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Aanika Biosciences, Inc.
67 35th St. Suite B-521
Brooklyn, NY 11232

Generally Recognized as Safe (GRAS) Determination

Watermarked *Bacillus subtilis* AA07-1 Spore Preparation

Submitted via the Electronic Submission Gateway

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AANIKA

Aanika Biosciences, Inc.
67 35th St. Suite B-521
Brooklyn, NY 11232

Contents

List of abbreviations

ADI	Advisable Dietary Intake
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
cGMP	current Good Manufacturing Practice
G	Gram
EDI	Estimated Dietary Intake
cEDI	cumulative Estimated Dietary Intake
EFSA	European Food Safety Authority
EU	European Union
FG	Femtogram
FDA	US Food and Drug Administration
FD&C	Food, Drug and Cosmetic Act, United States 1998a
GRN	GRAS Notice
IDF	International Dairy Federation
KO	Knockout
Mg	Milligram
MIC	Minimum Inhibitory Concentration
µg	Microgram
NGS	Next Gen Sequencing
NOAEL	No Observed Adverse Effect Level
ORF	Open Reading Frame
PPB	Parts per Billion
PPM	Parts per Million
PCR	Polymerase Chain Reaction
PG	Picogram
qPCR	quantitative Polymerase Chain Reaction
USDA	US Department of Agriculture
WGS	Whole Genome Sequence

AANIKA

Aanika Biosciences, Inc.
67 35th St. Suite B-521
Brooklyn, NY 11232

21 CFR §170.225; PART 1. GENERAL INTRODUCTION, STATEMENT AND CERTIFICATION

1.1 INTRODUCTION

Watermarked *Bacillus subtilis* AA07-1 Spore Preparation is a microbial food culture technology designed and constructed to benefit consumers in enhancing food safety, reducing food waste and ensuring compliance with food supply regulations. There is a reasonable certainty that the technology is safe for human consumption. The technology has no nutritive value, nor does it impart flavor, texture, color, or other technical functions in the food, e.g., flowability, and will be present on foods in insignificant amounts both in terms of microbial count and weight. Further, because the spores are by their nature resistant to the digestive conditions in the GI tract and the organism has been modified to ensure that it does not reproduce or enter an active vegetative state in either in the GI tract or in the environment, the technology is essentially inert.

As out lined in the US FDA *Welcome to the New Era of Smarter Food Safety*, FDA is taking an updated approach to food safety, leveraging technology and other tools to proactively create a safer and more digital, traceable food system. "Our ultimate goal is to bend the curve of foodborne illness in this country by reducing the number of illnesses."[1] The New Era of Smarter Food Safety Blueprint, announced in July 2020, outlines achievable goals to enhance traceability, improve predictive analytics, respond more rapidly to outbreaks, address new business models, reduce contamination of food, and foster the development of stronger food safety cultures.

Aanika Biosciences, Inc. (hereinafter Aanika) is taking a novel approach to participate in the overall food safety effort by developing a safe and suitable biological tool for the tracing foods from farm to fork. Aanika has developed a *Bacillus subtilis* strain, a species well-known for its non-pathogenic and non-toxigenic characteristics, that has a DNA "watermark" inserted in its chromosome, allowing the detection of the organism by existing rapid, robust genetic techniques such as real-time polymerase chain reaction (PCR) and Next Gen Sequencing (NGS). Aanika further envisions that a food producer will be able to "watermark" their foods at the farm or early production step level by addition of the watermarked strain to ensure that foods can be traced quickly and efficiently to the producer in the event of a food-associated illness outbreak.

Today, the identification of the source of a food-related disease outbreak can take weeks, if not months, of intense effort by FDA, CDC, local health officials, and farmers to identify the source of the outbreak. Because of the inherent delay in the current tracing protocols, consumers remain at risk as recalls cannot be employed until the source of the problem is identified. The Aanika technology described in this notice facilitates the epidemiological actions by public health authorities to identify the source of

[1] New Era of Smarter Food Safety Blueprint | FDA

the foodborne outbreak and seeks to reduce the tracing time from weeks to hours, making the reaction to an outbreak nearly real-time in nature and useful in reducing the number of illnesses. Further, in many cases the shelf-life of the problem foods is long over before the investigation determines the food that has caused the outbreak and the investigation is only useful over the long-term helping to identify problem regions, farms, farming practices or production issues related to the outbreak. The "watermarked" *B. subtilis* facilitates the reduction in the number of illnesses in an outbreak and a rapid response at the producer level to lower the risk of future outbreaks.

The Aanika technology is not the software-based solution that FDA envisioned when setting the food traceability goal, but the wedding of the biological tagging of foods with IT tools will greatly enhance the interests of the food industry and the FDA in enhancing food safety.

The Aanika technology could play a significant role in reducing food waste through supply chain management activities. Knowing how long fresh foods are in the supply chain would be a significant boon to producers and retailers. Often retail grocers tag fresh vegetables and produce, when possible, upon arrival in stores, but there is no system to tag the products at the point of origin. Aanika envisions that the "watermark" will act like a barcode for foods that are sold in bulk or as part of a packaged product. Such barcodes could be managed in a manner similar to the barcodes in common use on packaged products.

Other applications of the technology could include use as country-of-origin identifiers. It is a well-recognized problem in international commerce that the country of origin of agricultural products may be disguised by passing the products through intermediate markets before importation into the US, thereby skirting regulatory and trade controls. In summary, the need for this product spans the food safety, food waste and product traceability landscape which are priorities for US regulatory authorities.

1.2 EXEMPTION FROM PRE-MARKET APPROVAL

Pursuant to 21 CFR Part 170, subpart E, Aanika Biosciences Inc. submits a Generally Recognized as Safe (GRAS) notice and claims that the use of Watermarked *Bacillus subtilis* AA07-1 Spore Preparation is Generally Recognized as Safe under the conditions of its intended use and is, thereby, exempt from statutory premarket approval requirements.

1.3 NAME AND ADDRESS OF THE NOTIFIER

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

1.4 COMMON OR USUAL NAME OF THE SUBSTANCE

Watermarked *Bacillus subtilis* AA07-1 Spore Preparation; also referred to as AA07-1 in this GRAS notice.

1.5 INTENDED CONDITIONS OF USE

The Watermarked *Bacillus subtilis* AA07-1 Spore Preparation is intended for use in the production, storage, handling and shipping of the foods for the sole purpose of tracking and tracing foods through supply chains. The use in tracking food is intended to assist food manufacturers, health authorities, and regulatory agencies in their efforts to prevent or limit food related illnesses by becoming an integral part of food companies record keeping requirements under the proposed rule "Requirements for Additional Traceability Records for Certain Foods" under FSMA, which is a key element of FDA's New Era of Smarter Food Safety Blueprint[2]. Watermarked *Bacillus subtilis* AA07-1 Spore Preparation will play a role providing a Key Data Element (KDE) in documenting Critical tracking Events for a food. For example, a leafy green grower may have KDEs related to where the produce is grown, shipping records, etc. Adding a KDE providing for the application of Watermarked *Bacillus subtilis* AA07-1 Spore Preparation would allow for the tracking of a single head of lettuce as part of a shipping lot, providing traceability once boxes are opened at processors or retail outlets.

The use of Watermarked *Bacillus subtilis* AA07-1 Spore Preparation may limit the impact of food-related illnesses when they do occur by allowing health authorities to identify the food responsible for the outbreak and to identify the supplier of the identified food rapidly in a timeframe that may save lives and limit the scope of needed recalls to the foods in question rather than the very destructive practice of advising avoidance of an entire category of food.

The use of Watermarked *Bacillus subtilis* AA07-1 Spore Preparation may also be used in optimizing supply chains and to assist in limiting economic adulteration of foods by establishing the identity and origins of a food.

The spores will transit the GI tract of consumers without entering the vegetative and population growth phases of the organism's life cycle, thereby having no effect on consumers during passage through the GI tract.

Target foods include:

- Leafy greens such as lettuce, spinach and kale
- Grains such as rice, wheat and corn
- Oils such as palm, olive and coconut
- Dairy products such as milk, cream, butter and cheese

The Watermarked *Bacillus subtilis* AA07-1 Spore Preparation is not intended for use in infant formula or USDA regulated foods.

The intended addition of Watermarked *Bacillus subtilis* AA07-1 Spore Preparation is approximately 10^6 spores / g, which results in an Estimated Dietary Intake (EDI) of approximately 4 x10^8 spores per day (see

[2] FSMA Proposed Rule for Food Traceability | FDA

Section 3). Since spores are stable under envisioned storage conditions and no overage is needed to protect against loss during shelf life. The watermarked spores are enumerated by phase contrast microscopy rather than colony forming units (CFUs) since the spores do not germinate at detectable levels on agar plates and colonies are not formed.

1.6 BASIS FOR GRAS DETERMINATION IN ACCORDANCE WITH 21 CFR §170.30(B)

This GRAS determination is based upon scientific procedures in accordance with 21 CFR §170.30(b).

1.7 AVAILABILITY OF INFORMATION FOR FDA REVIEW

Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times (customary business hours) at a specific address set out in the notice or will be provided to FDA upon request (electronic format or paper).

1.8 DISCLOSURE AND CERTIFICATION

1.8.1 FOIA (Freedom of Information Act):

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA (Freedom of Information Act).

1.8.2 Trade secret or confidential

This notification does not contain any trade secret or confidential information.

1.8.3 Information included in the GRAS notification:

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Aanika Biosciences, Inc. and pertinent to the evaluation of the safety and GRAS status of the use of W 1 Spore Preparation.

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Kevin O. Gillies
Head of Regulatory and Scientific Affairs
Aanika Biosciences, Inc.

21 CFR §170.230; PART 2 - IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

2.1 IDENTITY OF THE GRAS ORGANISM

The subject of this notification is Watermarked *Bacillus subtilis* (*B. subtilis*) AA07-1 Spore Preparation (hereinafter AA07-1), a suspension in water of a gemination-deficient spore preparation of a genetically

modified strain of *B. subtilis* produced via submerged fermentation, that is to be applied to foods. AA07-1 has been engineered to delete genes required for spore germination and to contain a non-functional DNA watermark consisting of fewer than 200 nucleotides integrated into its genome. The watermarked strain was derived from *B. subtilis* strain 168. The complete genome sequence of the watermarked strain has been determined and the strain is 99.9% identical to strain 168 differing only in very small DNA sequence changes related to targeted deletion events and the addition of watermark DNA described herein. The strain has been deposited with the American Type Culture Collection (ATCC) as AAN000002.

2.2 SAFETY OF *BACILLUS SUBTILIS* AS A HOST SPECIES FOR GENETIC MODIFICATION OF STRAINS TO BE USED IN FOOD

Bacillus subtilis is comprised of numerous strains of aerobic, motile (in the vegetative state), Gram-positive, rod-shaped, spore-forming bacteria that have been demonstrated to be non-pathogenic and non-toxigenic, including strain 168 and its derivatives which are considered to be the model systems for the species. The species is found in virtually every natural environment examined including the Gastro-Intestinal (GI) tract of vertebrates, including humans (Logan, 2004 and Priest, 1993). The organism is generally found in the spore state in nature and only enters the vegetative state under limited, nutrient-rich conditions. Although, acknowledging the seemingly ubiquitous presence in the environment, Earl *et al*. have raised questions as to whether finding the spores of *B. subtilis* in the environment means that the organism was growing in that particular niche, given that the organism is isolated using techniques that depend on the organism being in the spore state and spores are easily carried by wind currents (Earl, Losick and Kolter 2008). One niche that is generally agreed to be site of natural *B. subtilis* growth is on plant material or in the rhizosphere where evidence exists for growth on decaying plant material.

The presence of *B. subtilis* on plant material results in its presence in the feces of plant-eating animals, including humans (Earl, Losick and Kolter 2008) (Hong, Khaneja, et al. 2009). The number of *Bacillus* species in human feces has been estimated to be approximately 10^4 CFU/g in populations where Bacilli are commonly present in traditional foods.

