2.6. Specifications

Table 3: Specifications of *B. subtilis* R0179

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Fine to granular, ivory to beige powder	Visual observation
B. subtilis count	>25x10^9 cfu/g Or >100x10^9 cfu/g	Bacteriological enumeration – in-house method*
Yeast and molds	<1000 cfu/g	MFHPB-22
Coliforms	<10 cfu/g	ISO 4831
Salmonella	Absent in 25 g	MFHPB-20
Escherichia coli	Absent in 1 g	ISO 7251
SOURCE: Lallemand Health Solutions, 2020 (unpublished).		

[redacted]

The technical data sheets of the products *Bacillus subtilis* R0179 Standardized at 25 billion and 100 billion cfu and the certificates of analysis of 3 non-consecutive batches of the 25 billion and 100 billion grades are available in Appendix 3.

2.7. Heavy Metals

Raw materials likely to contain these impurities are tested against established specifications and approved for use before they are entered into the manufacturing stream.

Data analysis of the content of heavy metals in two non-consecutive samples of *Bacillus subtilis* R0179 meet the specifications set forth in table below.

Table 4: Heavy Metals Analysis of *Bacillus subtilis* R0179

Test – Heavy Metals	USP Specifications 2232	Specifications (mg/kg) (EU regulation 1881/2006)	*Bacillus subtilis* R0179 (sample no)	
			U120140248	U120140250
Lead (mg/kg)	≤0.5	≤3	0.04	0.04
Cadmium (mg/kg)	≤0.5	≤1	0.09	0.09
Arsenic (mg/kg)	≤1.5	**	0.2	0.2
Mercury (mg/kg)	≤1.5	≤0.10	0.05	0.2
SOURCE: Lallemand Health Solutions, 2020 (unpublished).				

Bacillus subtilis R0179 samples standardized at 25 billion and 100 billion have been tested for the heavy metals content and, based on the quality systems matrix approach including the heavy metals content of pure strain, ascorbic acid, and lactose, and their exact quantity, we can confirm that *Bacillus subtilis* R0179, standardized at 25 billion and 100 billion, meets the requirements of the USP <2232> and EU regulation 1881/2006 (See statement in appendix 4). We have provided certificates of analysis (in appendix 3) for the pure strains (as demonstrated above) to show that the strains are not at risk for heavy metal contamination. Raw materials used in the blend of products are monitored based on the verification of raw material supplier's declaration.

2.8. Stability

2.8.1. Stability of Bacterial Powder

1. Stability of Non-Standardized Bacterial Powder

Bacillus subtilis R0179, like most live microorganisms, has a higher stability at lower temperature (as demonstrated by the first stability data at refrigerated temperature), and as such should be kept refrigerated whenever possible. Tables 5 and 6 display the stability results for 24 months at 4°C and 25°C for non-standardized *B. subtilis* R0179 powder (i.e., pure strain). These data are derived from 11 different stability lots of *B. subtilis* R0179 averaged together.

Table 5. Stability Data for Non-Standardized *Bacillus subtilis* R0179 at 4°C

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	3.57×10^{11}	3.24×10^{11}	3.10×10^{11}	2.78×10^{11}	2.27×10^{11}	2.15×10^{11}
Survival rate (%)	100	91	87	78	64	60
SOURCE: Lallemand Health Solutions, 2020 (unpublished).						

Table 6. Stability Data for Non-Standardized *Bacillus subtilis* R0179 at 25°C

Storage time (months)	0	1	3	6	9	12	18	24
Bacterial content (cfu)	3.57×10^{11}	3.02×10^{11}	2.99×10^{11}	2.53×10^{11}	2.27×10^{11}	2.29×10^{11}	1.66×10^{11}	1.36×10^{11}
Survival rate (%)	100	85	84	71	64	64	46	38
SOURCE: Lallemand Health Solutions, 2020 (unpublished).								

2. Stability of Standardized (25×10^9 cfu/g) Bacterial Powder

Tables 7 and 8 show stability results for 24 months at 4°C and 25°C for *B. subtilis* R0179 standardized at 25×10^9 cfu/g by addition of excipients. These data are derived from 7 different stability lots of *B. subtilis* R0179 standardized at 25×10^9 cfu/g averaged together.

