JHeimbach LLC

April 29, 2021

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

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, Lallemand Health Solutions, through me as its agent, hereby provides notice of a claim that the addition of *Bacillus subtilis* strain R0179 to conventional foods is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Lallemand Health Solutions has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

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

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

Sincerely,

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

Encl.

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Generally Recognized as Safe (GRAS) Determination for the Use of *Bacillus subtilis* Strain R0179

LALLEMAND HEALTH SOLUTIONS
Regulatory Affairs
17975 rue des Gouverneurs
Mirabel, Québec, Canada
J7J 2K7
Tel (450) 433-9139

And

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Port Royal VA 22535
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PART 1. SIGNED STATEMENTS AND CERTIFICATION

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1.1. GRAS Notice Submission

Lallemand Health Solutions of Mirabel, Québec, Canada (Lallemand) submits this GRAS notification through its agent James T. Heimbach, president of JHeimbach LLC, in accordance with the requirements of 21 CFR Part 170, Subpart E.

1.2. Name and Address of Notifier

Lallemand Health Solutions
17975 rue des Gouverneurs
Mirabel, Québec, Canada
J7J 2K7
Tel (450) 433-9139

Notifier Contact:
Solange Henoud – Regulatory Affairs Director
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+1 (514) 573-7067

Agent Contact:
James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
923 Water Street #66
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1.3. Name of GRAS Organism

The microorganism that is the subject of this Generally Recognized as Safe (GRAS) notice is the bacterial strain *Bacillus subtilis* strain R0179. This strain was deposited at the Pasteur Institute under number I-3471.

1.4. Intended Use, Estimated Daily Intake (EDI) and Consumer Exposure

Lallemand intends to use *Bacillus subtilis* R0179 as an ingredient in selected food products at a maximum level of 1 x 10^9 colony forming units (cfu) *B. subtilis* R0179 per serving after processing. The intended uses of *B. subtilis* R0179 include addition to the following 11 food categories as defined in 21 CFR §170.3: (n)(1) baked goods and baking mixes; (n)(3) beverage and beverage bases; (n)(4) breakfast cereals; (n)(6) chewing gum; (n)(9) confections and frostings; (n)(10) dairy product analogs; (n)(21) fruit and water ices; (n)(32) nuts and nut products; (n)(33) plant protein products; (n)(35) processed fruits and fruit juices; and (n)(37) snack foods. Since the fermentation medium contains soy and may contain milk products (depending on whether the version is dairy or non-dairy), foods to which strain R0179 is added require labeling to indicate the presence of these allergens.

These food categories are in addition to those included in a previous GRAS determination in 2012: whole-grain yeast breads and rolls and specialty breads; muffins and sweet quick breads; Kombucha; 100% fruit juices and nectars; 100% vegetable juices; and diet salad dressings.

The intended uses of the strain do not include infant formula or other foods targeted to infants or toddlers or any foods regulated by the U.S. Department of Agriculture.

The exposure calculation was performed by Exponent (the complete report can be found in Appendix 1). Exponent calculated that the estimated daily intake (EDI) of *B. subtilis* R0179 is lower than the level of 1.0x10^{10} cfu/day found to be without harm in clinical studies performed with *B. subtilis* R0179 as part of a finished multi strain product called Medilac and a 4-week safety study in humans with the pure strain *B. subtilis* R0179 at a level of 1x10^{10} cfu per day.

1.5. Statutory Basis for GRAS Status

Lallemand Health Solution's GRAS determination for the intended use of *Bacillus subtilis* R0179 is based on scientific procedures as described under 21 CFR §170.30(b).

1.6. Premarket Exempt Status

The intended use of *Bacillus subtilis* R0179 is not subject to the premarket approval requirements of the Federal Food Drug and Cosmetic Act based on Lallemand's conclusion that such use is GRAS.

1.7. Availability of Information

The data and information that serve as the basis for the GRAS determination will be sent to the FDA upon request or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHeimbach LLC, 923 Water Street, Port Royal Virginia 22535, telephone 804-742-5543 and e-mail jh@jheimbach.com.

