the study. There were no signs of gross toxicity, AEs or abnormal behavior. No gross abnormalities were noted upon necropsy. The LD_{50} was determined to be greater than 5000 mg/kg bw in female rats.

6.3.3. Human Studies

The safety of Bss-19 has not been formally investigated in healthy adult subjects. However, many recent human clinical studies have been and continue to be published on the strain of closest similarity, *B. subtilis* DE111 and on other *B. subtilis* strains.[51-54] Due to the large amount of published human studies, included below are clinical studies located in the literature published since EFSA's most recent Opinion in 2016, which addressed studies published up to June 2016.

6.3.3.1. B. subtilis DE111

In a human clinical trial investigating the safety of *B. subtilis* DE111, Maher (2019) gave 5 x 10^9 CFU *B. subtilis* DE111 in a single capsule or placebo daily to 41 healthy young adults for an average of 20 days.[55] Blood samples (comprehensive metabolic panel, lipid panel, C-reactive protein (CRP)) and stool samples were collected at the beginning and end of the study. Serum glucose levels were significantly lower in the treatment group when comparing pre to post capsule consumption. Triglycerides remained the same within the treatment group, while the control group displayed a significant increase from pre to post capsule consumption. There was no significant variation from the normal range of CRP. The authors discussed that the decrease in serum glucose had been observed in two animal studies where, in the first study, a compound isolated from *B. subtilis* (1-Deoxynojirimycin) helped to improve diabetic conditions in bovine calves and, in a second study, freeze-dried cultures of a combination of bacteria reduced blood glucose levels in rats with elevated glucose levels. The authors concluded that daily ingestion of one capsule containing approximately 5 x 10^9 CFU *B. subtilis* was well tolerated in healthy young adults.

Cuentas et *al.* (2017) investigated the use of 1 x 10^9 CFU *B. subtilis* DE111 or placebo daily for 105 days in 50 adults. Comprehensive metabolic panels, lipid panels, and CRP levels stayed within normal references ranges for the treatment and placebo groups with no significant serum level differences. Additionally, no AEs were reported.[56]

In addition to the safety study and clinical study above, the following two clinical studies were conducted which did not have any reported AEs. Toohey et *al.* (2018) investigated the effects of 5 x 10^9 CFU *B. subtilis* DE111 or placebo daily on 23 Division I female athletes for 10 weeks—no AEs were reported.[57] Townsend et *al.* (2018) gave 1 x 10^9 CFU *B. subtilis* DE111 or placebo daily to 25 Division I male athletes for 12 weeks—no AEs were reported.[58]

6.3.3.2. Other *B. Subtilis* Strains

Penet et *al.* (2019) gave 5 x 10^9 CFUs of *B. subtilis* MB40 or placebo daily to 100 subjects for four weeks.[59] There were no statistically significant differences between groups with regard to anthropometric, vital, hematological and clinical chemistry parameters. Reported AEs were similar between groups and consisted of constipation, diarrhea, flatulence, dry mouth, abdominal discomfort, increased appetite and paresthesia. All AEs were resolved before end-of-study. Soman and Swamy (2019) evaluated the safety in a combination of 2 x 10^9 CFUs of *B.* coagulans, *B. clausii,* and *B. subtilis* versus placebo in 60 subjects for 30 days.[60] No AEs were reported in any subject during the study period. Hatanaka et *al.* (2018) gave 2.2 X 10^9 *B. subtilis* C3102 spores or placebo daily to 88 healthy adults with loose stool for eight weeks and did not report any AEs.[51] Lefevre et *al.* (2017) described that after 40 total days of treatment with *B. subtilis* CU1 (2 x 10^9 spores/day) compared to placebo in 100 elderly human subjects, no undesirable physiological effects or biological safety concerns with regard to AEs, liver and kidney function markers, complete blood counts, hemodynamic parameters, and vital signs were noted.[52, 61] Alkaya et *al.* (2017) investigated the use of 5 x 10^7 CFU/day of combined *B. subtilis*, *B. megaterium*, and *B. pumilus* spores in subjects for eight weeks—no AEs occurred.[53]

Further, one study published before the most recent EFSA Opinion, Hanifi et *al.* (2015), is included because it evaluated oral dose-response tolerance at similar or higher daily dosages than the intended use levels of Bss-19. To evaluate oral dose-response tolerance, Hanifi et *al.* (2015) gave 81 subjects *B. subtilis* R0179 at doses of 0.1 x 10^9, 1 x 10^9, and 10 x 10^9 CFU/day or placebo for 4 weeks. The test article was well tolerated at all doses and survived passage through the human GI tract.[54]

6.3.4 Opportunistic Infections

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*.[62] 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*.[63] Overall, infections with *B. subtilis* occur at very low rates, and generally occur in hospital settings in immunocompromised patients and/or during medical procedures.[62-64]

6.4 Allergenicity

Bss-19 does not contain or have added any of the eight major allergens (milk, egg, fish, Crustacean shellfish, tree nuts, wheat, peanuts, 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.