Table 7. Stability Data for *Bacillus subtilis* R0179 Standardized at 25x10^9 cfu/g at 4°C

Storage time (months)	0	3	6	12	18	24
Survival rate (%)	100	>100	>100	>100	>100	>100
SOURCE: Lallemand Health Solutions, 2020 (unpublished).						

Table 8: Stability Data for *Bacillus subtilis* R0179 Standardized at 25x10^9 cfu/g at 25°C

Storage time (months)	0	3	6	9	12	18	24
Survival rate (%)	100	>100	>100	>100	>100	99	92
SOURCE: Lallemand Health Solutions, 2020 (unpublished).							

3. Stability of bacterial powder Standardized at 100x10^9 cfu/g

Tables 9 and 10 display stability results for 24 months at 4°C and 25°C for *B. subtilis* R0179 standardized at 100x10^9 cfu/g by the addition of excipients (low-pH lactose and l-ascorbic acid). These data are derived from 3 different stability lots of *B. subtilis* R0179 standardized at 100x10^9 cfu/g averaged together.

Table 9. Stability Data for *Bacillus subtilis* R0179 Standardized at 100x10^9 cfu/g at 4°C

Storage time (months)	0	3	6	12	18	24
Survival rate (%)	100	>100	>100	>100	>100	>100
SOURCE: Lallemand Health Solutions, 2020 (unpublished).						

Table 10. Stability Data for *Bacillus subtilis* R0179 Standardized at 100x10^9 cfu/g at 25°C

Storage time (months)	0	3	6	9	12	18	24
Survival rate (%)	100	87	77	91	90	>100	>100
SOURCE: Lallemand Health Solutions, 2020 (unpublished).							

2.8.2. Genetic Stability of the Strain R0179

The genetic stability of bacterial culture *B. subtilis* R0179 was evaluated over 20 transfers by RAPD PCR. The RAPD-PCR analysis on DNA extracted from *B. subtilis* R0179 transfer 1, 5, 10, 15, and 20 and compared with the DNA from the R0179 mother culture (T0) shows that there were no changes in the DNA profiles obtained using 5 different primers over 20 transfers (Figure 8). R0179 genetic integrity was not affected by storage or repeated cultivation. The RAPD-PCR profile assessment of *B. subtilis* R0179 indicates that it is genetically stable with repeated cultivation. The complete report is available in Appendix 5.