While found in the human GI tract but long considered a soil microorganism, there is evidence to suggest that some *B. subtilis* isolates may be commensal residents or able to geminate and complete their lifecycles in an animal GI tract. Results from genome sequence analysis and laboratory experience suggest that *B. subtilis* has the capacity to grow anaerobically using nitrogen as an electron acceptor and ingested spores can germinate in the GI tract (Earl, Losick and Kolter 2008) (Hong, To, et al. 2009). Feeding studies indicate that some isolates of the species pass through the GI tract within a few days and thereafter no fecal presence of the test strain was evident, indicating a lack of growth/or persistence in the human intestine.[3] Leser *et al*. reported that following a 2-week feeding study in pigs, the level of *Bacillus* in the feces of the test population decreased to background levels within 1 week (Leser, Knarreborg and Worm 2007). The weight of evidence suggests that *B. subtilis* is commonly found

[3] GRN 905

in the GI tract of humans and other plant-eating animals. Supplementation does not appear to increase the GI microbial load over time.

2.2.1 History of the use of *B. subtilis* in food

The International Dairy Federation (IDF) publishes an authoritative and peer-reviewed list of microorganisms known to have a safe history of use in food (International Dairy Federation 2018). The IDF list provides a valuable tool for both the food industry and regulatory authorities when establishing the safety of microbial food cultures for use in food ; a safe history of use of microbial species is the cornerstone of the regulatory allowance for microbial food cultures world-wide. For example, when a person decides to use *Streptococcus thermophilus* in a yogurt product, regulatory authorities do not ask "which strain" of *S. thermophilus* it is in order to determine its safety profile. The presence of *S. thermophilus* in fermented milk products predates our commercial addition of the culture to foods like fermented milks or yogurt as the culture was present in such foods for millennia prior to the advent of commercial starter usage; its safety is universally recognized at the species level and is so recognized in the IDF list. *B. subtilis* is listed on the IDF list and is thereby recognized as having a safe history of use in food based upon its use in the production of Natto and other traditional Asian foods (de Boer and Diderichsen 1991). The species is also intended for use in numerous food products and dietary supplements (Hong, Duc and Cutting 2005) (Permpoopattana, et al. 2012), including at least one (1) supplement product that contains a genetically modified *B. subtilis* strain ZB183™[4,5].

The IDF expert group routinely updates the list as the microbial components of fermented foods in various countries become better characterized. The IDF list is based upon a scientific evaluation of the documented history of use at the species taxonomic level and represents an expert review of safety of the included microorganisms. Results of clinical studies and use in food provide the overwhelming evidence of safety of the organisms on the IDF list. The findings of safety in clinical trials involving premature infants for the prevention of necrotizing colitis demonstrate the safety in the most sensitive of at-risk populations (AlFaleh 2012).

In addition to the safe use in human food, *B. subtilis* has documented safe use in animal feed for various species including fish, beef cattle, pigs, and poultry (EFSA 2020). We are unaware of published reports in the scientific literature that indicate or suggest safety concerns related to feeding B. subtilis strains to animals[6].

2.2.2 Regulatory Reviews of the use of *B. subtilis* in food

US

[4] GRN 831, 905, 955, 969 (incorporated herein by reference)
[5] ZBiotics
[6] PubMed search 2017-2022 accessed January 20, 2022; found 353 papers reporting on the use of *B. subtilis* in animal feed, including EFSA safety reviews of numerous *B. subtilis* strains. There were no reports of adverse effects on the subject animals, while many of the reports indicated positive effects on the animals in the studies.

- The US FDA has issued "no questions" letters to applicants of GRNs relating to the use of *B. subtilis* as a production organism for food enzymes[7]
- The US FDA has issued "no questions" letters to applicants of GRNs relating to the use of *B. subtilis* strains as microbial food ingredients in a wide range of foods at inclusion rates of up to 10^{10} CFU/ serving and EDIs of approximately 1.8 x 10^{11} spores per day for the average adult male[8].
- The Center for Veterinary Medicine found no safety concerns for *B. subtilis* when used as a direct fed microbial in animal feed. This finding is general in nature and applies to all isolates of the species and to animal species in general, including beef, poultry, swine, and fish without restriction as to feed inclusion rates or intake.[9]

EU

- EFSA considers *B. subtilis* to be suitable for Qualified Presumption of Safety (QPS) and has so listed the species beginning in 2007 and has approved numerous strains of *B. subtilis* for use in animal feed (EFSA 2020). The QPS listing is not limited by inclusion rates or estimates of intake.

Canada

- Safe and suitable bacteria are allowed under Canadian regulations as ingredients in food[10]
- *Bacillus subtilis* ToC46 may be used as an enzyme production organism[11]
- *Bacillus subtilis* is listed on the Natural Health Products Ingredient Database as an acceptable medicinal ingredient in Natural Health Products[12]
- Numerous Licensed Natural Health Products containing *Bacillus subtilis* as a medicinal ingredient are approved[13]

Japan

- Safe history of use the production of the traditional food "natto" (de Boer and Diderichsen 1991) and designated a Food for Specified Health Use (FOSHU)[14]

[7] GRN 714, 649, 592, 579, 476, 406, 274, 205, 114, and 20 (incorporated herein by reference)
[8] GRN 905, 955, 969
[9] AAFCO (2019) Official Publication of the Association of American Feed Control Officials (AAFCO). Available at: https://www.aafco.org/Publications (Accessed: November 9, 2021.
[10] Food and Drug Regulations PART B DIVISION 1 Section B.01.010
[11] Food and Drug Regulations; Part B Foods; Division 16 food Additives; Section B.16.100 Table V
[12] Ingredient Search Results (hc-sc.gc.ca); Schedule 1 of the Natural Health Product Regulation; accessed November 9, 2021
[13] Search results (canada.ca); accessed November 9, 2021
[14] https://www.mhlw.go.jp/site_kensaku_english.html?q=Bacillus%20subtilis; Ministry of Health, Labor and Welfare, Japan; accessed November 12, 2021

Australia/New Zealand

- Numerous enzymes produced by genetically modified *Bacillus subtilis* are approved for use[15]

B. subtilis is a "workhorse organism" used in laboratory research being the primary Gram-positive model organism, and in industrial enzyme production (Zeigler *et al*., 2008), including food-grade enzymes[16]; it has a history of use as a food including dietary supplements, as animal feed (de Boer and Diderichsen, 1991) and is generally considered not toxic to humans, animals, or plants by regulatory agencies . Thus, the species is a safe and suitable source for the construction of the watermarked strain.

2.2.3 Safety of *B. subtilis* strain 168 as a host strain for use in the production of food

B. subtilis strain 168, the parent strain for chassis strain[17] AA07, is an isolate of *B. subtilis* that is a well-known laboratory strain auxotrophic for tryptophan that has been in common laboratory use for forty years. Strain 168 was derived from a naturally occurring strain via chemical mutagenesis and its genome has been sequenced (NCBI RefSeq: NC_000964.3). *In silico* genome sequence analysis indicates that *B. subtilis* 168 is a "legacy strain" derived from *B. subtilis* Marburg (ATCC 6051T), the type strain of both *B. subtilis* and *B. subtilis* subsp. *subtilis* (Zeigler et al., 2008) (Kunst et al. 1997).

B. subtilis strain 168 is widely used as a research tool and is considered a Bio Safety Level 1 organism by NIH indicating that the strain is non-toxigenic and non-pathogenic and poses little risk to healthy adults[18]. *B. subtilis* strains used in industrial enzyme production and for food ingredient use in the US market are derived from or are closely related to strain 168 as documented in GRAS notices 714, 905, and 955. Both strain MB40 (GRN 955) and strain SG188 (GRN 905), are greater than 98% similar to strain 168, based upon New Generation Sequencing (NGS) genome sequence comparison. *B. subtilis* BEST 195, a strain used for Natto production, is also highly related to strains 168, MB40, SG188 and AA07-1 (Mayumi, et al. 2014) .

Since the genome sequence of strains168, MB40, SB188 and BEST 195 are highly related *B. subtilis* isolates, they may be considered substantially equivalent and the data substantiating the safety of one strain applies to the other highly related strains. Therefore, it is not necessary to repeat the details of the comprehensive safety determinations by the FDA in GRNs 709, 905 and 955; however, the findings are summarized herein . We note specifically that strain MB40 has been shown to be well tolerated in humans at approximately 10^9 / CFU per day in a clinical trial and well tolerated with a No Observed

[15] **Australia New Zealand Food Standards Code – Schedule 18 – Processing aids (legislation.gov.au)**; accessed January 4, 2022

[16] Organisation for Economic Cooperation and Development, Safety Evaluation of Foods Derived by Modern Biotechnology, 1993.

[17] "…reusable biological frame where non-native components can be plugged in and out to create new functionalities…." In the case of strain AA07, AA07 is a foundation microorganism where various modifications can be made to express large numbers of identifying watermarks for use in the track and trace function.

[18] NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH GUIDELINES) April 2019; Department of Health And Human Services; National Institutes of Health

Adverse Effect Level (NOAEL) level of approximately 10^{11} CFU /kg bw/day in a rat model system (Spears, et al. 2021), equivalent to approximately 10^{13} CFU / 70kg male / day or an Advisable Dietary Intake (ADI) of approximately 10^{11} CFU/70 kg male/day.

EFSA addressed the human trials and other literature related to the safety of *B. subtilis* in 2016 and 2020 (EFSA 2016) (EFSA 2020) and did not report any relevant safety issues. A comprehensive literature search of publications from 2020-2021 did not identify any references to safety concerns related to *B. subtilis* 168 or closely related organisms when consumed by humans and other animals[19]. Thus, strains 168, MB40, SG188 have been demonstrated by scientific procedures to be:

- Well-characterized strains and confirmed to be *B. subtilis* isolates by phenotypic and *in silico* genomic analysis
- Non-pathogenic by virtue of being isolates of the species *Bacillus subtilis* that are non-pathogenic to humans, other animals and plants
- Non-toxigenic based upon history of safe consumption, *in silico* bioinformatic analysis and toxicological testing of strains derived from 168, MB40 and SG188, including testing in animal models and *in vitro* cell toxicity tests
- Free of genetic determinants for resistance to clinically important antibiotics by *in silico* bioinformatic analysis; and confirmed by antibiotic MIC testing
- Free of extrachromosomal elements that may facilitate horizontal gene transfer
- Free of genetic determinants for known bacterial virulence factors by *in silico* bioinformatic analysis.

Based on the publicly available information summarized above, Aanika has determined that repeating the animal toxicology studies performed with MB40 is not warranted and finds that strain 168 is a safe and suitable organism with the appropriate life cycle characteristics for use as a strain background for the development of the watermarked organism.

Following the guidance provided by Codex Alimentarius that the focus of safety assessments performed on genetically modified organisms should focus on changes to the safe and suitable host background, the four (4) AA07 deletion mutant DNA sequences inserted into the *B. subtilis* 168 plus the single watermark DNA sequence added to AA07 and putative peptides produced from the DNA are the focus of the following safety evaluation.

2.3 CONSTRUCTION OF THE GRAS ORGANISM

2.3.1 Construction of germination-deficient Strain AA07

In order to limit the potential for growth of our watermarked strain when its spores are added to foods, developed a chassis strain of *B. subtilis* with an undetectable germination rate. This germination-

[19] PubMed, National Center for Biotechnology Information (last accessed December 29, 2021); keywords: Bacillus subtilis AND safety, Bacillus subtilis AND pathogen

deficient strain (AA07) was made by deleting four genes critical to spore germination in *B. subtilis* (*ger*D, *sle*B, *cwl*D and *cwl*J) (Setlow, 2014).

The AA07 germination-deficient strain was derived from *B. subtilis* 168. *B. subtilis* 168 and four single-gene deletion mutants of *B. subtilis* 168 were obtained from the Bacillus Genetic Stock Center (BGSC)[20]. Strains BKE01550 (trpC2 ΔgerD::erm), BKK01530 (trpC2 ΔcwlD::kan), BKK22930 (trpC2 ΔsleB::kan) and BKE02600 (trpC2 ΔcwlJ::erm) are part of a large well-documented knockout library of *B. subtilis* strain 168 created and deposited in the BGSC by Dr. Carol Gross University of California at San Francisco. Each of the four strains has had a single gene required for spore germination deleted and replaced with a cassette containing a gene conferring either kanamycin resistance or erythromycin resistance flanked by lox71 and lox66 sites and primer sites that can be used for verification of the knockout via Sanger sequencing of a PCR product (Figure 1).