1.8. Freedom of Information Act Statement

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

1.9. Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information as well as favorable information known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of *Bacillus subtilis* R0179.

1.10. FSIS Statement

Not applicable.

1.11. Name and Title of Signer

[redacted]

James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to Lallemand Health Solutions

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

Sections:

2.1. Name of the GRAS Organism

The notified substance is *Bacillus subtilis* strain R0179.

2.2. Source of the GRAS Organism

Bacillus subtilis R0179 was isolated from a Korean commercial product. The bacteria in the Korean product were supplied by a Japanese company. It is our understanding that the strain was originally isolated in Japan from the fermented soy product natto.

The strain *Bacillus subtilis* R0179 is deposited:

- Under number I-3471 in the *"Collection Nationale de Cultures de Microorganismes"* (CNCM, Paris), operated by Institut Pasteur.
- Under number LMG S-29845 in the LMG Bacteria Collection of the Belgian Coordinated Collections of Micro-Organisms (BBCM/LMG) operated by the Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University.

2.3. Description of the GRAS Organism

The *Bacillus* genus *sensu stricto* includes about 90 established species with new species appearing frequently due to increasing numbers of publications (Logan 2004). These are endospore-forming, rod-shape, aerobic or facultative aerobic, usually Gram positive, catalase positive, mesophilic for the most part, and sometimes mobile by peritrichous flagella. Due to the endospore-forming ability of members of this genus, these bacteria can tolerate adverse conditions such as heat, acidic media, and harsh environmental conditions. Spores are dormant life forms which can exist in a desiccated and dehydrated state indefinitely (Nicholson et al. 2002). Because of their ubiquitous distribution, spores have had an impact on human activity for a long time. They can be found in foods, cosmetics, and pharmaceutical products.

Spores of bacilli have been marketed since at least 1960 in Europe and Southeast Asia, where they are employed for bacterioprophylaxis and bacteriotherapy in order to treat and prevent various gastrointestinal disorders including infectious and antibiotic-related diarrhea in humans (Mazza 1994).

The properties of *Bacillus subtilis* are supported by the positive impact of intake on the lactic-acid bacteria (LAB) population. The capacity of *B. subtilis* to produce catalase and subtilisin has been reported to promote growth of *Lactobacillus* species. When co-cultured, *Bacillus subtilis* (natto) enhanced the growth

and viability of *L. reuteri, L. acidophilus*, and *L. murinus* (Hosoi *et al. 20*00). Several reports in birds and mammals show that *B. subtilis* intake can modify the intestinal microflora (Hosoi *et al. 19*99; Teo and Tan 2007; Maruta et al. 1996). These studies reveal that addition of *B. subtilis* to the diet can improve the intestinal microbiota. Fecal microbiota changes were reported in mice. While no changes of *Enterobacteriaceae* and *Enterococcus* species were noted, changes in the *Bacteroidaceae* and *Lactobacillus* population were observed depending on the diet. Monosaccharides and oligosaccharides seem essential for production of metabolites during germination and outgrowth (Hosoi *et al.* 1999). Similar results have been reported in chickens and a reduction of numbers of *Clostridium* species and *E. coli* compared to negative controls has been observed (Teo and Tan 2007). In sows and gilts, after a 3-week test period, an increase in *Lactobacillus* species and *Bifidobacterium* species has been observed while lower levels of *Streptococcus, Enterobacteriaceae*, *Clostridium perfringens* and Bacteroidaceae were reported (Maruta et al. 1996).

2.3.1 Phenotypic Identification of *B. subtilis* R0179 Strain

B. subtilis R0179 is a Gram-positive rod (see Figure 1) appearing as individual rods or occasionally in short chains. They are weakly motile and spore forming bacteria producing ellipsoidal endospores (see Figure 2).

B. subtilis R0179 forms large beige rough colonies on selective media Tryptic Soy Agar (TSA) (see Figure 3). The optimal growth temperature is 37°C under aerobic conditions. It can also grow in the absence of oxygen using nitrate ammonification and various fermentation processes.