No reports of allergic reactions to *B. subtilis* were found in our investigations. Given total exposure together with results of toxicological and clinical studies, the allergic potential of *B. subtilis* can be considered very low.

6.5 Past Sales and Reported Adverse Events

Bss-19 has never been released on the market and therefore has no past sales or past reported AEs to account for. No FDA letters regarding concern for safety to companies that market products containing *B. subtilis* were located. A search of FDA's Recalls, Market Withdrawals, & Safety Alerts search engine and FDA's Center for Food

Safety and Applied Nutrition Adverse Event Reporting System did not uncover any mention of *B. subtilis* products. All databases were accessed on June 2, 2020.

6.6 Basis for the GRAS Conclusion

Dupont's Bss-19 has been the subject of a thorough safety assessment as described above. The totality of evidence supporting safety is comprised of data and information that establish the safety of Bss-19 under the conditions of its intended use and data and information that is corroborative of safety. The general availability and general acceptance, throughout the scientific community of qualified experts, of the data and information that establish the safety of Bss-19 under its intended conditions of use establish the general recognition of this data and information. Together, the establishment of safety based on scientific procedures and its general recognition form the basis for DuPont's conclusion of GRAS status of Bss-19 for its intended use.

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 16S rRNA sequence as well as complete genome sequencing, demonstrating unequivocally that it is a strain of the *B. subtilis* subspecies *inaquosorum* with established phenotypic characteristics;
- The analyses and resulting data showing Bss-19 lacks resistance to clinically relevant antibiotics per European Food Safety Authority (EFSA) minimal inhibitory concentration cut-offs and guidelines, with the exception of chloramphenicol, where further investigation by DuPont showed that the resistance is not expected to be transferrable;
- The lack of potential of Bss-19 to produce toxins or virulence factors that have been demonstrated to be virulent to hosts (via comparison of genomic sequences to known virulence sequences in the DBETH exotoxin protein database);

- The methods of manufacture, specifications, as well as batch analyses, showing that all specifications are met for each batch, demonstrating safe production methods and robust quality control standards for Bss-19;
- The intended use as an ingredient in foods at an addition level of up to 1 x 10^{10} CFU per serving, which is in line with addition levels for other GRAS microbial ingredients (including *B. subtilis* in GRN 831) as well as with levels of fermenting bacteria found naturally in various fermented foods, with an estimated exposure of 9.1 x 10^{10}–1.4 x 10^{11} CFU/day (1.3–2.0 x 10^{9} CFU/kg bw/day) by conservatively assuming consumption at the maximum intended use addition level in 50% of all food servings daily;
- A previous GRAS notice to FDA (GRN 831) for a very similar strain, *B. subtilis inaquosorum* strain DE111, received a no questions letter from FDA for use as an ingredient in cow's milk and soy-based non-exempt infant formula for term infants at a maximum level of 2 x 10^{8} CFU/100 mL and in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fats and oils; fruit juices; frozen daily deserts and mixes; fruit and water ices; gelatins; puddings and fillings; grain products and pastas; soft/hard candy and cough drops; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk and milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at addition levels from 1 x 10^{6} to 1 x 10^{10} CFU/serving. The estimated daily intake (EDI) for the strain was determined to be 1.3 x 10^{11} CFU/day which FDA did not object to.
- Another previous GRAS notice to FDA (GRN 905) for another *B. subtilis* strain, *B, subtilis* DSM 32444, received a no questions letter from FDA for use as in ingredient in beverages (milk drinks, protein high energy sports drinks, hot beverages, and juices) and dry and shelf-stable products (cereals, cookies, gums, and confectionary) at a maximum level of 1 x 10^{9} CFU/serving. The EDI for the strain was determined to be 5.0 x 10^{9} CFU/day which FDA did not object to.

6.6.2 Data and Information that are Corroborative of Safety

- *B. subtilis'* EFSA QPS status for food and feed use, at any reasonable dose/intended use, suggesting no further regulatory review prior to introduction of new 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;
- The documented long history of safe human consumption of *B. subtilis* as a common bacterial species in fermented foods,[38] such as in natto (with

concentrations of approximately 1 x 10^8 CFU/gram, equivalent to approximately 1 x 10^{10} CFU/ 100 g serving), over decades without known concerns for safety;[48, 49]

- The lack of serious adverse events reported in clinical trials using *B. subtilis* at daily dosages up to 1 x 10^{10} CFU/day;
- 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;[65-67]
- An unpublished acute oral toxicity study showing the acute oral LD_{50} of *B. subtilis* Bss-19 is greater than 5000 mg/kg bw in female rats.

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 Bss-19 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 Bss-19 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 our conclusion of GRAS status.

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 July 2019 and again on June 2, 2020.

7.1 Data and Information that are *not* Generally Available

An unpublished acute oral toxicity study was provided by DuPont and is part of the basis of the determination of safety (subpart 6.3.2).

7.2 References that *are* Generally Available

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