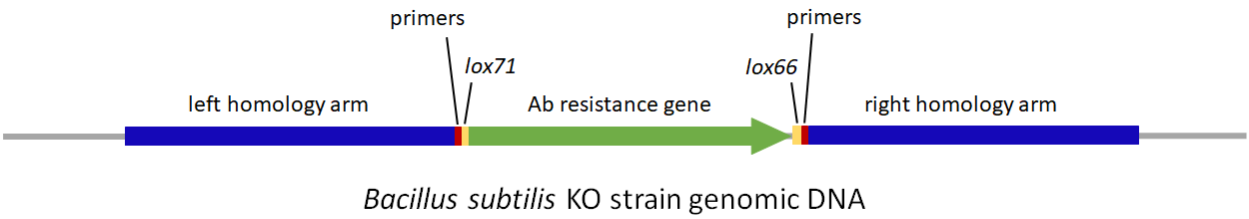

Figure 1. Diagram of germination-related gene KO sites in strains BKE01550, BKK01530, BKK22930 and BKE02600. The germination-related gene has been replaced by an antibiotic resistance gene cassette as described (5). Primer sites facilitate Sanger sequencing of PCR products and NGS.

The lox71 and lox66 sites are recognition sites for Cre recombinase, which can be used in subsequent genetic engineering steps to remove the antibiotic resistance gene within the cassette, replacing it with a lox72 'scar' region that the Cre recombinase no longer recognizes (Yan, et al. 2008). This results in a deletion mutant that no longer has antibiotic resistance but has the target gene knocked out and replaced by a nonfunctional piece of DNA that is 150bp in length and is composed of the lox72 scar and primer sites (Figure 2).

[20] https://bgsc.org/; Bacillus Genetic Stock Center, Department of Microbiology in the College of Arts and Sciences, The Ohio State University, Columbus, OH

Aanika Biosciences, Inc.
67 35th St. Suite B-521
Brooklyn, NY 11232

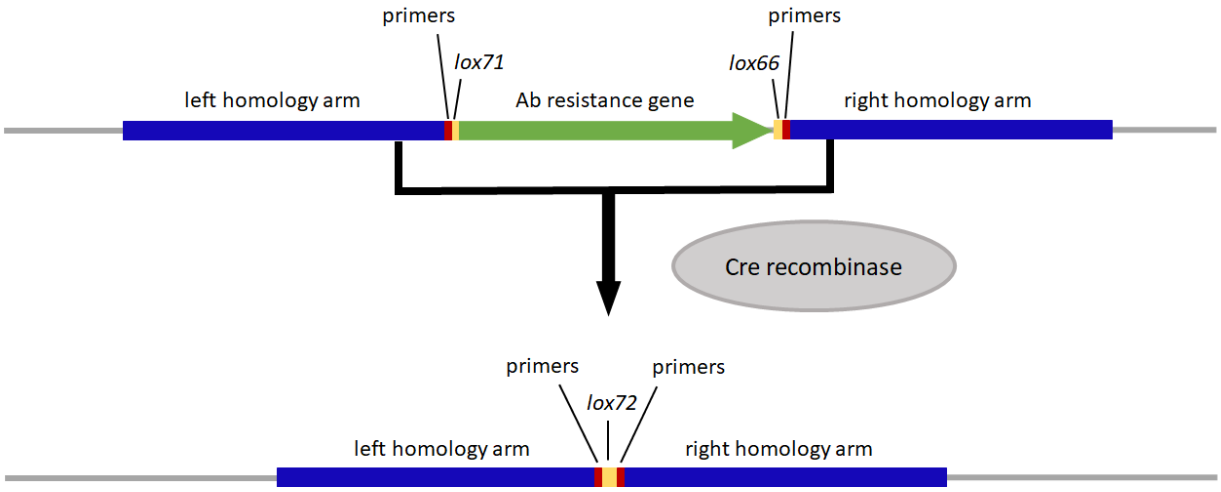

Figure 2. Diagram of Cre-loxP method of removing antibiotic resistance genes from KO strains

The first step in engineering the AA07 strain was to remove the antibiotic resistance gene from strain BKE01550 (trpC2 ΔgerD::erm) using the Cre-loxP system. Because the antibiotic resistance marker gene is flanked by lox71 and lox66, it can be removed using Cre recombinase through transformation with the plasmid pDR244 which was obtained from the BGRC and which contains genetic instructions for synthesis of the Cre recombinase protein. pDR244 contains a spectinomycin resistance gene and is susceptible to heat-cure (Yan, et al. 2008). Transformants are selected on spectinomycin-containing agar plates, incubated overnight at 42C, and then screened for absence of spectinomycin resistance to confirm loss of the plasmid. The resultant strain was designated AA01 (trpC2 ΔgerD::lox72). This system takes advantage of the low affinity of the Cre recombinase to the lox72 site that remains after the marker is removed following recognition of the lox71 and lox66 sites. This facilitates multiple deletions in the same strain. Figure 3. shows the workflow for this process.

Figure 3. Workflow for removal of antibiotic resistance genes

The workflow in Figure 4. was repeated to generate the quadruple germination deficient mutant strain: Genomic DNA was extracted from the remaining three purchased strains: BKK01530 (ΔcwlD::kan), BKK22930 (ΔsleB::kan) and BKE02600 (trpC2 ΔcwlJ::erm). To perform the addition of each knockout, genomic DNA was extracted from the strain containing the desired single knockout. The deletion cassette and the genomic regions flanking it (the 'homology arms') representing about 1000bp on either side were amplified via polymerase chain reaction (PCR). The PCR product was purified and this linear

double-stranded DNA used to sequentially transform the strain AA01 (ΔgerD::lox72) to achieve a strain with four different germination gene deletions: ΔgerD, ΔcwlD, ΔsleB and ΔcwlJ. The antibiotic resistance gene in the center of the cassette was removed following each deletion by transfection with pDR244 as described above. pDR244 was then removed by heat-cure. Antibiotic-sensitive strains were identified by comparing growth on media +/-antibiotics, and the deletions confirmed by DNA sequencing.

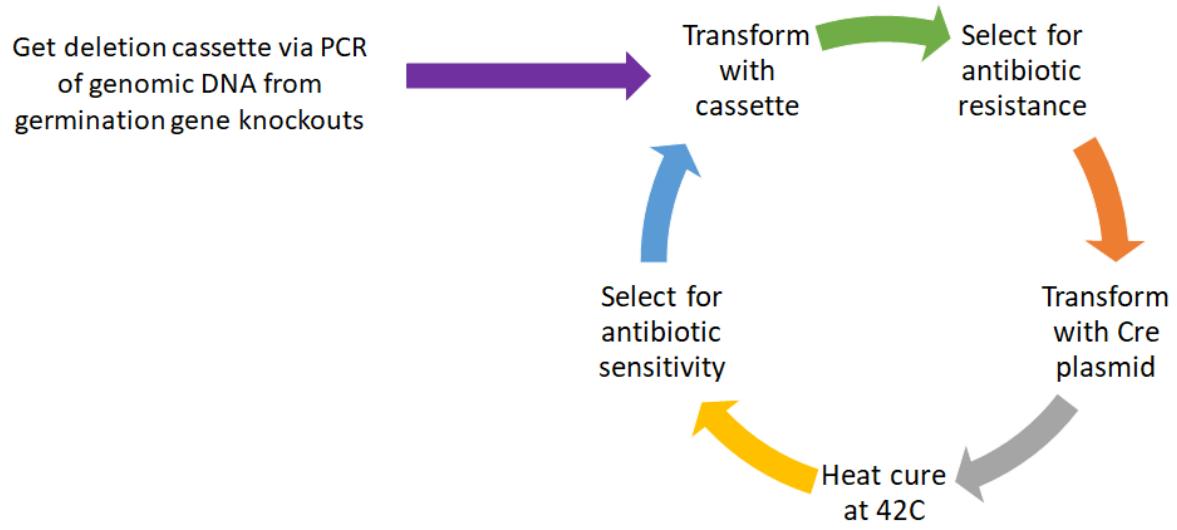

Figure 4. Workflow for creating the quadruple KO germination deficient strain AA07.

The final strain AA07 (trpC2 ΔgerD::lox72 ΔcwlD::lox72 ΔsleB::lox72 ΔcwlJ::lox72) is a tryptophan auxotroph since it is derived from strain 168, and it has four genes critical to spore germination deleted and replaced with 150bp DNA consisting of primer sites and the lox72 'scar'. Thus, AA07 can be maintained in the vegetative state, cultured on agar plates for enumeration and in liquid culture. but once the spore state is induced, the strain has reached a life cycle dead end and can no longer be grown on media for growth of *Bacillus subtilis*.

Strain	Germination rate[1]
Wild type (*B. subtilis* 168)	100 %
Single deletion mutant	0.01482 %
Double deletion mutant	0.01320 %
Triple deletion mutant	0.0024 %
Quad-deletion mutant (Chassi strain AA07)	Below detection

[1]Presence of spores was confirmed by staining with the Schaeffer and Fulton method and microscopy. Germination rates were determined from cultures with an average

of 1x10⁹ spores/mL. Numbers represent the average of at least 3 individual experiments. Germination rate: percentage of spores that generated colonies on LB agar plates after a 24-hour incubation at 37° C.

Table 1. Germination rate of AA07 and precursor strains

Table 1 shows the sequential reduction in the germination rate of spores as genes are deleted in the wild-type 168 and the subsequent mutants to produce AA07. Germination could not be detected in spore preparations of the final mutation to produce AA07. These data were corroborated by plating serial dilutions of heat treated[21] watermarked strain AA07-1 spore preparations on LB[22] medium, which is commonly used to stimulate germination of *Bacillus* spores. No colonies were observed on the LB plates, including plates containing approximately 10^9 spores indicating that the germination rate for the preparation is < 1 germination event per billion spores. These data provide strong evidence for the view that the gene deletion events virtually eliminate spore germination in the most favorable environments and are almost certainly ensuring that germination will not occur in more nutrient-limited environments such as the human GI tract or in the environment.

2.3.2 Construction of watermarked strain AA07-1

Strain AA07-1 was constructed using the quadruple germination gene knockout strain *B. subtilis* AA07 as its base as described in Section 2.3.1. A strain-specific synthetic watermark DNA was inserted into the AA07 genome at the site of *lysA*, a gene coding for meso-2,6-diaminopimelate decarboxylase, an enzyme critical for the synthesis of the amino acid L-lysine . The resulting watermarked strain AA07-1 is auxotrophic for both tryptophan and lysine. Again, the strain can only be grown on culture medium in the vegetative state. Once the sporulation state is induced the strain cannot be cultured or return to the vegetative state.

The method of construction of strain AA07-1 is graphically displayed in Figure 5. A linear piece of DNA containing a watermark cassette flanked by homology arms was derived using a PCR-based process. Assembly PCR was used to join a kanamycin resistance gene flanked by watermarks BC1 and BC2 (the "watermark cassette") to left and right homology arms (homologous to 1kb 5' and 3' regions flanking the *lysA* gene). The resulting linear DNA was gel-purified and used to transform strain AA07, and transformants selected on kanamycin containing agar plates. The *lysA* gene is replaced with the watermark cassette by homologous recombination in a manner similar to the germination-related gene knockouts described above in section 2.2.1, creating an additional auxotrophy for lysine beyond the tryptophan dependence of the parent 168 strain. Cre recombinase is then used to delete the KanR gene as shown in Figure 5. via transformation with pDR244 followed by heat-cure at 42C as described in the workflow diagrammed in Figure 3. The BC1 and BC2 regions contain primer sequences that can be used for Sanger sequencing of PCR products, isothermal amplification techniques, and NGS. All DNA sequences added to the organism were first screened as described in Section 2.2.3 below.