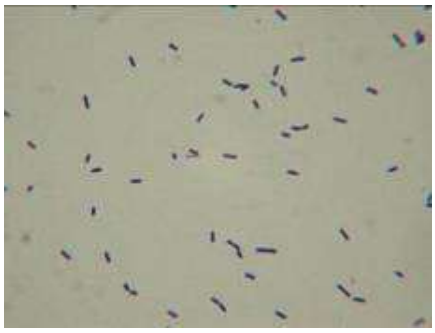

Figure 1: Microscopic observation of Gram-stained *B. subtilis* R0179 (Magnification 100X).

Figure 2: *B. subtilis* R0179 (Magnification 10,000x) Scanning Electron Micrograph by Dr. A. Smith, U. of Guelph, (Ont), Canada.

Figure 3: Colonies of *B. subtilis* R0179 on TSA agar.

B. subtilis R0179 can grow on different sugars. Based on the API 50CHB gallery (specific for the identification of *Bacilli*, Biomérieux, France) results (see Table 1), the strain R0179 is identified with 98.6% certainty as being *Bacillus subtilis*:

Table 1: Results of API 50 CHB gallery.

API 50 CHB (37ºC, 48 hours)									
Control	-	Galactose	-	α-methyl-D-mannoside	-	Melibiose	+	D-turanose	+
Glycerol	+	D-glucose	+	α-methyl-D-glucoside	+	Sucrose	+	D-lyxose	-
Erythritol	-	D-fructose	+	N-acetylglucosamine	-	Trehalose	+	D-tagatose	-
D-arabinose	-	D-mannose	+	Amygdalin	+	Inulin	+	D-fucose	-
L-arabinose	+	L-sorbose	-	Arbutin	+	Melezitose	-	L-fucose	-
Ribose	+	Rhamnose	-	Esculin	+	D-raffinose	+	D-arabitol	-
D-xylose	-	Dulcitol	-	Salicin	+	Starch	+	L-arabitol	-
L-xylose	-	Inositol	+	Cellobiose	+	Glycogen	+	Gluconate	-
Adonitol	-	Mannitol	+	Maltose	+	Xylitol	-	2-ketogluconate	-
β-methylxyloside	-	Sorbitol	+	Lactose	-	β-gentiobiose	+	5-ketogluconate	-

2.3.2 Genotypic Identification of *B. subtilis* R0179 Strain

We used the whole genome sequence to properly identify and taxonomically group this strain. Specific for this strain, we wanted to ensure that it did not demonstrate significant homology with any potential pathogenic *Bacillus* species.

The *Bacillus* genus, which includes over 200 species, harbors a high level of genomic diversity; species are markedly different from one another at the genome level and express a different set of proteins (proteome). Several *Bacillus* species, including *B. subtilis*, have Generally Recognized as Safe (GRAS) status for various applications, and were shown to be devoid of pathogenic toxins (Hong et al. 2005); only two species of *Bacillus* are known to be human pathogens with different levels of virulence (*Bacillus cereus* causing food poisoning and *Bacillus anthracis* causing anthrax). Wang and Ash (2015) used whole genome data and the feature frequency profile method in order to determine that *Bacillus subtilis* strains form a monophyletic clade that is genetically distant from the *B. anthracis* and *B. cereus* clades, as shown in Figure 4 (Wang & Ash 2015).

The feature frequency profile method can be more adaptable in detecting a large number of small genetic variabilities between sequences, such as intron deletions, exon sequence indels, transposable element

insertions, coding sequence base transversions, and short interspersed element/long interspersed element insertions.

