Vishaal Bhuyan
Aanika Biosciences, Inc.
86 34th St. Suite D-605
Brooklyn, NY 11232

Re: GRAS Notice No. GRN 001095

Dear Mr. Bhuyan:

The Food and Drug Administration (FDA, we) completed our evaluation of GRN 001095. We received Aanika Biosciences, Inc.'s (Aanika) notice on June 22, 2022, and filed it on March 1, 2023. Aanika submitted amendments to the notice on December 12, 2022, June 2, 2023, and June 14, 2023, which included information regarding the identity, manufacturing method, stability, specifications, including revised heavy metal specifications, results from additional batch analyses, clarification regarding the intended use, a revised dietary exposure assessment, and an updated literature search.

The subject of the notice is *Bacillus subtilis* ATCC AAN000002 spore preparation for use as a tracer at a level of approximately 10^6 spores/g in fruit, vegetables, grains, oils, dairy products, nuts and seeds, and seafood to track and trace these foods through supply chains.[1,2] The notice informs us of Aanika's view that these uses of *B. subtilis* ATCC AAN000002 spore preparation are GRAS through scientific procedures.

Aanika describes *B. subtilis* ATCC AAN000002 spore preparation as a beige slurry. Aanika states that *B. subtilis* ATCC AAN000002 is a non-pathogenic, non-toxigenic, spore-forming, Gram-positive, rod-shaped bacterium. *B. subtilis* ATCC AAN000002 is genetically engineered from the host strain, *B. subtilis* strain "168." Four genes[3] required for spore germination were deleted from the host strain, resulting in the germination-deficient "chassis" strain, *B. subtilis* strain "AA07." A non-functional DNA "watermark" consisting of fewer than 200 nucleotides was stably integrated into the genome of the "chassis" strain, resulting in *B. subtilis* ATCC AAN000002. The strain's identity was confirmed using whole genome sequencing and deposited in the strain

[1] Aanika states that *B. subtilis* ATCC AAN000002 spore preparation is not intended for use in infant formula, any products under the jurisdiction of the U.S. Department of Agriculture, or in foods for which standards of identity do not permit its addition.

[2] Aanika intends to produce additional non-pathogenic, non-toxigenic, germination-deficient strains of *B. subtilis* spore preparation, derived from *B. subtilis* strain "AA07," but containing a different chromosomally integrated, non-functional DNA "watermark" subject to the same manufacturing and safety standards described in GRN 001095.

[3] Four genes critical to spore germination in *B. subtilis* were deleted (*gerD*, *sleB*, *cwlD*, and *cwlJ*), resulting in a "chassis" strain of *B. subtilis* with an undetectable germination rate.

collection of the American Type Culture Collection (ATCC) in Manassas, Virginia. Aanika discusses the results of phenotypic and genotypic characterization used to confirm that any antibiotic resistance genes used during construction of *B. subtilis* ATCC AAN000002 were removed. Aanika states that *B. subtilis* ATCC AAN000002 is poorly mobilized for genetic transfer to other organisms.

Aanika describes the manufacture of *B. subtilis* ATCC AAN000002 spore preparation by fermentation of a pure culture. When logarithmic growth of the culture is achieved, the fermentation media is replaced with starvation media, which results in sporulation within 72 hours. Residual vegetative cells are heat-killed at 80 °C, followed by washing with water to remove residual media components, heat-killed vegetative cells, and cellular components.[4] The preparation is resuspended in water resulting in the final spore preparation. Aanika states that no components of the fermentation medium are allergens or are derived from allergenic sources. Aanika states that *B. subtilis* ATCC AAN000002 spore preparation is manufactured under current good manufacturing practices using approved food additives, substances that are GRAS for their intended purpose, or are otherwise appropriate for the intended use.

Aanika provides specifications for *B. subtilis* ATCC AAN000002 spore preparation that include spore count (> 10^9 spores/mL); heavy metals, including lead (< 0.05 mg/kg); aerobic colonies (no colonies); yeast and mold (≤ 300 colony forming units (CFU)/g); coliforms (< 30 CFU/g); *Escherichia coli* (< 10 CFU/g); *Staphylococcus aureus* (< 10 CFU/g); *Salmonella* serovars (negative/25 g); and *Listeria* spp. (negative/25 g). Aanika provides the results from the analyses of four non-consecutive batches to demonstrate that *B. subtilis* ATCC AAN000002 spore preparation can be manufactured to conform with the provided specifications. Aanika states that results of stability testing indicate that *B. subtilis* ATCC AAN000002 spore preparation is stable for 12 months at 4 °C and at room temperature.