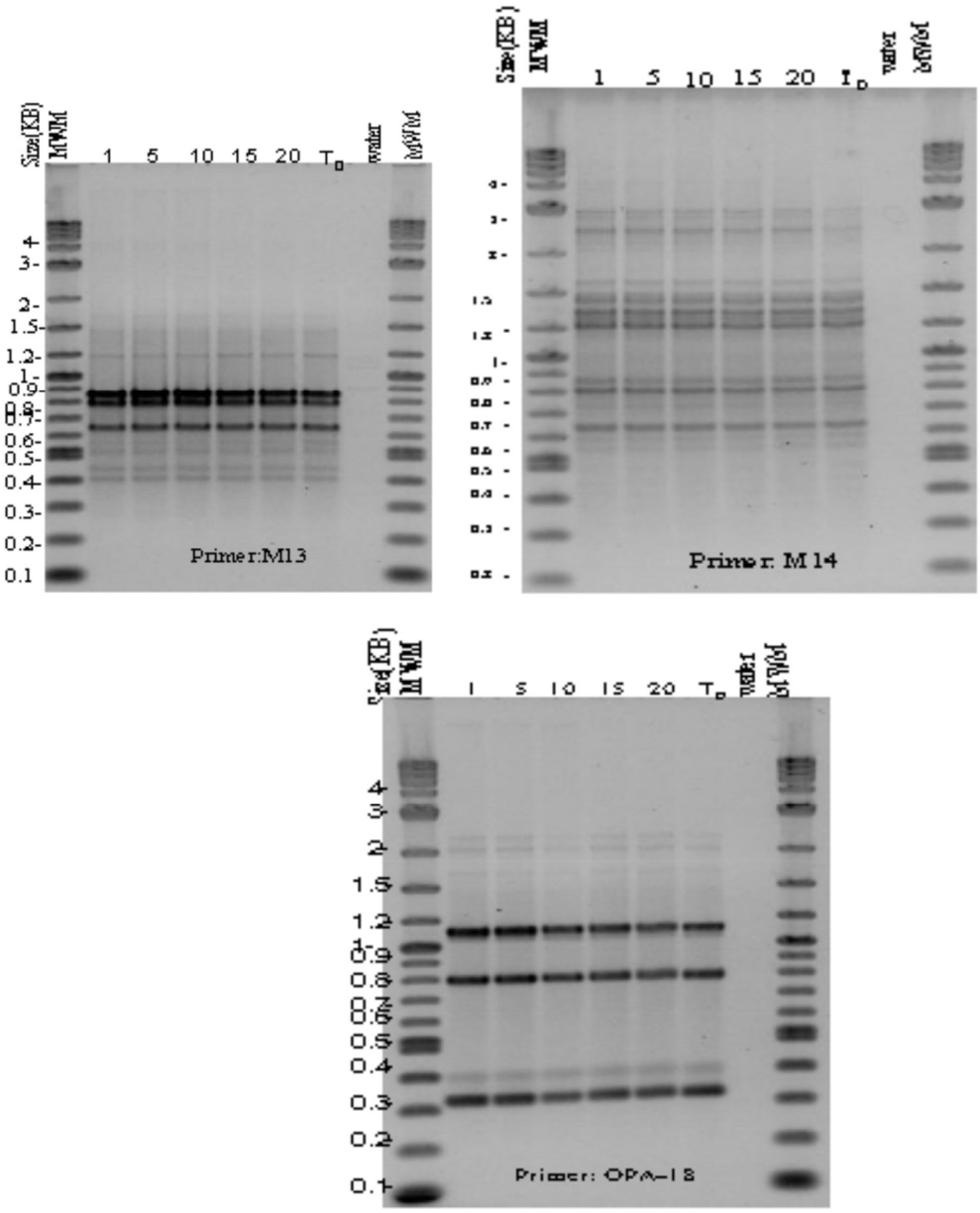

Figure 8: Photos of RAPD-PCR gels using primers: M13, M14 and OPA-18.

2.8.3. Stability in Different Food Categories

An advantage of adding *B. subtilis* spores to food is their excellent stability in many food matrices and their resistance to multiple food processes. Extensive stability testing has been performed to determine addition levels needed to assure the presence of target levels of viable bacteria throughout the shelf life of different foods.

The microorganism has been added at levels as high as $5x10^9$ cfu/serving in order to obtain a remaining concentration of $1x10^8$ cfu/serving after processing (cooking, pasteurization, homogenization, blending, etc.) and storage and throughout the shelf-life.

Stability testing in the food matrices listed in Table 11 showed that the bacterial concentration in the finished product remains stable through the shelf life (no increase or significant drop in population has been observed). The Table 11 shows the survival rate of the strain in different food categories after processing, storage, and shelf life. The analytical protocol for enumeration of *B. subtilis* R0179 in food matrices is given in Appendix 6.

Table 11: Starting dose, shelf life, and storage condition for five food categories.

Food Categories	Starting concentration in order to obtain a remaining concentration of 10^8 cfu/serving	Shelf life	Storage conditions
Whole grain-based bread	2-10x10^{8} *	21 days	room temperature (22-25°C)
Whole wheat muffins	20-40x10^{8}*	14 days	room temperature (22-25°C)
Kombucha	70-100x10^{8}*	3 months	refrigerated (4-8°C)
100% fruit or vegetable juices	70-100x10^{8}*	1 year	room temperature (22-25°C)
Diet salad dressing	50-80x10^{8}*	2 years	room temperature (22-25°C)
SOURCE: Lallemand Health Solutions, 2020 (unpublished).			

* The survival rate depends on the processing conditions that may vary betweens manufacturers.

PART 3. DIETARY EXPOSURE (EDI)

Lallemand intends to use *B. subtilis* R0179 as an ingredient in select food products at a maximum level of 1 x 10^9 cfu *Bacillus subtilis* R0179 per serving after processing. The EDIs represent potential intakes of *B. subtilis* R0179 per day for the US population age 2 years and older.

The intended additional 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. The proposed food use categories, a description of foods included in each use category, and the serving sizes of foods in each use category are listed in Table 12 below.

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.