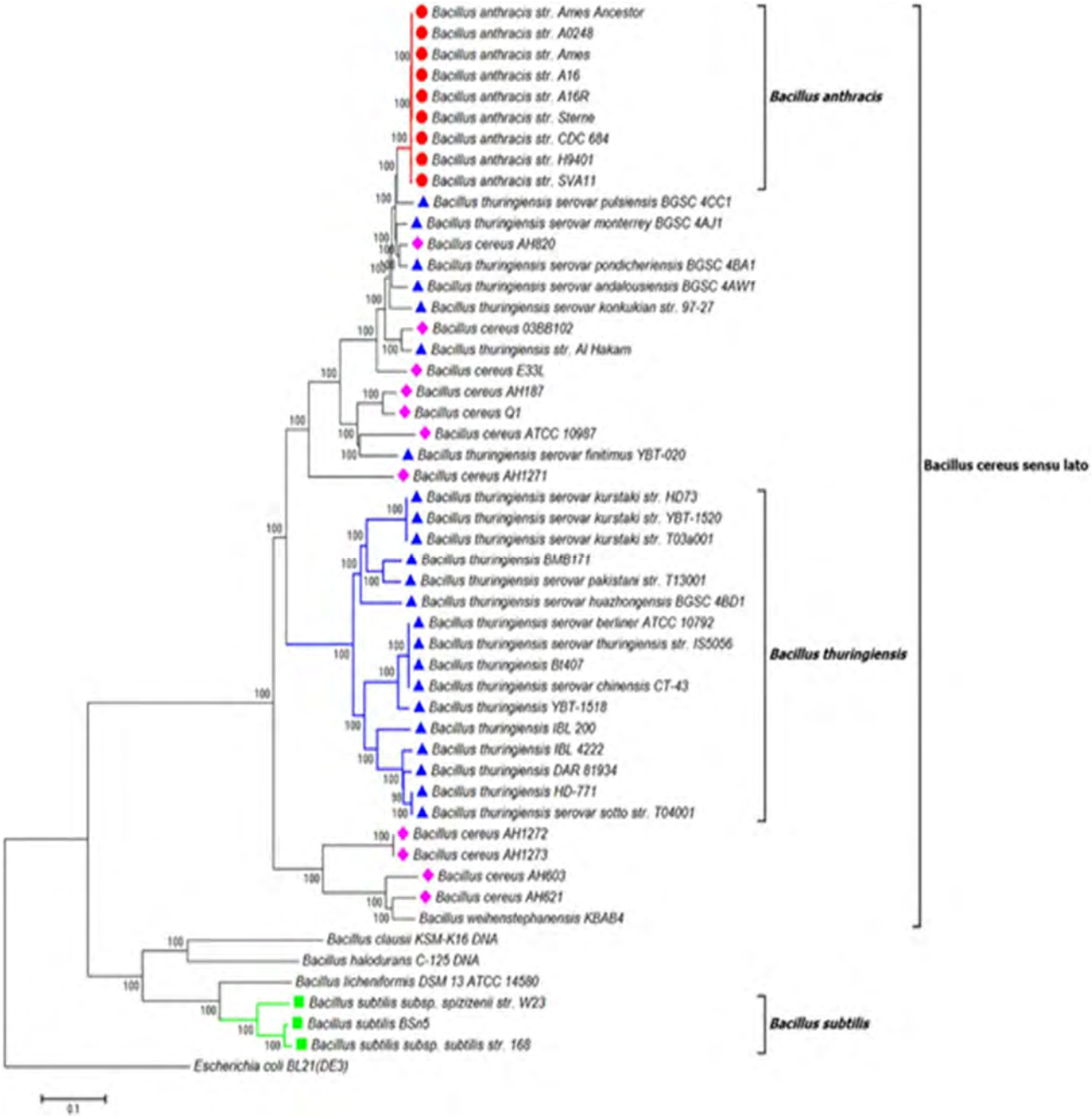

Figure 4 - Phylogenetic analysis of Bacillus species showing the monophyly of B. subtilis and genetic separation from B. cereus and B. anthracis (Wang & Ash. 2015).

From the publication "Neighbor joining (NJ) tree inferred from the kSNP analyses of the whole genome. The monophyly of *B. anthracis* was confirmed with high bootstrap support (100). A monophyletic clade containing 16 *B. thuringiensis* isolates was recognized (clade *Bacillus thuringiensis*). All the *B. anthracis*, *B. cereus, B. thuringiensis* and *B. weihenstephanensis* form a monophyletic clade (*Bacillus cereus sensu lato*), which is separated from the remaining *Bacillus* species examined in this study. Outside this major clade, the monophyly of *B. subtilis* was confirmed (100 bootstrap support)." (Wang & Ash 2015).