[21] Heat treatment eliminates any residual vegetative cells carried over from the fermentation step.
[22] Luria Broth; Luria Broth (LB) and Luria Agar (LA) Media and Their Uses | ASM.org

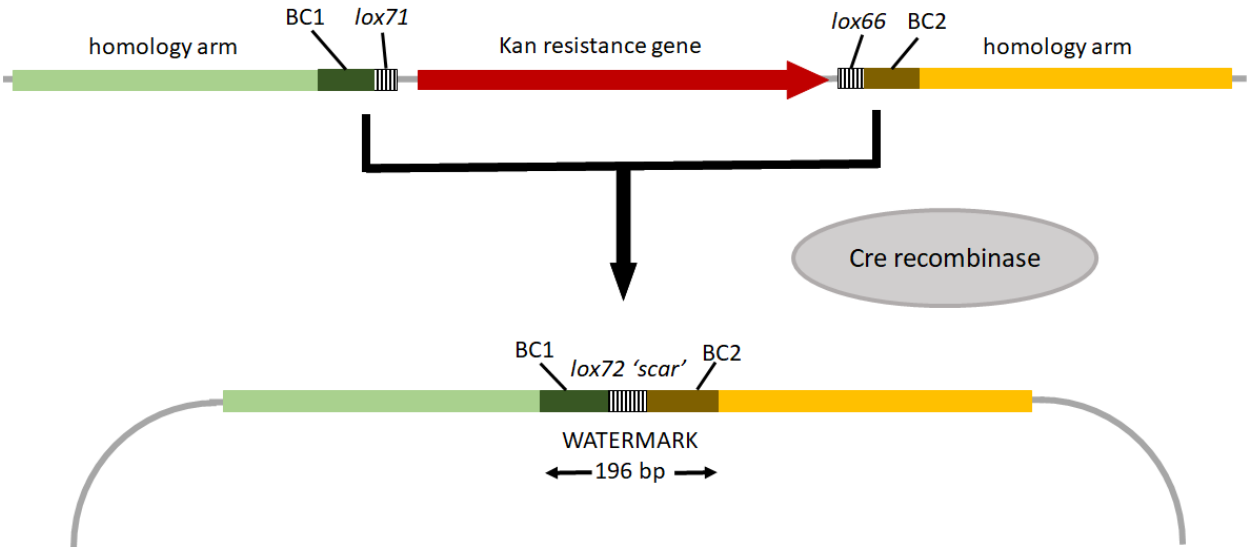

Figure 5. Addition of the watermark DNA to the genome of strain AA07.

The resulting strain AA07-1 (while in the vegetative state) was screened on agar plates +/- antibiotics to ensure that it was not resistant to erythromycin, kanamycin or spectinomycin. Additionally, whole genome sequence analysis confirmed the absence of these sequences in AA07-1.

2.3.3 Stability of introduced genetic sequences

All introduced genetic sequences are stably integrated into the *B. subtilis* chromosome rendering it poorly mobilized for genetic transfer to other organisms. Opportunity for strain mutation is minimized through limiting the time in the vegetative state. Aanika maintains a collection of validated seed cultures grown from single-colony isolates and stored at -80°C. Strain identity of all seed stocks is confirmed by whole genome sequencing. Propagation, preservation and storage are monitored and controlled.

2.3.4 safety of the introduced genetic sequences and their putative peptide products

As strain 168 is recognized as non-toxigenic and non-pathogenic and strains closely related to 168 have been shown to be non-toxigenic and well tolerated in acute animal toxicological studies and human feeding studies and clinical trials, Aanika has analyzed only those aspects of the organism that have been changed in the strain development process. We note that complete genome sequencing has been employed to ensure that only the desired modifications have been made and that their respective locations and structure are known.

There are five genomic regions that differ in strain AA07-1 compared to parent strain 168 and the chassis strain AA07 derived from 168 (described in 2.3.2 and 3). Four open reading frames (ORFs) have been deleted in 168 to produce AA07 (Δ*gerD,* Δ*cwlD,* Δ *sleB,* Δ*cwlJ*) and 159-nucleotide markers have been added to each locus to simplify the identification of the mutants. The deletion of ORF at *lys*A and

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the insertion of a 196-nucleotide watermark in AA07 yields strain AA07-1 with five modified ORFs (*gerD, cwlD, sleB, cwlJ and lys*A). The loci for the five gene knockout regions are shown in Table 2.

Gene	Genomic Location	length of ORF (nts)	length of protein (aas)	Function
cwlD	156,612 - 157,325	714	237	spore cortex peptidoglycan synthesis
cwlJ	282,469 - 282,897	429	142	peptidoglycan hydrolysis, cortex lysis
sleB	2,399,152 - 2,400,069	918	305	peptidoglycan hydrolysis, cortex lysis
gerD	158,515 - 159,072	558	187	clustering of germinant receptors
lysA	2,436,947 - 2,438,266	1320	439	diaminopimelate decarboxylase

Table 2. Genomic regions deleted in strain AA07-1

In all five knockout regions the entire open reading frame of the deleted gene is removed except for the start and stop codons. This was done rather than removing the entire gene (promoter, ribosome binding site and open reading frame) in order to avoid affecting the expression of other genes that may potentially be part of the same operon. This approach is a highly conservative strategy that ensures that only the modifications described in this notice result from described strain construction.

The knockout and gene insertion process includes the insertion of watermark DNA sequences (primers) plus the lox scar and universal primers present at each deletion/insertion locus. Although Aanika does not have evidence for the transcription/translation of the transformed knockout regions, i.e., knockout region plus the described DNA inserts, because of the construction, the primer and lox72 scar regions in all engineered sites could theoretically be transcribed and translated using the promoter and ribosome binding sites that originally controlled expression of the deleted ORFs. The five short peptides that could theoretically be produced are shown in Table 3.

Gene KO site	Peptide length (aas)	mw	Peptide sequence
cwlD	51	5.6kD	MAGEKGELKNHNRPEGGKAGYRSYSIHYTNGRRGSCHCRLKLAKHRRICAL
cwlJ	51	5.6kD	MAGEKGEPDPKDAHEGGKAGYRSYSIHYTNGRRGSCHCTNTKSQTRRICAL
sleB	51	5.7kD	MAGEKGESMPPTVNEGGKAGYRSYSIHYTNGRRGSCHCKIKRQGRRRICAL
gerD	51	5.8kD	MAGEKGETYNNYTTEGGKAGYRSYSIHYTNGRRGSCHCTIQQKDNRRICAL
lysA	52	5.7kD	LAGEKGENASSVRTYEGTQTAWEGGKAGYRSYSIHYTNGREHRYGAPTSRTT

Table 3. Putative peptide sequences of encoded by watermark DNA ORFs.

Because AA07-1 differs from *Bacillus subtilis* 168 and other substantially equivalent isolates only in the five regions described Tables 2 and3, Aanika has focused the strain safety evaluation on the putative peptides listed in Table 3. The DNA is not derived from another organism that is known to present a risk to consumers and codes for no known protein. In addition, the exposure to consumers related to the consumption of the spores and, thereby the presumptive peptides, is likely to be extremely low because of the inert qualities of the spore. These conditions indicate that traditional animal toxicological studies

are unlikely to yield meaningful results related to the putative peptides and will not provide guidance for a safety assessment.

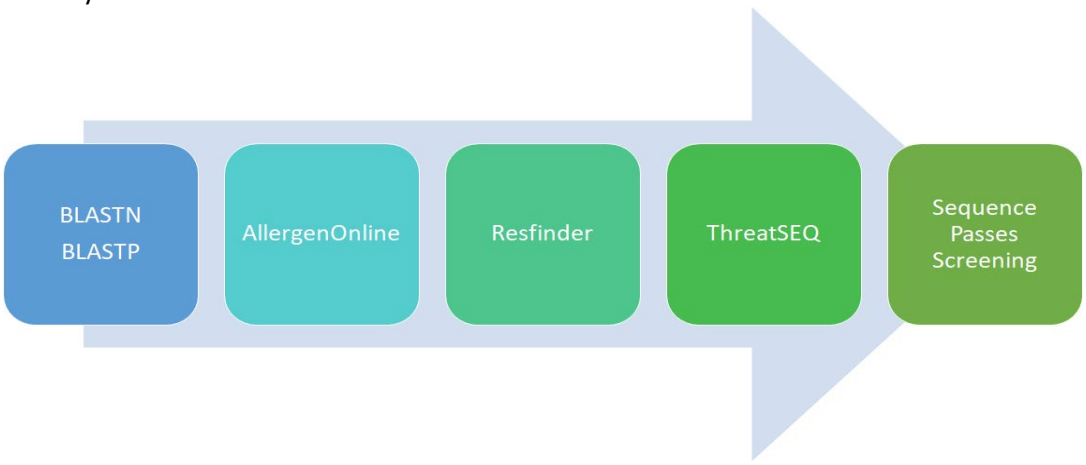

Figure 6. *In silico* peptide screening process.

The multi-step analysis of the putative peptides described above (Figure 6) involves a predictive toxicological approach employing publicly available and validated bioinformatic tools. This step satisfies the recommendation of Pariza et al. (Pariza, et al. 2015) and Codex Alimentarius (FAO 2009) for appropriate safety studies to be conducted if the inserted DNA is not derived from a food source.

To be functional in a track and trace system the inserted watermarks must be unique and distinguishable from known sequences by PCR and/or NGS. In order to ensure that the watermark sequences are unique and to qualify sequences for use as watermarks prior to strain construction, the DNA insert sequences plus 100bp upstream and downstream were analyzed using BLASTN and BLASTP[23] to determine if there is significant homology between the watermark sequence and any known nucleotide or amino acid sequence (other than the canonical lox72 'scar' region). In order for the watermark to be useful, it should not be a sequence that is present in other organism to avoid "false-positive" identification.

Once the uniqueness of the watermark DNA is determined, the putative protein aa sequences are queried using web-based tools designed for use of advanced proteonomics methodologies to identify potentially harmful peptide sequences:

1. Allergen Online employing a total sequence alignment FASTA search with a 35% homology cutoff, which is considered the most predictive method for determining potential IgE cross-reactivity for the identification of potential protein allergens

[23] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch; National Institutes of Health National Center for Biotechnolgy Information, Bethesda, MD.

(Pearson and Lipman 1988). The database is curated to contain only those proteins that have been scientifically verified to be allergens[24].

2. ResFinder [25] for the identification of potential DNA coding sequences associated with acquired antimicrobial resistance. (Bortolaia, et al. 2020)
3. ThreatSEQ a software tool developed by Battelle, based on broad-based threat identification algorithms and proprietary "Sequence of Concern Database". The database compiles more than 10,000 sequences of concern comprising 850 types of sequences of concern from 75 species of bacteria, 96 viruses, 12 eukaryotic pathogens and other risk-factor contributors. These factors include (but are not limited to) virulence factors, antibiotic resistance, immune evasion factors, human bioregulators, protein toxins and others (e.g., opioid enzyme pathways). It covers 100 percent of U.S. Select Agents and Australia Group Lists (Tier 1) and virtually all known bacterial human/zoonotic agents. It also screens against the full genomes of organisms derived from the National Center for Biotechnology Information (NCBI) database and other select agent registries around the world[26].
4. Virulence Factor Database is a BLAST-based search tool for comparative pathogenomics. The database is curated to contain only experimentally verified virulence factor protein sequences (Yang, et al. 2008)[27].

Bacillus subtilis is not known to be allergenic when used as a food or production organism for food ingredients. A survey of recent literature supports the conclusion that sensitization of consumers by *Bacillus subtilis* via the oral route is unlikely.[28] It is known that enzyme products produced by microorganisms, including *Bacillus subtilis*, can be sensitizers via inhalation or skin exposure to concentrated enzyme powders, but this risk is primarily an industrial safety concern[29] and not applicable to the food described here as the watermarked *Bacillus subtilis* spore preparation is produced as a liquid suspension.