Table 12: List of Intended Uses

21 CFR §170.3 Food Categories Proposed for Addition of *Bacillus subtilis* R0179			Description of Proposed Uses	Serving Size (as consumed)[a]
1	Baked goods and baking mixes	All ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving	Biscuits, cornbread, scones, hush puppies, crumpets; cakes, lightweight; coffee cakes, crumb cakes, doughnuts, Danish, sweet rolls, sweet quick type breads; croissants; English muffins; pizza crust; soft pretzels; tortillas	55 g
			Breads (excluding sweet quick type), rolls	50 g
			Brownies; grain-based bars	40 g
			Cakes, mediumweight	80 g
			Cookies; crackers that are usually used as snacks; taco shells, hard	30 g
			Crackers that are usually not used as snack, melba toast, hard bread sticks, ice cream cones	15 g
			Croutons	7 g
			French toast, crepes, pancakes, variety mixes; bagels; muffins	110 g
			Pies, cobblers, fruit crisps, turnovers, other pastries; cakes, heavyweight	125 g
			Waffles	85 g

21 CFR §170.3 Food Categories Proposed for Addition of *Bacillus subtilis* R0179			Description of Proposed Uses	Serving Size (as consumed)[a]
3	Beverage and beverage bases	Non-alcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks	Carbonated and noncarbonated beverages, water	360 ml
			Juices, nectars, fruit drinks	240 ml
4	Breakfast cereals	Ready-to-eat and instant and regular hot cereals	Breakfast cereals (hot cereal type), hominy grits	1 cup prepared
			Breakfast cereals, ready-to-eat, weighing less than 20 g per cup	15 g
			Breakfast cereals, ready-to-eat, weighing 20 g or more but less than 43 g per cup; high fiber cereals containing 28 g or more of fiber per 100 g	40 g
			Breakfast cereals, ready-to-eat, weighing 43 g or more per cup	60 g
6	Chewing gum	Chewing gum, all forms	Chewing gum	3 g
9	Confections and frosting	Candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars	Baking candies	15 g
			Baking decorations (e.g., colored sugars and sprinkles for cookies, cake decorations)	4 g
			Confectioner's sugar, marshmallows	30 g
			Frosting or icing	2 tbsp
			Sugar	8 g
10	Dairy product analogs	Nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products	Cream or cream substitutes, fluid	15 mL
			Cream or cream substitutes, powder	2 g
			Milk-substitute beverages	240 mL
			Non-dairy whipped toppings	2 tbsp
21	Fruit and water ices	All frozen fruit and water ices	Frozen flavored and sweetened ice and pops	2/3 cup
32	Nuts and nut products	Whole or shelled tree nuts, peanuts, coconut, and nut and peanut spreads	Nut butters, pastes, or creams	2 tbsp
			Nuts, mixtures, all types	30 g
33	Plant protein products	"Reconstituted vegetable protein" category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins	Bacon substitutes	15 g
			Cheese, all others	30 g
			Mixed dishes: Measurable with cup	1 cup
			Mixed dishes: Not measurable with cup	140 g
			Substitute for luncheon meat, meat spreads, Canadian bacon, sausages, frankfurters, and seafood	55 g
			Tofu, tempeh	85 g
			Yogurt	170 g

21 CFR §170.3 Food Categories Proposed for Addition of *Bacillus subtilis* R0179			Description of Proposed Uses	Serving Size (as consumed)[a]
35	Processed fruits and fruit juices	All commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, "ades", and drink substitutes made therefrom	All other fruits (except those listed as separate categories), fresh, canned or frozen	140 g
			Candied or picked fruit	30 g
			Dried fruit	40 g
			Fruit relish	70 g
			Fruits for garnish or flavor (e.g., maraschino cherries)	4 g
			Juices used as ingredients (e.g., lemon juice, lime juice)	5 mL
			Juices, nectars, fruit drinks	240 mL
37	Snack foods	Chips, pretzels, and other novelty snacks	All varieties, chips, pretzels, popcorn, extruded snacks, fruit and vegetable-based snacks (e.g., fruit chips), grain-based snack mixes	30 g

[a] Serving size correspond to values in Table 2 – Reference Amounts Customarily Consumed per Eating Occasion: General Food Supply as cited in FR Vol 81, No. 103, Friday, May 27, 2016, pp 34000-47. Available at: https://www.govinfo.gov/content/pkg/FR-2016-05-27/pdf/2016-11865.pdf. Serving size as a volume measure (e.g., cup, mL, tbsp) assumed that 1 mL = 1 g.