Further analysis was warranted to cement the placement of our strain within this *Bacillus subtilis* clade, and so phylogenetic trees were generated with *B. subtilis* 168, which is a type strain of this species used in the Wang & Ash (2015) study. Many other strains (e.g., NCIB 3610, W23, 168) are also type strains of *B. subtilis* due to significant homology between these strains (Zeigler *et al*. 2008). We included *B. subtilis* NCIB 3610 in our phylogenetic trees to ensure that our strain was well placed within the *B. subtilis* species.

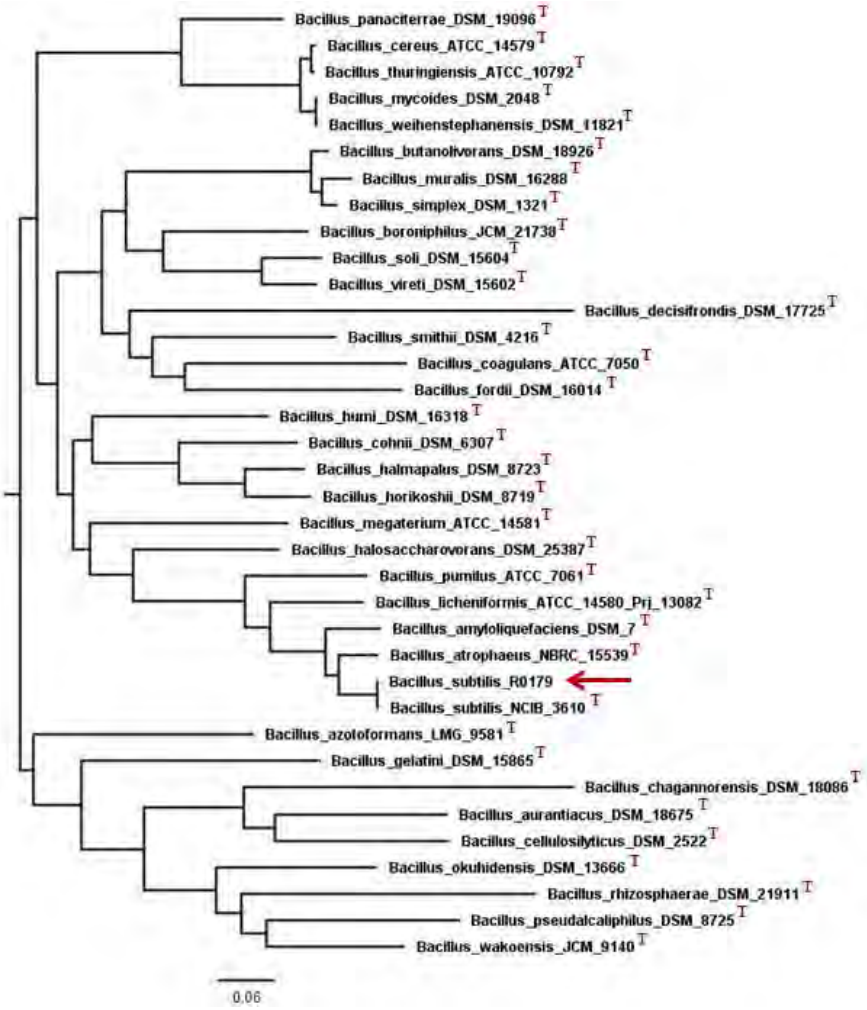

Figure 5 - Phylogenetic analysis of the B. subtilis R0179 strain (red arrow) in comparison to other Bacilli type strains (T).

Our analysis has determined that the strain is well placed within the *B. subtilis* species, and that it is outside the *B. cereus* or *B. anthracis* clades.

To demonstrate its similarity with the type strains of *B. subtilis* we created the phylogenetic tree shown in Figure 6 based on the whole genome sequence of the publicly available sequences of other *B. subtilis* strains.

Figure 6 -Phylogenetic analysis of the *B. subtilis* R0179 strain (red arrow) in comparison to other *Bacillus subtilis* strains with a *B. amyloliquefaciens* outgroup. Type strain indicated with red T.