Table 3 summarizes the results of the watermark DNA homology searches described above. Each putative peptide sequence was analyzed for sequence homology, using the most comprehensive tools for protein risk factors and none were found to have significant homology to any toxins or toxin-related gene sequences (e.g., *B. cereus* emetic toxin genes), toxin-producing peptide synthetases (e.g. cereulide synthetase), virulence factors, allergenic epitopes or other proteins of concern. We note that significant identity to sequences in *B. subtilis* AA07 precursor strain proteins (putative)were found in the

[24] http://www.allergenonline.org/; version 21 released February 14, 2021; Food Allergy Research and Resource Program, University of Nebraska-Lincoln.
[25] http://cge.cbs.dtu.dk/services/ResFinder/;
[26] https://www.battelle.org/markets/health/chemical-and-biological-threats/biosecurity-pandemic-preparedness/threatseq
[27] VFDB: Virulence Factors of Bacterial Pathogens (mgc.ac.cn)
[28] PubMed search January 6, 2021; Key words: *Bacillus subtilis* AND allergenicity; *Bacillus subtilis* AND allergen
[29] Microsoft Word - 75972776_1 (enzymetechnicalassociation.org)

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BLASTP and ThreatSEQ searches as expected, as these precursor sequences (*cwl*D, *cwl*J, *sle*B, and *ger*D inserts) have been entered into the relevant databases accessed by the two search algorithms. These results can be considered positive controls for the search protocols.

Sequence	Database	Significant Homology to Known Sequences
*cwl*D Insert		
	BLASTP	*Bacillus subtilis* AA07 precursor putative protein [1] (Appendix 7.2.8)
	Allergen Online	None (Appendix 7.2.3)
	ResFinder	None[2]
	ThreatSEQ	B*acillus subtilis* AA07 precursor putative protein(Appendix 7.2.13)
	VFDB	None (Appendix 7.2.12)
*cwl*J Insert		
	BLASTP	*Bacillus subtilis* AA07 precursor strains(Appendix 7.2.7)
	Allergen Online	None (Appendix 7.2.4)
	ResFinder	None[2]
	ThreatSEQ	B*acillus subtilis* AA07 precursor putative protein
	VFDB	None (Appendix 7.2.13)
*sle*B Insert		
	BLASTP	*Bacillus subtilis* AA07 precursor putative protein(Appendix 7.2.9)
	Allergen Online	None (Appendix 7.2.5)
	ResFinder	None[2]
	ThreatSEQ	B*acillus subtilis* AA07 precursor putative

		protein(Appendix 7.2.13)
	VFDB	None (Appendix 7.2.12)
*ger*D Insert		
	BLASTP	*Bacillus subtilis* AA07 precursor putative protein(Appendix 7.2.10)
	Allergen Online	None (Appendix 7.2.6)
	ResFinder	None[2]
	ThreatSEQ	B*acillus subtilis* AA07 precursor putative protein(Appendix 7.2.13)
	VFDB	None (Appendix 7.2..12)
*lys*A *Watermark*		
	BLASTP	*Bacillus subtilis* AA07 precursor putative protein(Appendix 7.2.11)
	Allergen Online	Yes[3] (Appendix 7.2.2)
	ResFinder	None[2]
	ThreeatSEQ	*Bacillus subtilis* AA07 precursor putative protein(Appendix 7.2.13)
	VFDB	None (Appendix 7.2.12)

[1]Homologous to *B. subtilis* strain AA07 hypothetical peptides resulting from deletion modification events at *cwl*D, *cwl*J, *sle*B, and *ger*D knockout regions and related lox scars and primers.

[2]None in addition to strain 168 and the chassis strain AA07

[3]Single positive report but not statistically significant Table 4. Analysis of putative peptides encoded by DNA inserted in germination knockout loci.

2.3.4.1 Allergen Online Results

As noted in Table 3, one positive query result was obtained from the Allergen Online sequence homology FASTA search using the *lys*A watermark putative protein sequence as the query subject (Appendix 7.2.2). The query sequence was reported to have sequence identity of 36.4% to the Fra a 1

allergen from strawberry, i.e., slightly higher that the Codex threshold of 35% sequence identity that is recommended to be an indicator of potential cross-reactivity with IgE antibodies elicited by a known allergen[30]. Based upon the Allergen Online statistical analysis algorithms, however, this presumptive identify is not significantly different from an expected random identity match with an E-value (Expectation-value) of 0.73 and a Bit value of 24.6. Based upon the query statistical analysis, Aanika considers the query result to be a false positive. This conclusion is in keeping with the Codex recommendation that levels of 35% sequence identity and higher should be subject to additional evaluation. Supporting evidence for the assertion that the query results represent a false positive, we note that the query did not return positive sequence identity to any of the *Roseace* family PR-10 protein group allergens, e.g. apple, that have known cross-reactivity to Bet v. 1 IgE and have significant high % sequence identity to the Fra a 1 allergen in strawberry nor did the query return a positive sequence homology to white birch pollen antigens that have a high sequence identity with Fra a 1 and other PR-10 proteins plus is known to have elicit significant cross-reactivity with proteins from the *Roseace*. Further, when the Fra a 1 amino acid sequence was used as a query protein, in contrast, virtually all of the known *Roseace* family allergens (PR-10 members) were returned as having sequence identity values of higher than 50%, E-values of $<2.2^{-5}$ and Bit values > 100; all indicators of high sequence identity and a high level of statistical significance of the result (Pearson, 2013).

Because the *Roseace*, including strawberry, share highly conserved sequences, we conclude that the absence of sequence homology betweeen the *lys*A wartermark and the *Roseace* allergen sequences, the indications that the search result from the Allergen Online database search showing limited identity to Fra a 1 was not statistically different than an expected random match, and the absence of significant homology to any other known allergens in the Allergen Online database that are related to Fra a 1 indicates that the *lys*A watermark is highly unlikely to be related to Fra a 1 or other known allergens and highly unlikely to elicit an allergenic response in consumers.

2.3.4.2 ResFinder Results

Although resistance to some antibiotics is a general trait of bacteria, the key question is whether the bacteria are resistant to clinically used antibiotics and do they have the capacity to transfer such resistance to other bacteria as a result of their use in food. Whole genome sequence comparison of strain 168, AA07 and AA07-1 demonstrates that the strains exhibit antibiotic resistance traits similar to other *Bacillus subtilis* that have been reviewed by FDA that are currently sold in the US market[31] and do not raise additional concerns related to the traits. On a procedural note, we do not report Minimum Inhibitory Concentrations (MIC) for antibiotic sensitivity for the spore preparation, which is common for safety assessments of microbial food cultures, as AA07-1 spores do not germinate to a measurable degree and grow on the MIC medium, making this widely used method unsuited for the purpose. We note that it is highly unlikely that strain AA07-1 can transfer such antibiotic resistance to other organisms because it is only in the food supply as the spore state and lacks cellular components, e.g.,

[30] Report of the Third Session Of The Codex Ad Hoc Intergovernmental Task Force On Foods Derived From Biotechnology (ALINORM 03/34); Codex Alimentarius Commission, Rome 30 June-5 July 2003.
[31] GRNs 905, 955

plasmids, that are likely to participate in horizontal gene transfer, and is therefore, highly unlikely to be able to participate in such transfer of antimicrobial traits.

2.3.4.3 ThreatSEQ

ThreatSEQ database is comprised of protein sequences from a wide array of pangenomic protein data related to established risk criteria. This database overlaps with the VFDB database where known bacterial virulence factor proteins are the focus as the VFDB database is incorporated in the ThreatSEQ Sequences of Concern database. The threat SEQ query, for the five (5) putative sequences in Table 3, returned no hits of significance to sequences in the database.

2.3.4.4. VFDB

The Virulence Factor Database " is an integrated and comprehensive online resource for curating information about virulence factors of bacterial pathogens."[32]. The VFDB was searched using each of the putative peptides query subjects and no hits were returned.

In summary, the host strain background 168 and the quad mutant AA07 derived from 168 have been shown to have a safe strain lineage and, thereby, safe for use as a chassis strain(s) for the introduction of DNA to produce the watermarked *Bacillus* subtilis AA07-1 strain. Following guidance provided by OECD, Codex, and peer reviewed scientific literature, Aanika has used state of the art proteonomics / pathogenomic methodology in determining that the DNA introduced into the chassis strain is unrelated to known DNA sequences archived in pangenomic databases and the putative proteins that may be produced from the inserted DNA are not significantly related to any known proteins that may present a hazard to consumers. In addition to the safety of the strain demonstrated by history of use, safety of the chassis strain due to its substantial equivalence to known safe strains and the safety of the inserted DNA products that may be produced from the inserted DNA sequences, the spores under the conditions of use described in this notice are present on the food in insignificant quantities as measured both in spore count and weight and are germination deficient, this and the fact that the spores are resistant to GI tract conditions and are likely to pass through the human GI tract unchanged make it highly unlikely that consumers will be exposed to the putative proteins described above.

Aanika envisions supplying the food industry with many watermarked *Bacillus subtilis* strains as part of the food industries tracking programs. New watermarked strains will undergo the same quality and quantity of safety evaluation as described in Section 2.3 above, including:

- Evaluation of the watermark DNA to establish its uniqueness
- Insertion of the watermark DNA into safe and suitable *Bacillus subtilis* host backgrounds based upon strain 168 using genetic techniques appropriate for the manufacture of food microorganisms
- Complete DNA sequencing of the watermarked strain

[32] VFDB: Virulence Factor Database (mgc.ac.cn)

- Bioinformatic *in silico* safety evaluation of the watermarked strain gene sequence, including the inserted sequence as well as upstream and downstream adjacent DNA sequences
- Establishing appropriate QC specifications
- Employing safe and suitable manufacturing practices in accordance with cGMP as established in 21 CFR §117.

2.4. METHOD OF MANUFACTURE

Submerged fermentation was used to produce batches of Watermarked *Bacillus subtilis* AA07-1 Spore Preparation at Aanika Biosciences consistent with current Good Manufacturing Practices for Food as provided for in 21 CFR § 117.

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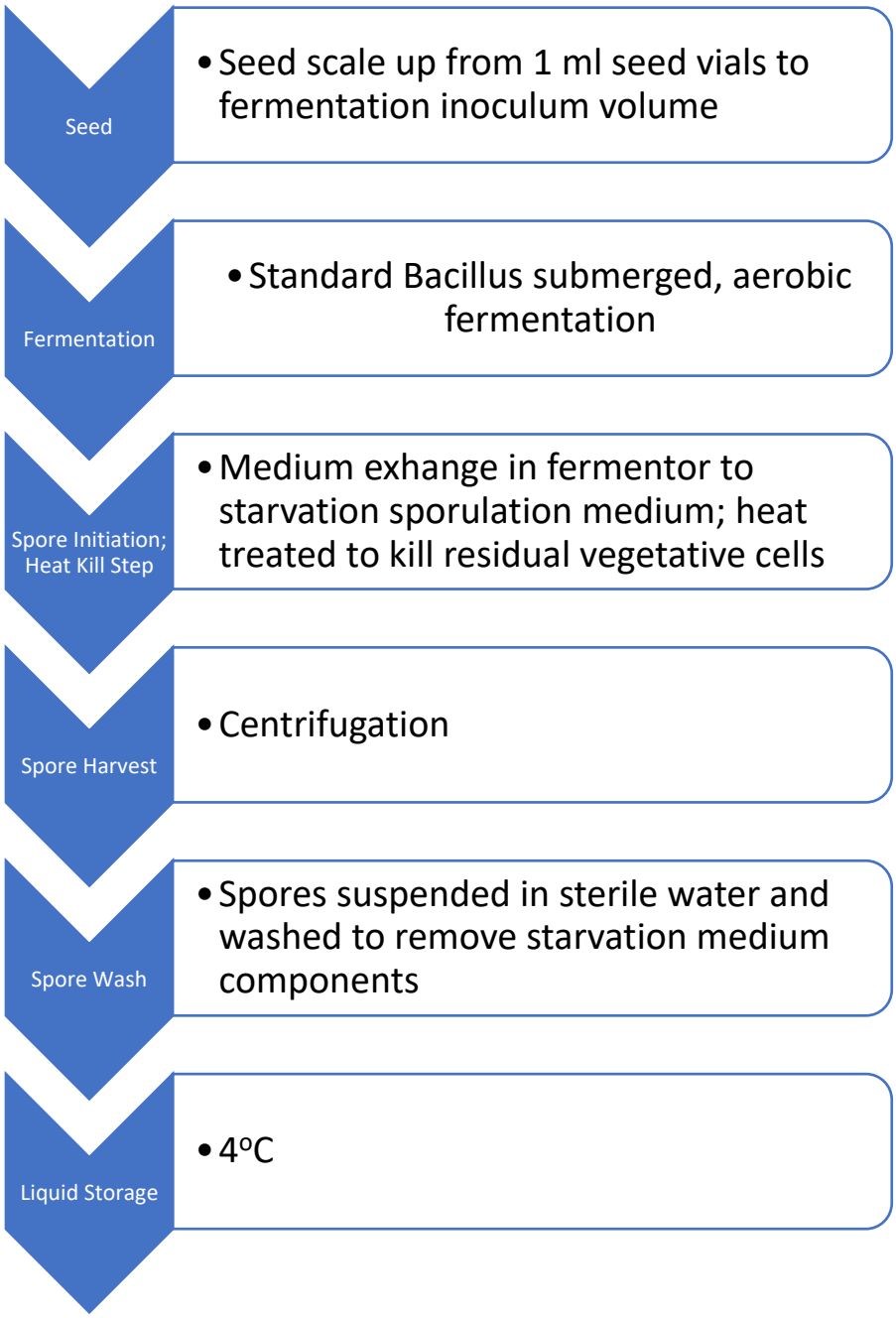