The estimated daily intake (EDI) of *B. subtilis* R0179 from the proposed food uses is based on food consumption records collected in the What We Eat in America (WWEIA) component of the National Health and Nutrition Examination Survey (NHANES) conducted in 2013-2014 and 2015-2016 (NHANES 2013-2016). A total of 13,600 individuals age two years and older provided 2 complete days of dietary recalls. Based on the two-day data provided by consumers of target foods, the 2-day average EDI of *Bacillus subtilis* R0179 at the mean and 90th percentile of intake is 5.6 x 10^9 cfu/day and 9.6 x 10^9 cfu/day, respectively, see Table 13. The full report from Exponent may be found in Appendix 1.

Table 13: Two-day Average Estimated Daily Intake of *Bacillus subtilis* R0179 by the U.S. Population 2 years and Older Based on Intended Uses in Select Categories of Foods; NHANES 2013-2016

			Bacillus subtilis R0179 (CFU/day)			
			Per Capita		Per User	
Population	N	% Users	Mean	90th Percentile	Mean	90th Percentile
U.S. population 2+ y	13,561	100	5.5E+09	9.6E+09	5.6E+09	9.6E+09

The 90th-percentile EDI of *B. subtilis* R0179 from the food categories included in the 2012 GRAS determination (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) is 2.8x10^8 cfu. The maximum 90th-percentile daily intake from all uses combined is the sum of the two EDIs, or 9.9x10^9 cfu.

PART 4: SELF-LIMITING LEVELS OF USE

There is no meaningful technological or organoleptic limitation to the concentration of *B. subtilis* R0179 which may be added to foods.

PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD

The conclusion that the intended use of *Bacillus subtilis* R0179 is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958.

PART 6. NARRATIVE

6.1. Recognized Safety of Aerobic Endospore-Forming bacteria and *Bacillus* species
6.2. History of consumption
6.3. Safety Parameters
6.4. Human studies
6.5. Safety Evaluations by Authoritative bodies
6.6. Decision-Tree Analysis of the Safety of the Notified Strain
6.7. Safety Assessment and GRAS determination
6.8. Statement regarding information inconsistent with GRAS
6.9. Conclusion of the Expert Panel

The human intestinal tract is inhabited by over a thousand billion bacteria from over 400 different species (Zetterstrom et al. 1994; Edwards and Parrett 2002). When purposely administered for their associated benefits, bacteria must be evaluated for safety. As recommended by authoritative bodies and experts in the field (EFSA 2005, EFSA 2007, EU-PROSAFE (Vankerckhoven et al. 2008), FAO/WHO 2001, Marteau et al. 2001, SCAN 2000, Sorokulova 2008, Wassenaar and Klein 2008), this section will review the literature on aerobic spore-forming bacteria (AEFB) and *Bacillus* species and evaluate the virulence and infectivity risk, antibiotic resistance profile and potential for transfer, antibiotic and toxin production, absence of plasmids, bile salt deconjugase activity, acid and bile stability, and ability to adhere to intestinal cells, including *in vivo* studies in adults, children, and infants, and studies in animals.

6.1. Recognized Safety of Aerobic Endospore-Forming Bacteria and *Bacillus* Species

The *Bacillus subtilis* species used to be identified as a saprophyte soil microorganism, but recent works show the ability of *Bacillus subtilis* strains to form biofilms (Hong et al. 2009), to sporulate anaerobically (Tam et al. 2006; Nakano et al. 1997; Casula and Cutting 2002) and to secrete antimicrobial compounds which inhibit the growth of pathogens (Hong et al. 2009). These findings suggest that *Bacillus subtilis* may possesses features to survive, grow, and sporulate in the human intestine. *B. subtilis* has been isolated from the human GIT (Hong et al. 2009; Fakhry et al. 2008), but also from pigs (Leser et al. 2008) and chickens (Cartman et al. 2008). A recent study compared the number of spores carried by the soil (~10^6 spores/g) versus the levels found in human feces (~10^4 spores/g). The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination. Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal (Hong et al. 2009).