These data show that *B. subtilis* R0179 is well placed within the *B. subtilis* species, and outside of other *Bacillus* species. Note that *B. amyloliquefaciens* DSM 7 is used as an outgroup here in Figure 6, whereas in Figure 5, showing the wider *Bacillus* phylogenetic tree, this strain is grouped quite near to the *B. subtilis* group. This indicates that the *B. subtilis* strains are quite homogeneous and are clearly distinct from other species within the *Bacillus* genus (i.e., *cereus* and *anthracis*).

2.4. Genomic Analysis

2.4.1. Sequencing

Whole genome sequencing of *B. subtilis* R0179 was done at the Centre of Innovation at Genome Quebec (740 Doctor-Penfield Ave., Montreal, QC, Canada H3A 1A4) using 454 Life Sciences paired-end pyrosequencing (Roche Applied Science). Coverage was 9.6-fold. The reads were assembled into five scaffolds with 613 intra-scaffold gaps using Celera software. The mean sequence gap length was only 82

bases. The genome comprised 4,021,566 bp. This represents greater than 95% of the expected genome based on the size of previously sequenced *B. subtilis* genomes available on GenBank. (http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2&type=1&name=Bacteria%20Complete%20Chromosomes).

2.4.2. Annotation of the Genome

Annotation of the genome R0179 sequence was done online using Rapid Annotation using Subsystem Technology pipeline (RAST; http://rast.nmpdr.org/rast.cgi). The RAST server was developed to annotate microbial genomes. It works by projecting manually curated gene annotations from the SEED Viewer database onto newly submitted genomes.

The genome annotation statistics are displayed in the table below. There were 47% (1928) open-reading frames (ORFs) or protein encoding genes (PEG) with assigned functions and 53% (2233) ORFs with unknown functions or hypothetical proteins.

R0179 Genome Annotation Statistics

Element	Quantity
ORFs	4161
RNA coding sequences	116
ORFs in subsystem	1928
ORFs not in subsystems	2233

2.4.3. Identification of Plasmids

B. subtilis R0179 was examined for the presence of plasmid DNA. The genomic evaluation found no DNA sequence that might be associated with a plasmid. An attempt to isolate plasmids using the Perfectprep® Plasmid Maxi Prep Kit according to the methods articulated in the accompanying protocol also failed to identify any plasmid DNA in *B. subtilis* R0179 (Tompkins *et al.* 2008).

2.5. Production Process

The manufacturing process presented in the following sections comprises the production of the lyophilized bacterial strain as a powder.

Information regarding the facilities involved in the manufacture and testing of this strain, including the responsibilities of each, is provided in Table2.

The culture medium contains soy products and may contain cow milk products, depending on whether the version is dairy or non-dairy. All foods to which *B. subtilis* R0179 is added require labeling to indicate the presence of these allergens.

Table 2: Facilities and Responsibilities (Lallemand 2019).

Name and Address	Activity
LALLEMAND HEALTH SOLUTIONS INC. 17975 rue des Gouverneurs Mirabel, Quebec, J7J 2K7 Canada and affiliated production sites	Production of dried powder of bacteria: Culture Collection, Fermentation, Concentration, Freeze-Drying, Standardization, Grinding and packaging, Quality Control, Storage

Bacillus subtilis R0179 is produced in compliance with current Good Manufacturing Practice (cGMP).

The facility LALLEMAND HEALTH SOLUTIONS INC, is compliant with the requirements for cGMP set by the local authority (Health Canada) for the manufacturing and handling of the strains under Part 3 of the *Natural Health Products Regulation* of 2004 and requirements for food production under the Safe Food for Canadians Regulations, as well as registered as a food facility with the FDA.

The steps of the manufacturing process of the bacterial strain powder *B. subtilis* R0179 are listed and described schematically in Figure 7.

The manufacturing process comprises the following steps:

1. Culture Collection

The strains are deposited in an international culture collection which guarantees always having an isolate of the strain in a safe and secure place. As a further safeguard, additional culture tubes are stored at -80°C at Lallemand's R&D laboratory at the National Research Council Canada (CNRC) in Montreal. The Master Cell Bank, composed of freeze-dried ampoules stored at an appropriate temperature according to SOP, is kept at Lallemand Health Solutions.