Figure 7. Flow chart of the manufacturing process, which is described in more detail in Sections 2.3.1-2.3.6 below.

2.4.1 Summary of the production process

Pure seed cultures (AA07-1 maintained in the vegetative state) are used to inoculate small batches that are grown overnight and used to inoculate larger batches grown using submerged fermentation. When the larger batches achieve logarithmic growth, the media is changed from standard LB broth to starvation media. The culture is grown in starvation media for 72h which results in sporulation. Residual vegetative cells are heat-killed at 80° C, and the spores washed repeatedly with water to remove media carryover, killed vegetative cells and cellular components before resuspending in water to create the final product.

2.4.2 Raw materials

The raw materials used in the production process are standard ingredients used in microbial fermentation and are GRAS, approved food additives, or otherwise appropriate for the use. The raw materials conform to Food Chemicals Codex (FCC) specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made consistent with FCC requirements. The raw materials are subjected to the appropriate quality control analyses to ensure conformance to specifications. No major food allergens[33] are used in the process or formulation.

2.4.3 Control of production organism *B. subtilis* AA07-1

Bacillus subtilis strain AA07-1 has been deposited in the American Type Culture Collection (ATCC) strain repository as AAN000002. Aanika Biosciences maintains a master seed stock culture in parallel as a vegetative cell glycerol stock. All seed stock cultures begin with a single colony from a streaked plate from the master seed stock. Using aseptic conditions, multiple seed stock aliquots are made from a single culture flask and stored at -80° C to limit genetic drift. Each new batch of seed stock culture is thoroughly controlled for identity and purity.

2.4.4 Spore production process

Each batch of the fermentation process is initiated with a seed vial from the validated seed stock culture collection The seed vial is used to inoculate a seed culture of the production organism, *B. subtilis* AA07-1, described in Part 2 and this seed culture is then scaled up in volume to the final production fermentation. Spores are produced by pure culture submerged fermentation of the genetically modified strain of *B. subtilis* AA07-1. Production requires two stages, initial fermentation followed by sporulation achieved by starvation accomplished via transfer of the culture to minimal media. The spore preparation is then heated to kill any remaining vegetative cells. The multi-step recovery process is designed to recover spores from the culture broth and separate them from the vegetative cells. Centrifugation or filtration is used to recover spores and vegetative cells. This is followed by washes to remove nutrient broth components.

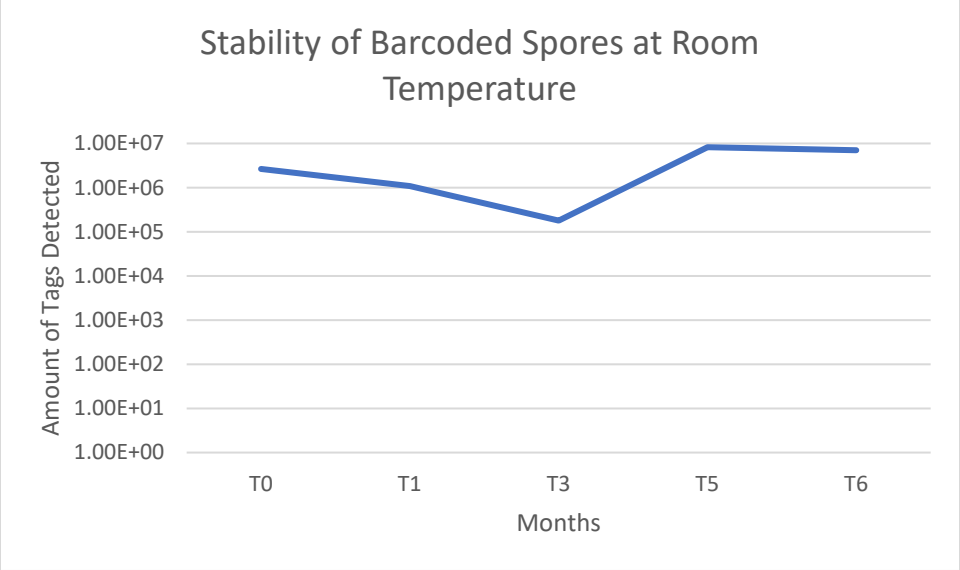
All equipment is designed, constructed, operated, cleaned, and maintained to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures

[33] Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004

are employed, and microbiological analyses are conducted to ensure absence of foreign microorganisms.

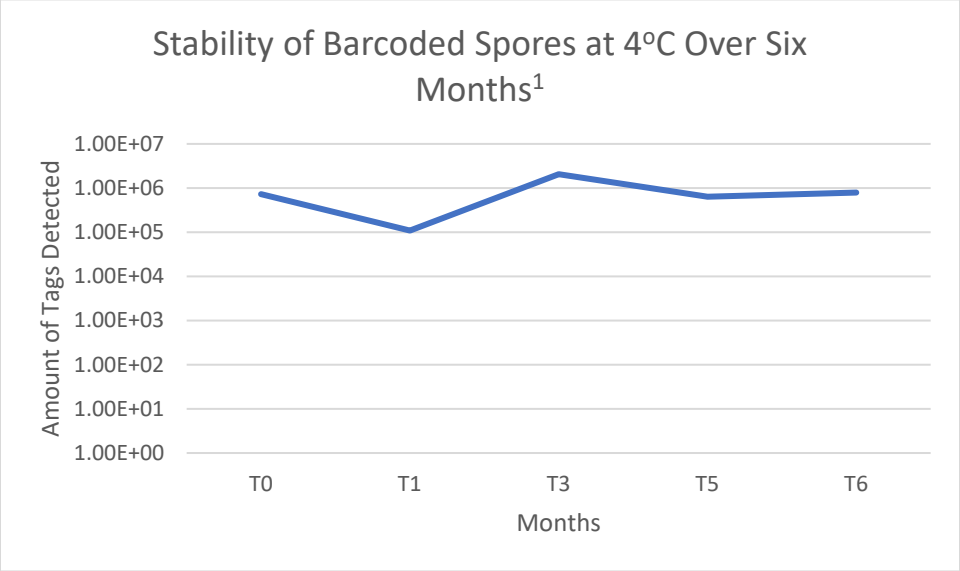
2.5 AA07-1 SPORE PREPARATION SHELF LIFE

Figures 8 and 9 show that there is essentially no change in spore count of the Watermarked *Bacillus subtilis* AA07-1 Spore Preparation after 6 months storage at room temperature and 4°C . Shelf-life studies are ongoing, and the spore stability is expected to exceed one (1) year.



[1] The construction of the strain ensures one copy of the barcode per spore thus the barcode "tag" detected equals the number of spores in the preparation.

Figure 8. Stability of the spore preparation at room temperature



[1] The construction of the strain ensures one copy of the barcode per spore thus the barcode "tag" detected equals the number of spores in the preparation.

Figure 9. Stability of the spore preparation stored at 4 °C

2.6 COMPOSITION AND SPECIFICATIONS

2. 6.1 Specifications

Food grade specifications for QC release of *B. subtilis* strain AA07-1 (Table 3) conform to food industry norms and contained in GRAS notices reviewed by FDA[34].

PHYSICAL AND CHEMICAL PARAMETERS	SPECIFICATION (ACCEPTABLE TARGET/RANGE)	TEST METHOD
Appearance	Beige Slurry	Visual
Identity	Barcode Present	DNA Sequencing[1]
Spore Count	> 10^9 / mL	Microscopy
Heavy Metals Limits	PPM	
Lead	<1	AOAC 999.10, modified[2]
Mercury	<0.5	EPA Method 7471B
Cadmium	<0.5	AOAC 999.10-2005
Arsenic	<0.3	AOAC 2011.14, Modified
Microbiological Limits		
Yeast and Mold	≤ 300 CFU/g	AOAC 997.02
Salmonella	Negative in 25 g	AOAC2013.02
Coliforms	≤ 30 CFU/g	AOAC 997.02

[34] GRNs 969, 955, 905, 831

Escherichia coli	Negative in 25 g	AOAC 991.14
Listeria	Negative in 25 g	AOAC 997.03
Staphyloccus aureus	<10 CFU/g	AOAC 2003.07

[1] Sanger sequencing of barcode region
[2] AOAC Official Methods, 21st Ed., George Latimer, J. Editor, 2019.

Table 5. Food Grade Specifications for *B. subtilis* strain AA07-1

Quality Control test results summarized in Table 4 for 3 lots of AA07-1 demonstrating that the production process described in Section 2.3 is capable of producing product to the established food grade specifications in Table 4.

PHYSICAL AND CHEMICAL PARAMETERS	SPECIFICATION	Lot P21-084A	Lot P21-084B	Lot P21-063A	
Appearance	Liquid spore suspension	Conforms	Conforms	Conforms	
Identity	Barcode Present	Conforms	Conforms	Conforms	
Spore Count	> 10^9 / mL	Conforms	Conforms	Conforms	
Heavy Metals	PPM				
Lead	< 1	0.693	0.965	<0.5	
Mercury	< 0.5	<0.025	<0.025	<0.5	
Cadmium	< 0.5	<0.5	<0.5	<0.5	
Arsenic	< 0.3	<0.5	<0.5	<0.5	
Microbiological Limits					
Yeast and Mold	≤300 CFU/g	< 10 CFU/g	< 10 CFU/g	<10 CFU/g	
***Salmonella* species**	Negative in 25 g	Negative in 25 g	Negative in 25 g	Negative in 25g	
Coliforms	≤30 CFU/g	< 10 CFU/g	< 10 CFU/g	<10	
E. coli	<10 CFU/g	< 10 CFU/g	< 10 CFU/g	<10	
Listeria	Negative in 25 g	Negative in 25 g	Negative in 25 g	Negative in 25g	
S. aureus	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	

Table 6. Quality Control test results for three (3) lots of AA07-1 spore preparation

2.7 PHYSICAL OR TECHNICAL EFFECT

B. subtilis strain AA07-1 Spore Preparation functions solely as a tool for the tracking and tracing of a food through the supply chain and has no discernable physical or technical effect (making no impact on the weight, appearance, flavor, color, texture or shelf-life) on the food nor it is it intended to have any discernible effect on the consumer of foods containing the strain.

The spores are essentially inert to digestion and, because of strain construction to remove genes required for germination, the strain does not increase in number in the GI tract. In addition, based upon

experimental data in pigs (Leser, Knarreborg and Worm 2007), the strain whether in the spore or vegetative states are likely to be transient microbes, making no contribution to the overall gut microflora.