Several articles have evaluated the fate and dissemination of *B. subtilis* spores in animal models: white Leghorn chicks (Cartman et al. 2008); BALB/c mice (Casula and Cutting 2002; Hoa et al. 2001); crossbred Yorkshire and Danish Landrace pigs (Leser et al. 2008). These studies, independently of the animal model, showed that *B. subtilis* spores germinate early in the proximal sections of the gastrointestinal tract. In mice the germination occurred in the jejunum and ileum but in chickens and pigs it appeared to occur much earlier. In addition, messenger RNA (mRNA) from a genetically engineered chimeric gene strongly expressed only by vegetable cells in a murine model was detected at significant levels in the jejunum and, to a lesser degree, in the ileum (Casula and Cutting 2002). Finally, Hoa et al. (2001) showed that when spores are orally administered to mice, more spores are eliminated in feces then are ingested,

demonstrating that germination, reproduction, and re-sporulation occurs. Re-sporulation was also demonstrated by Tam et al. (2006) by measuring the reverse transcriptase-PCR expression of sporulation-specific genes. It is thus apparent that *B. subtilis* bacteria can complete their entire life cycle in their host. Those combined findings demonstrate that *Bacillus subtilis* spores are more than passive transient passengers in the gut but are part of the normal animal and human microbiota which can interact with host cells and provide beneficial effects.

The aerobic saprophytic *Bacilli* comprise a large group of sporulating Gram positive bacteria widely distributed in nature, mainly in soil, whose members are found ubiquitously. Except for *B. anthracis* and *B. cereus*, *Bacillus* species are generally regarded as safe. Furthermore, *B. subtilis* has been broadly studied for biotechnology applications. It is recognized as the type species for the genus without pathogenic potential to humans (De Boer et al. 1991). Because of the ubiquitous dispersion of the bacterium, it is inevitable to find it in association with other microorganisms in immunocompromised patients (21 cases of bacteremia, all in patients with catheters, lumbar puncture, or other intervention [Kiss et al. 1988]; 8 cases in patients with cancer, head trauma, or stroke, or patients that have undergone surgery [Richard et al. 1988]; 2 infections in blood cancer patients [Pennington et al. 1976], and in drug abusers [Reller 1973]). Cases of infections reported before 1970 are not considered because of the lack of discrimination between *B. subtilis* and all other aerobic endospore-forming organisms (Gordon 1973).

B. subtilis has been hypothetically implicated in several cases of food-borne illness (Kramer and Gilbert 1989). The main symptoms were vomiting and diarrhea, and the bacterial load was estimated at 10^5 to 10^9 cfu/g. The causative agent was identified as a strain of *B. subtilis* carrying the 3 genes of HBL enterotoxin normally produced by *B. cereus* (Rowan et al. 2001). Another case was reported of emesis from an infant cereal product (Duc et al. 2004). Two *Bacillus* species were isolated, *B. cereus* and *B. subtilis*. It is most likely that *B. cereus* was responsible for the food-poisoning, but no emetic toxin was detected. The disease may have been caused by an unidentified cereulide-type toxin or simply by the bacterial load. As mentioned before, *B. subtilis* strains are often consumed in high concentration and no adverse effect has been reported, so it seems improbable that this case was caused by the bacterial load of *B. subtilis*.

A 73-year-old man with chronic lymphocytic leukemia died from an infection putatively caused by a commercial product, Enterogermina (Oggioni et al. 1998). While this product claimed to contain *B. subtilis*, the microorganism was later identified as *B. clausii* (Spinosa et al. 2000).

Three cases of diarrhea were reported after usage of a product commercialized as Bactisubtil®. *B. cereus* was isolated from the stool and from the product (Kniehl et al. 2003). The product was first labeled as containing a strain of *Bacillus subtilis* but has been confirmed to contain *B. cereus* (Hong et al. 2005).

Recently, seven cases of allergic reactions were reported after consumption of natto-fermented soybeans (Inomata et al. 2007). The symptoms associated were generalized urticaria and dyspnea for all patients as well as loss of consciousness (5 patients), collapse (2 patients), vomiting (2 patients), and diarrhea (2 patients). In these cases, the causative agent was not *B. subtilis*, but allergy to fermented beans; this diagnosis was confirmed by positive skin-prick tests with fermented soybeans by all patients.

Other articles by Mazza (1994), Sanders et al. (2003), Hong et al. (2005), and Van et al. (2009) have reviewed the use of *Bacillus subtilis* sp. and its safety. These reviews examined the potential health benefits from consuming *B. subtilis* and concluded that there were no safety concerns.