Genomic and phenotypic identifications are performed to guarantee the identity of the strain. Strain purity is confirmed by the absence of bacteriological contamination. From the Master Cell Bank, the Working Cell Bank cryovials are prepared and stored at -80°C.

From the Master Cell Bank, Working Cell Bank frozen cryotubes are prepared according to the production requirements and stored at -80°C. For each production vial used, many analyses such as microscopic aspect, colony aspect, and ID verification (API strips), are performed to guarantee strain identity. Strain purity is confirmed by the absence of microbiological contamination upon the microbiological analyses.

2. Pre-Culture

A frozen cryotube from the Working Cell Bank, previously kept at -80°C, is thawed and transferred into a test tube containing sterilized culture medium. Each strain's culture is incubated for a given time period at a determined temperature without agitation.

3. Sub-Culture

The revivified bacterial strain is transferred to an Erlenmeyer flask containing sterilized culture medium. Each strain's subculture is incubated for a given time period at a determined temperature without agitation.

An aliquot is taken at the end of the incubation period to verify specific parameters such as microscopic aspect, pH, and optical density.

4. Inoculum

An aliquot from the sub-culture is transferred to a large Erlenmeyer flask containing sterilized culture medium. Each strain's seed is incubated for a given time period at a determined temperature without agitation. Quality Control (QC) analysis at this step includes pH, optical density, microscopic aspect, colony aspect, total microbiological count, and absence of microbiological contaminants.

5. Culture Medium Preparation

The raw materials released by QC following inspection and meeting specifications are weighed per culture media recipe, then dissolved in water using a high shear mixer or added directly in the fermenter. The culture media are either treated using a UHT system, sterilized, or pasteurized *in situ* and cooled to the incubation temperature. The pH is adjusted prior to inoculation with the seed culture. The temperature is continuously monitored during preparation, sterilization, and cool-down.

6. Fermentation

A pre-fermentation step may be performed in a smaller fermenter prior to the fermentation when larger inoculum volume is required.

The inoculum is transferred from the flask or the pre-fermenter to the sterilized culture media for biomass production ("fermentation"). During the fermentation, the culture is gently agitated and temperature is controlled. The bacterial strain is grown in the fermenter until the targeted parameters are reached, which corresponds to the late exponential phase of the bacterial growth.

At the end of the fermentation, a sample is sent to the QC lab for testing, including pH, optical density, microscopic aspect, colony aspect, total microbiological count, and absence of microbiological contaminants. The fermentation broth is cooled before concentration.

7. Concentration

The fermentation broth is concentrated by ultra-filtration or centrifugation.

8. Cryopreservation and Freeze-Drying

Food-grade approved cryoprotectants are blended with the concentrated bacterial culture until a homogeneous solution is obtained. A sample of the cryoprotected concentrated bacterial culture is brought to the QC to test for the total count and the absence of microbiological contaminants. Sterile containers are filled with the mixture and introduced into the freeze-dryer. The mixture is then deposited onto trays.

The trays are freeze-dried for a determined time; the cycle is completed when the targeted parameters are reached. Temperature and pressure of the freeze-dryer and of the concentrated culture are monitored

throughout the process. The freeze-drying process consists of a primary drying phase under a controlled vacuum to sublimate free water. The freeze-dried cake-like bacterial culture is either ground directly or collected in bags and stored in the freezer or under refrigerated conditions until grinding. Samples are collected by QC and tested for different parameters such as bacteriological count and microbiological contaminants.

As indicated in Sections 1.4 and 2.5, the product is produced in both dairy and non-dairy versions. The cryoprotectants in the dairy version include milk while those in the non-dairy version do not.

9. Grinding and Packaging

The freeze-dried bacterial concentrates are ground and collected in bags. The bags are weighed, labelled, and inventoried. They are then placed in covered bins for storage.

Samples of the bacteria powder are brought to QC for testing. The total count, the absence of microbiological contaminants, water activity, humidity, and physical aspects are performed for QC release of the lot.

10. Storage

The freeze-dried ground bacterial cultures are kept in refrigerated storage (2°C to 8°C) or frozen (-20°C).