2.8 SUMMARY

Sections 2.1. through 2.8 describe in detail the analysis of the base organism 168, the chassis strain AA07 and the modifications made to strain 168 to produce the watermarked strain AA07-1. *Bacillus subtilis* 168 has been shown to be a safe and suitable host strain for the development of the watermarked strain AA07-1 and the modifications leading to AA07-1 have been exhaustively analyzed to insure with reasonable certainty the safety of the resulting strain. Because Aanika envisions the generation of many watermarked strains to be used in or on foods to provide unique identifiers of the food, it will employ the above safety evaluation for all newly created strains.

21 CFR §170.235; PART 3 – INTENDED USES AND ESTIMATED DIETARY EXPOSURE

The *B. subtilis* watermarked spores are intended to be added to foods for the sole purpose of tracking and tracing foods through supply chains. The spores have no technical or functional effect in the food. The spores will transit the GI tract of consumers without entering the vegetative and population growth phases of their life cycle.

Target foods include, but are not limited to:

- Leafy greens such as lettuce, spinach and kale
- Grains such as rice, wheat and corn
- Oils such as palm, olive and coconut
- Dairy products such as milk, cream, butter and cheese

The spores will be added at approximately 10^6 spores per gram of food. At this level the spores are present at exceedingly low amounts (by weight) based on the following calculation (using the weight of a *Bacillus* cell as an approximation for the weight of a single spore) and are present at orders of magnitude below the uses described in GRAS Notices 969, 955, 905 for other *Bacillus subtilis* strains based upon estimated CFU addition.

The Estimated Dietary Intake for the relevant food categories is
Based on US Department of Agriculture (USDA) intake estimates using the What We Eat In America (WWEIA) 2007-2008 dietary component of the National Health and Nutrition Examination Survey (NHANES).[35]

[35] Mean Amounts of Retail Commodities Consumed per Individual1, Estimated From Dietary Intake Data, by Gender and Age, in the United States, WWEIA, NHANES 2007-2008

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Given:

1. The approximate weight of a single *Bacillus* cell is 150 femtograms or $1.5x10^{-13}$grams (Burg, et al. 2007) and will be used as an estimate of spore weight[36]
2. 10^6 spores per gram of food

Then:

10^6 spores per gram of food x 0.15pg/spore = $1.5x10^5$ pg of spores per gram of food

$1.5x10^5$ pg of spores per gram of food = 0.15 µg of spores per gram of food = 150 ppb

Thus, on a purely mass inclusion basis, the presence of the spores presents an insignificant amount on the final food.

When compared with the Estimated Dietary Intake (EDI) envisioned for other *B. subtilis* strains in GRAS notices that have been reviewed by FDA and where "no questions" letters have been provided to submitters (approximately 10^{10} to 10^{11} spores per day), the number of spores ingested in the above uses is likewise, insignificant.

Leafy greens such as lettuce, spinach and kale

21 g of leafy greens consumed per day for males and females 2 years and older
10^6 spores per gram; 21 g x 10^6 spores per gram
2.1×10^7 spores per day per persons 2 years and older

Grains such as rice, wheat and corn

117 g of grains consumed per day for males and females 2 years and older
10^6 spores per gram; 117 g x 10^6 spores per gram
1.2×10^8 spores per day per persons 2 years and older

Oils such as palm, olive and coconut

20 g of oils consumed per day for males and females 2 years and older
10^6 spores per gram; 20 g x 10^6 spores per gram
2.0×10^7 spores per day per persons 2 years and older

Dairy products such as milk, cream, butter and cheese

288 g per day for males and females 2 years and older
10^6 spores per gram; 288 g x 10^6 spores per gram
2.9×10^8 spores per day per persons 2 years and older

[36] A conservative estimate given that spores contain less water than vegetative cells due to the replacement of water by dipicolinic acid (Setlow, 2006).

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Based upon the data for EDI from the individual good categories above, the Cumulative EDI is:

446 gram of food/person/day x 10^6 spores per gram = 4.46 x 10^8 spores/person/day

The cumulative EDI of spores is highly conservative as it assumes that the spores will be added to all foods in the category, which is unlikely for any food ingredient use. In addition, consumers are likely to wash some products prior to consumption thus reducing the number of spores on the food, e.g., leafy greens. We also note that the cumulative EDI is approximately 0.1% of the EDI envisioned for other uses of *B. subtilis* spores in the food supply[37].

The low cumulative EDI indicates that the uses envisioned in this notice are unlikely to materially increase consumers exposure to *B. subtilis*. The actual exposure to consumers is likely to be far lower for the watermarked strain than these calculations suggest compared to other *B. subtilis* strains in the food supply as the Aanika strain development strategy of deleting germination genes means that the strain likely does not reproduce and increase in number on or in food or in the GI tract given that <1 spores are likely to germinate from a daily exposure of approximately 10^9 spores (see Table 1). Thus, the cumulative EDI and mass calculations above are the maximal amounts to which consumers will be exposed unlike other *B. subtilis* spores in the food supply that have not similarly been constructed and, thus, may increase in number in foods or in the GI tract depending on the uses.

[37] GRNs 969, 955, 905, 833

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21 CFR §170.240; PART 4 - SELF-LIMITING LEVELS OF USE

The level of use of the technology is limited by the amount necessary to be detected in or on the food. As there are no functional or technical advantages to be gained from adding the watermarked strain, food producers will likely use the minimum amount of spore preparation that allows for detection.

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21 CFR §170.246; PART 5 - COMMON USE IN FOOD BEFORE 1958

This part does not apply.

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21 CFR §170.250; PART 6 - NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The information summarized in the following sections is the basis for our determination of general recognition of safety of the AA07-1 watermarked spore preparation and Aanika Biosciences' conclusion that the technology is GRAS for the uses described when manufactured in accordance with cGMP. To conclude that the watermarked spore preparation is reasonably certain to be safe for use in food, we have rigorously followed the recommendations of Pariza et al. (Pariza, et al. 2015) outlined in decision tree format (Appendix 1). Thus, our safety evaluation summarized in Part 6 includes an evaluation of the *B. subtilis* AA07 chassis strain, the introduced DNA, and the spore form of *B. subtilis*. We note that the data and information cited in this notification is publicly available and does not contain any data or information that is exempt from disclosure under the FOIA.

6.1. *B. SUBTILIS* SAFE HISTORY OF USE IN FOOD

The soil and plant living saprophyte *Bacillus subtilis* is recognized as non-pathogenic and non-toxigenic for humans, animals and plants (Priest 1993)[38]. It is classified as a Risk Group 1 organism according to the National Institutes of Health Guidelines for Research Involving Recombinant Molecules and is generally considered to be non-pathogenic and non-toxigenic since the definition of Risk group 1 organisms is that they are not associated with disease in healthy adult humans.

The microorganism is commonly present in foods eaten by humans and animals as an environmental component and has been consumed in large quantities when eating the Japanese food natto (de Boer and Diderichsen 1991) and other fermented vegetables in Asia and this consumption is the basis for the listing on the IDF Inventory of Microorganisms with a Documented Safe History of Use in Food. *B. subtilis* is used as a food ingredient in compatible foods and in dietary supplements for humans, and as a direct fed microbial in animal feed.

Industrial strains belonging to the *Bacillus subtilis* species have been used for decades in the production of enzymes, and more than a decade as recombinant organisms to produce a variety of bio-industrial products like food grade enzymes, vitamins, antibiotics, and additives (Schallmey, Singh and Ward 2004).

B. subtilis strain 168, from which the chassis strain AA07 is derived, is a tryptophan auxotroph (trpC2) and therefore requires the addition of tryptophan to the growth media , including media containing acid-hydrolyzed protein components such as casein. It is widely used as a safe laboratory organism in genetic engineering. Strains highly related to 168 (>98% by genome sequence homology analysis) are used in food enzyme production and as food ingredients whose uses have been reviewed by FDA and "no questions" letters have been issued to the submitters of the relevant GRAS notices.

[38] EPA Final risk Assessment of Bacillus subtilis; February 1997.

6.2 SAFETY OPINIONS FROM REGULATORY BODIES

Regulatory authorities, including US, EU, Canada, Australia and New Zealand, have concluded that *B. subtilis* is safe for use in food, recognizing that the species is non-pathogenic and non-toxigenic with a safe history of use in food. EFSA has listed *B. subtilis* on the QPS list since 2007 and has reviewed that listing as a part of the Agency's ongoing updating of QPS status, most recently in 2019. Health Canada has authorized Natural Health Products containing *B. subtilis* strains. FSANZ (Food Safety Australia New Zealand) has authorized health claim labeling for *Bacillus subtilis* DE111 in food.

The US EPA has approved numerous *B. subtilis* strains for use as soil inoculants and biopesticides for use on food crops in recent years as the organism has been shown to inhibit the growth of saprophytic spoilage bacteria on plants. The EPA safety review[39] notes specifically that the organism is likely to be consumed as a result of the application to vegetable crops and specifically concludes that the overwhelming scientific evidence for the safety of the use is the basis for the approval.

Numerous genetically modified *Bacillus subtilis* strains are used to produce enzymes, vitamins, and active pharmaceutical ingredients, globally.

6.3 SAFETY OF THE *B. SUBTILIS* CHASSIS STRAIN AA07

An evaluation of the genetically modified strain AA07 embodying the concepts out lined by IFBC in 1990 (IFBC 1990) , the OECD in 1993 (OECD, 1993), ILSI Europe 1996 (ILSI Europe April 1997), FAO/WHO in 1996 (FAO/WHO 1996), Pariza and Johnson in 2001 (Pariza and Johnson, 2001), Pariza et al. 2015 (Pariza, et al. 2015), and Sewalt, *et al*. (Sewalt, et al. 2016) demonstrates the safety of this genetically modified strain. The components of this evaluation include the identity of the host strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain are given in Parts 2 and 3. By extension, the AA07 strain may be used as a "chassis" strain for future modifications for various functionalities.

B. subtilis 168 is one of the most studied Gram-positive bacteria as it has been a model system for the study of Gram-positive bacteria for over 40 years. The strain is also utilized in the development of industrial and food enzyme preparations which have been the subject of safety evaluations and GRAS Notices to the FDA. The safety of the use of 168 and its derivatives in food have further been demonstrated by toxicological testing of the highly related MB40 strain and feeding studies and human clinical trials. The chassis strain AA07 is derived directly from strain 168 by the sequential deletion of four (4) genes that are known to be required for germination of *Bacillus* spores. Each deletion event employs standard methods for the deletion of the target gene ORF and replacement by a synthetic DNA sequence to mark the deletion site and to provide a recognition sequence for the deletion. The deletion / insertion events are well-characterized by whole genome sequencing and have been demonstrated to be site-specific with no unintended chromosomal modifications. The deletion strategy was chosen to

[39] EPA Final risk Assessment of *Bacillus subtilis*; February 1997.

maintain operon structures ensuring that no unintended pleotropic effects would be created, e.g., frame shift ORFs, by the deletions.

AA07 is derived from 168 and, thereby, substantially equivalent to the legacy strain and very closely related to strains MB40 and SG198, which are the subject of GRAS notices to the US FDA and "no questions" letters have been provided to the submitters by the agency. Strains MB40 and SG198 and, by virtue of the close relationship between the 168 derivatives, AA07 have been shown to be non-toxigenic, lack known bacterial virulence factors, are sensitive to antibiotics used in clinical treatment of disease, show no evidence of acquired antibiotic resistance and lack cellular mechanisms known to facilitate horizontal gene transfer.

Aanika concludes based upon the information provided that the *Bacillus subtilis* strain AA07 is a safe and suitable chassis strain for development of spore preparations to be used in food.