6.2. History of Consumption of *Bacillus subtilis* Strain R0179

Human populations around the world have been in contact with *Bacillus subtilis* for many centuries because it is commonly found in soil. It is used in many fields of application like biotechnology, sanitation, pharmaceuticals, and food. *Bacillus subtilis* is a model organism for laboratory studies as a Gram-positive bacterium. Moreover, its ability to grow rapidly and produce enzymes in specific conditions makes *B. subtilis* the perfect microorganism for commercialization of enzymes like amylase or antibiotics like Bacitracin. However, the subject of interest of this GRAS notification is human consumption in food applications which will be described in the following paragraphs.

Natto is a popular traditional meal in Japan made by fermentation of soybeans with *Bacillus subtilis* (natto). Natto is not recognized as a subspecies even if it is often referenced in the literature. Fermented soybeans with *Bacillus subtilis* are also popular in Asia; they can be found in China under the name "dou-chi", "kinema" in Nepal and Myanmar, "tua nao" in Thailand, and "chungkukjang" in Korea. The fermented soybeans are consumed directly or can be added as an ingredient to salads, soups, sushi, and rice. Natto-fermented soybeans contain high levels of *Bacillus subtilis*, 10^8 cfu/g. Regular intake of natto can change the composition and metabolic activity of the human microbiota by increasing the number of *B. subtilis* and *Bifidobacterium* species (Terada et al. 1999). This meal also contains high levels of vitamin K_2 produced by the bacterium; it is a therapeutic agent in treatment of osteoporosis in Japan (Katsuyama et al. 2004).

Bacillus products have been marketed for human use since 1960 in Europe and Southeast Asia. The stability of spore-based products at room temperature represents a massive advantage compared to *Lactobacillus* or *Bifidobacterium* species. They contain *Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus, Bacillus clausii* and *Bacillus coagulans*. Many products are marketed to prevent or cure gastrointestinal disorders, particularly diarrhea with rotavirus infections or as an adjunct to antibiotic therapy. In Ukraine, Biosporin contains a mixture of *B. subtilis* and *B. licheniformis* (Dong et al. 2009).

B. subtilis is also used in animal feed in the United States; two products are marketed as Calsporin™ by the Calpis Co. and Growgen by the Eisai Co. Many clinical studies have been published showing the impact of *B. subtilis* C3102 (Calsporin™) on chickens and pigs (http://www.calsporin.com/english02/index.html). Two animal-feed products containing *Bacillus subtilis* are sold in Europe, one in the United Kingdom (BioGrow) for use in poultry, calves, and swine, and the other in Denmark (Bioplus) for fattening piglets, chickens, and turkeys. This species is recognised for unlimited use in the European Union (Dong et al. 2009).

Fermented soybean natto is reputed to have come to Japan by way of Korea approximately 400 years ago following the Japanese invasion of the peninsula (Prajapati and Nair 2003).

B. subtilis was first isolated and characterized from natto in 1913 by S. Sawamura of the Imperial University of Tokyo; he called it *Bacillus natto* (Hosoi and Kiuchi 2003). Today, *Bacillus subtilis* is recognized as a fermentative agent in the popular natto vegetables in Japan. The fermented soybeans showed an increase of consumption during 1990's and generated sales of 160 billion yen in 1996 (Statistic Bureau, Ministry of Public Management, Home Affairs, Post and Telecommunications, Japan 2001). A market survey of the purchase of Foods for Specified Health Use (FOSHU) products in Japan was conducted by the Japan 21 Health Promotion forum in 2001. The study involved 500 adults and showed natto as one of the top products purchased (Gibson 2005). FOSHU products are the foods approved by the Ministry of Health, Labor and Welfare as effective for preservation of health by adding certain active ingredients or removing undesirable ones. They are designed to be safe and effective for the maintenance and improvement of health by incorporating them into one's diet.