Note: For some applications, the freeze-dried bacterial powder may be standardized (blended with maltodextrin and ascorbic acid) before further use. A flow chart of the manufacturing process is provided in Figure 7.

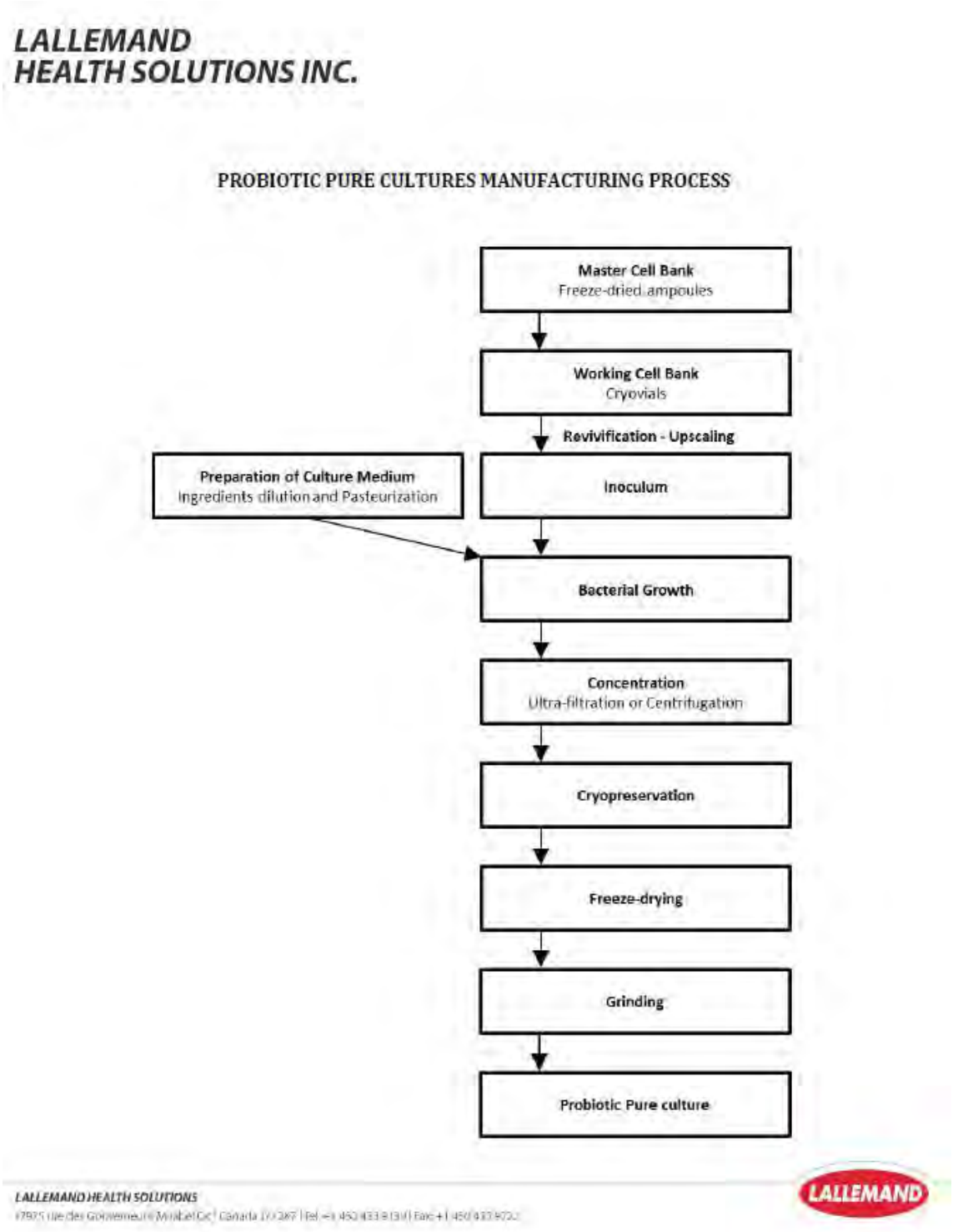

Figure 7: Flow Diagram of Manufacturing Process of *B subtilis* R0179.