Aanika estimates the dietary exposure to *B. subtilis* ATCC AAN000002 spore preparation based on the intended use level and the estimated per capita mean consumption of the intended food categories derived using the 2017-2018 National Health and Nutrition Examination Survey. Aanika estimates the dietary exposure to *B. subtilis* ATCC AAN000002 spore preparation from the intended uses to be 8.16 x 10^8 spores/person/d for the adult U.S. population.

Aanika describes the history of safe use of *B. subtilis* in human food, specifically in fermented food products, and explains that *B. subtilis* has been isolated from soil, decomposing plant matter, and from the feces of plant-eating animals, including humans. Aanika performed a literature search through May 2023 and summarizes published literature and governmental reviews that support the safety of consumption of *B. subtilis*, including strains that are closely related to *B. subtilis* strain "168," with no

[4] Aanika states that production batches are analyzed using microscopy to confirm that the preparation only contains *B. subtilis* spores, and by sequencing to confirm the identity as *B. subtilis* ATCC AAN000002. The final spore preparation is plated on rich microbiological media to confirm the absence of other viable bacterial species and vegetative *B. subtilis* cells. Aanika explains that *B. subtilis* ATCC AAN000002 is germination-deficient and is not able to generate colonies on agar plates.

serious adverse effects reported. Aanika explains that infection linked to *B. subtilis* has been reported in immunocompromised patients; however, because *B. subtilis* ATCC AAN000002 spore preparation is germination-deficient, it exists in a metabolically inactive spore state, minimizing its ability to cause infection in immunocompromised or at-risk patients. Aanika states that the species is generally regarded as non-pathogenic and non-toxigenic.

Aanika explains that a bioinformatic *in silico* safety evaluation was performed on the DNA "watermark," including the inserted sequence as well as upstream and downstream adjacent DNA sequences. Aanika's evaluation determined that the DNA "watermark" shared no homology to known and catalogued DNA sequences, including an absence of sequence homology to known peptide toxins, virulence factors, or allergens; that the insertion sites and the resulting genomic construction were well-characterized using DNA sequencing; and that no unintended modifications were reported in the strain. Aanika states that the putative polypeptide sequence was compared to comprehensive databases of polypeptide sequences and was not found to be homologous to any known hazardous proteins.

Based on the available data and information, Aanika concludes that *B. subtilis* ATCC AAN000002 spore preparation is GRAS for its intended use.

Standards of Identity

In the notice, Aanika states its intention to use *B. subtilis* ATCC AAN000002 spore preparation in several food categories, including foods for which standards of identity exist, located in Title 21 of the CFR. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Potential Labeling Issues

Under section 403(a) of the Federal Food, Drug, and Cosmetic Act (FD&C Act), a food is misbranded if its labeling is false or misleading in any way. Section 403(r) of the FD&C Act lays out the statutory framework for labeling claims characterizing a nutrient level in a food or the relationship of a nutrient to a disease or health-related condition (also referred to as nutrient content claims and health claims). If products containing *B. subtilis* ATCC AAN000002 spore preparation bear any nutrient content or health claims on the label or in labeling, such claims are subject to the applicable requirements and are under the purview of the Office of Nutrition and Food Labeling (ONFL) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety did not consult with ONFL on this issue or evaluate any information in terms of labeling claims. Questions related to food labeling should be directed to ONFL.

Section 301(ll) of the FD&C Act

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of

the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In our evaluation of Aanika's notice concluding that *B. subtilis* ATCC AAN000002 spore preparation is GRAS under its intended conditions of use, we did not consider whether section 301(ll) or any of its exemptions apply to foods containing *B. subtilis* ATCC AAN000002 spore preparation. Accordingly, our response should not be construed to be a statement that foods containing *B. subtilis* ATCC AAN000002 spore preparation, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that Aanika provided, as well as other information available to FDA, we have no questions at this time regarding Aanika's conclusion that *B. subtilis* ATCC AAN000002 spore preparation is GRAS under its intended conditions of use. This letter is not an affirmation that *B. subtilis* ATCC AAN000002 spore preparation is GRAS under 21 CFR 170.35. Unless noted above, our review did not address other provisions of the FD&C Act. Food ingredient manufacturers and food producers are responsible for ensuring that marketed products are safe and compliant with all applicable legal and regulatory requirements.

In accordance with 21 CFR 170.275(b)(2), the text of this letter responding to GRN 001095 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Susan J. Carlson -S

Digitally signed by Susan J. Carlson -S
Date: 2023.09.11 14:21:13 -04'00'

Susan J. Carlson, Ph.D.
Director
Division of Food Ingredients
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