Bacillus subtilis R0179 was isolated from a Korean commercial product. The bacteria in the Korean product were supplied by a Japanese company. *Bacillus subtilis* R0179 has been sold worldwide as a powder since 2007, or in products marketed in Korea since 1985 and in China since 1994. Indeed, in China and South Korea, a combination of *Enterococcus faecium* R0026 and *Bacillus subtilis* R0179 has gained wide acceptance and government approval. This combination comes in a variety of different formulations for adults, children, and newborn infants. The microorganism is marketed by Hanmi Pharmaceutical Company Limited (Seoul, South Korea) under the trade name Medilac® and has been available on the Korean market since 1994 and in China since 2000. The strains and the composition have not been changed since their introduction. The adult preparations, Medilac-S® and Medilac-DS®, contain 5.0×10^8 cfu and 1.0×10^9 cfu per capsule, respectively (DS referring to 'double strength'). Preparations for children and infants are also available under the trade name Medilac-vita® or Mamiai®, containing vitamins and minerals in addition to the bacteria. The strains and the products have been subjected to preclinical studies *in vitro* and *in vivo*. See sections 6.4.1. Studies in Adults and 6.4.2 Studies in Children and Infants for more information about those studies.

6.3. Safety parameters

6.3.1. Ability to Adhere to Intestinal Cells

The ability of beneficial microbes to adhere to intestinal cells has not been confirmed to be required for them to exert their benefit in the host, although it has been considered to be necessary for prolonged residence, the modulation of the host immune system, and competition with potential pathogens (Sherman et al. 2009). High adhesion capacity is typically associated with potential pathogenicity and may facilitate platelet aggregation or infectivity. *In vitro* adhesion assays are commonly used to predict *in vivo* adherence.

The ability of *B. subtilis* R0179 to adhere to human colon adenocarcinoma grade II cells (HT-29) that produce mucin was compared to other bacteria, *Enterococcus faecium* R0026 and *Lactobacillus helveticus* R0052. This was done according to the protocol outlined in Tompkins et al. (2008). Under these conditions, *B. subtilis* R0179 was the least adherent (21.2 cfu / HT-29 cell), *E. faecium* R0026 was moderately adherent (89.8 cfu / HT-29 cell), and *L. helveticus* R0052 was the most adherent (111.2 cfu /

HT-29 cell). This result correlates well with the findings of Hong et al. (2009), which indicated that *B. subtilis* strains were much less (10-100x less) adherent than the pathogenic *B. cereus*.

The poor ability of *B. subtilis* R0179 to adhere to the human cell confirms the prediction of low adherence based on the genome analysis and the lack of any functional adhesin genes. In fact, genomic analysis did not identify any genes encoding putative adhesin proteins to collagen or mucin. Two open-reading frames encoding two parts of a possible fibronectin-binding protein were identified but the sequence contained a stop-codon rendering the gene inactive. Therefore, the strain is unlikely to encode specific genes for adhesion to human cells.

6.3.2. Undesirable Metabolic Activity

6.3.2.1. D-lactate production

The UV test kit for the determination of D-/L-lactic acid from Xygen Diagnostics Inc was used for the quantification. The strain was grown in M12 broth for 16-18 hours at 37°C. It was found that *Bacillus subtilis* R0179 produces only L (+)-lactate (1.07 g/L). The D (-) optical isomer is not produced, so the potential of lactic acidosis as a complication of treatment in children with short bowel syndrome is not a concern for strain R079.

6.3.2.2 Bile Salt Deconjugase Activity

Various bacteria can hydrolyze bile salts, and this has been targeted as an important criterion for selection. An agar plate assay is used to detect bile salt hydrolase activity in lactic acid bacteria by supplementing the media with taurodeoxycholic, taurocholic, or taurochenodeoxycholic acids. Bile salt hydrolysis is manifested at two intensities, either the formation of precipitate halos around colonies or the formation of opaque granular white colonies.

The bacterial culture *Bacillus subtilis* R0179 was evaluated for its ability to produce bile salt hydrolase activity, which appears to be absent. The strain's growth and production of exopolysaccharides was markedly inhibited by the bile salts (see

for results). Results showed that colonies of *B. subtilis* R0179 are generally large, spreading, and irregularly shaped due to the exopolysaccharides they form, as observed in plate A. However, in the presence of sodium taurodeoxycholate hydrate (TCA), the strain forms small colonies with no precipitate halos. Therefore, *Bacillus subtilis* R0179 does not produce bile salt hydrolase. The bile salt inhibited the growth of the strain and prevented the production of exopolysaccharides.

The complete report of the analysis is given in Appendix 7.