6.4 SAFETY OF INTRODUCED WATERMARK CASSETTES AND THEIR PUTATIVE PROTEIN PRODUCTS

Five gene deletions and subsequent introduction of DNA sequences that can be used for strain identification and tracking (watermarks) have been determined to be safe and suitable modifications to the chassis strain AA07. Because AA07 has been shown to be substantially equivalent to strains 168, MB40 and SG188 (strains known to be safe and suitable for use in food) in all respects except the modifications described herein, Aanika focused on the safety of such changes in making our safety assessment following the FAO/WHO and Codex Alimentarius guidance that acknowledges the limitations of traditional animal toxicological testing when applied to whole organisms rather than the individual chemical moieties for which they were designed and validated (FAO 2009) and following the Pariza et. al. (Pariza, et al. 2015) guidance that appropriate safety testing be employed. A bioinformatic, predictive toxicology strategy was developed for the safety assessment of putative proteins and only considered employing animal toxicity testing if safety issues were raised by the bioinformatic analysis results. The bioinformatic strategy takes advantage of the vast, and growing, body of protein and DNA sequence data available as a result of the WGS and NGS revolution and the rapid advances in bioinformatic technologies designed to access the richness of the database resources.

Specific bioinformatic tools designed to identify protein or DNA sequences that are known to present risks to consumers regardless of mode of action or route of exposure were used. The databases include not only those sequences known to be toxic to animals from exposure to food but other harmful proteins from a variety of sources such as snake venoms. By looking for relatedness of the putative proteins to this broad spectrum of sequences from eukaryotic and prokaryotic sources containing allergen, toxin, and virulence factor-related sequences, one can assess with reasonable certainty that the sequences are not related to risk-related sequences in primary sequence but also in evolutionary homology and thus are highly unlikely to pose a risk to consumers.

The results of bioinformatic studies and the publicly curated and validated databases upon which they depend are described in detail in this notice . It may be concluded with reasonable certainty that there is no relationship between the putative proteins that may be produced in Watermarked *Bacillus subtilis* AA07-1 Spore Preparation and sequences that are associated with a risk to consumers and therefore animal toxicity studies are not warranted.

The watermark DNA (with no homology to known and catalogued DNA sequences) , the insertion sites and the resulting genomic construction are well characterized by DNA sequencing and no unintended modifications were reported in the strain. The deletions of the genes associated with spore germination and lysine synthesis were designed in such manner to maintain the overall gene expression mechanism of the loci to prevent polar expression changes. The result of this strategy is that the mechanism for expression of any DNA sequences within the deletion zones is intact and likely functional. Aanika has not confirmed that the watermark encoded polypeptide is produced in the watermarked strain, but it is possible because of the construction of the watermark cassette within a functional ORF that the polypeptide is produced from the watermark DNA sequence. Two lines of evidence suggest that there is little risk from the presence of the polypeptide in the watermark strain; (1) absence of sequence homology to known peptide toxins, virulence factors, or allergens and (2) likelihood of exposure to consumers is very low.

The putative polypeptide sequence has been compared to comprehensive databases of polypeptide sequences (Allegen Online, ResFinder, ThreatSEQ, and VFDB) that have the potential to be hazardous to consumers if ingested and the putative sequences were not found to be homologous to any of the known hazardous proteins. The sequences are homologous only to those found in the chassis strain AA07 as expected.

In addition to the uniqueness of the putative protein sequences, the exposure of consumers to the putative protein is likely to be very low in the application as the spores containing the protein are not likely to be digested in the human intestinal tract nor do they release intra-cellular contents into the intestinal lumen since the spores remain in the inert spore state and pass through the GI tract essentially intact.

The putative peptide products of the inserted DNA have been determined not to be a risk to consumers by virtue of the bioinformatic *in situ* analysis of the DNA and amino acid sequences. The spores are likely to pass unchanged through the GI tract and not exchange cellular contents with their environment, thus virtually eliminating the likelihood of exposure of consumers to the putative peptides. The spore coat of the modified strain is identical to the spore coat of un-modified strains highly related to strain 168 where toxicological studies did not demonstrate adverse events related to strain 168. Aanika has determined that animal toxicology studies on the modified strain are not warranted and unlikely to provide additional meaningful results to that of the MB40 strain studies whose safety has been reviewed and affirmed by FDA.

It is Aanika's intention to produce many unique watermarked *Bacillus subtilis* strains by the methods described in this notice, including the use of the chassis strain AA07 and synthetic DNA sequences that have been demonstrated with reasonable certainty to be safe using the predictive bioinformatic tools described in detail herein. The safety evaluation scheme discussed in detail in Section 2 is summarized below:

- Evaluation of the watermark DNA to establish its uniqueness
- Insertion of the watermark DNA into safe and suitable *Bacillus subtilis* host backgrounds based upon strain 168 using genetic techniques appropriate for the manufacture of food microorganisms following accepted international guidelines
- Complete DNA sequencing of the watermarked strain
- Bioinformatic *in silico* safety evaluation of the watermarked strain gene sequence, including the inserted sequence as well as upstream and downstream adjacent DNA sequences
- Establishing appropriate QC specifications
- Employing safe and suitable manufacturing practices in accordance with cGMP as established in 21 CFR §117.

6.5 INTENDED USES AND ESTIMATED DIETARY INTAKE

The AA07-1 spore preparation is intended to be used as an incidental additive, based upon the FDA decision tree for determining the regulatory category of a food substance[40] and is envisioned to be present in or on the foods at approximately 150 ppb by weight and the cumulative EDI for the described food categories, based upon the USDA *Mean Amounts of Retail Commodities Consumed per Individual, Estimated From Dietary Intake Data, by Gender and Age, in the United States, WWEIA, NHANES 2007-2008*, is 4.46 x 10^8 spores/person/day.

Aanika has concluded based upon these calculations and the fact that significantly higher exposure to *B. subtilis* spores (approximately 10^{11} spores/day or 10^3 times higher) is envisioned in GRAS notices submitted to FDA in recent years that the spores are present in the foods at insignificant amounts relative to these other uses of *Bacillus subtilis* in food. The spore preparation is only a tracking and tracing tool utilized during the manufacturing, packaging, storing and handling of food, employing an inherent DNA sequence property.

The cumulative EDI is highly conservative in that (1) the estimate is based upon the assumption that all foods in the listed categories will contain the technology, and (2) fresh foods such as leafy greens containing the technology will most likely be washed after purchase by consumers, which will reduce the amount of the watermarked strain on the food. The dietary intake of the watermarked strain may not reflect the actual exposure of consumers to the technology in the same was as intake of a readily absorbable chemical entity may be thought of as an indication of meaningful exposure, i.e., there is opportunity for interaction of the chemical moity with cellular processes. Because the watermarked

[40] https://www.fda.gov/food/food-ingredients-packaging/determining-regulatory-status-food-ingredient

strain AA07-1 has been modified to ensure that it does not germinate and enter the vegetative state and is thus likely to remain in the spore state and be eliminated from the GI tract as a spore, there is limited opportunity for the spores to interact with cellular processes.

6.6 MANUFACTURING

Bacillus subtilis AA07-1 is manufactured using well-known fermentation, sporulation induction, and spore harvesting processes to produce the *Bacillus subtilis* spore preparation in compliance with cGMP requirements under 21 CFR §117. No food allergens named in FALCPA [41]are used in the production of the preparation. Quality control testing of three (3) lots of the spore preparation demonstrate that the manufacturing process is capable of producing product that meets established specifications.

6.7 SUMMARY

Bacillus subtilis is recognized as a non-pathogenic and non-toxigenic species and is used in food production, as a food ingredient, and as a dietary supplement for humans and other animals. The strain lineage based upon *B. subtilis* 168 has been confirmed by animal feeding studies and clinical trials to be non-toxigenic and well tolerated in humans and various and other animals. The genetic modifications to the well characterized, safe and suitable *B. subtilis* AA07 (derived from strain 168) are well characterized by whole genome sequencing, utilize well-known genetic modification tools, and the introduced genetic material does not encode and express any known harmful or toxic substances.

The safety of this *Bacillus subtilis* AA07-1 spore preparation was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients and the decision tree of Pariza *et al*.. (Pariza, et al. 2015). The strain is genetically modified by rDNA techniques as discussed in Part 2 following established guidelines for the use of genetic modification techniques in developing organisms to be used in food and has been thoroughly characterized by genetic sequence analysis. The spore preparation is free of DNA encoding transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is stably integrated into the chromosome and the incorporated DNA does not encode and express any known toxins, virulence factors or known allergens or allergenic epitopes.

Based on known history of safe use of *B. subtilis*, the demonstration of safety of highly related strains by human and animal testing, the limited and well defined nature of the genetic modifications to create the desired watermarks, the absence of homology between the DNA watermark amino acid sequences and protein sequences known to present a risk to consumers, the limited exposure to AA07-1 cellular components including the putative peptides produced from the watermark DNA sequences because of the virtually non-existent germination capacity of the strain, and the manufacturing process comporting with cGMP, Aanika Biosciences concludes the proposed use of their Watermarked *Bacillus subtilis* AA07-1 Spore Preparation, when manufactured consistent with cGMP and meeting the specifications

[41] US Food Allergen Labelling and Consumer Protection Act, 2004

presented in this dossier, is Generally Recognized as Safe based on scientific procedures . Aanika Biosciences believes that qualified experts are likely to agree with this assessment.

A qualified panel of experts reviewed the GRAS notice and agrees that the Watermarked *Bacillus subtilis* AA07-1 Spore Preparation is Generally Recognized as Safe for the uses described when manufactured in accordance with good Manufacturing Practices. The panel report is attached (Appendix 7.2.15)

PART 7 – SUPPORTING DATA AND INFORMATION

7.1 BIBLIOGRAPHY

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7.2 APPENDICES

7.2.1. Pariza et al. Decision Tree

Decision Tree Question	Response
1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).	YES
2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)	YES
3. Is the strain genome free of genetic elements [iv] encoding virulence factors [v] and/or toxins [v] associated with pathogenicity? (If YES, go to 4. If NO, go to 15.)	YES
4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? (If YES, go to 5. If NO, go to 15.)	YES
5. Does the strain produce antimicrobial substances? (If NO, go to 6. If YES, go to 15.)	NO
6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7a or 7b. If NO, go to 8a or 8b.)	YES
7a. For strains to be used in human food: Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food? (If YES, go to 8a. If NO, the expressed product(s) must be shown to be safe before proceeding to 8a.)	NO
7b. For strains to be used in animal feed [ix]: Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in feed for the target animal species? (If YES, go to 8b. If NO, the expressed product(s) must be shown to be safe for the target animal species before proceeding to 8b.)	N/A
8a. For strains to be used in human food: Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial [xi] and characterizing [xii] component (not simply an 'incidental isolate')? (If YES, go to 9a. If NO, go to 13a.)	NO
8b. For strains to be used in animal feeds: Was the strain isolated from a feed (for example, silage) that has a history of safe consumption by target animals, for which the species, to which the strain belongs, is a substantial [xi] and characterizing component (not simply an 'incidental isolate')? (If YES, go to 9b. If NO, go to 13b.)	N/A
9a. For strains to be used in human food: Has the species, to which the strain belongs, undergone a comprehensive peer-	YES

reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts? (If YES, go to 10a. If NO, go to 13a.)	
9b. For strains to be used in animal feeds: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for feed use by an authoritative group of qualified scientific experts? (If YES, go to 10b. If NO, go to 13b.)	N/A
10a. For strains to be used in human food: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food? (If YES, go to 11a. If NO, go to 13a.)	YES
10b. For strains to be used in animal feeds: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9b continue to support the conclusion that the species, to which the strain belongs, is safe for use in feed? (If YES, go to 11b. If NO, go to 13b.)	N/A
11a. For strains to be used in human food: Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in “traditional” food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12a. If YES, go to 13a.)	NO
11b. For strains to be used in animal feeds: Will the intended use of the strain expand exposure to the species beyond the target animals that typically consume the species in “traditional” feed(s) in which it is typically found (for example, will a strain that was isolated from silage be used in swine feed)? (If NO, go to 12b. If YES, go to 13b.)	N/A
12a. For strains to be used in human food: Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14a. If YES, go to 13a.)	NO
12b. For strains to be used in animal feeds: Will the intended use of the strain expand intake of the species (for example, increasing the number of feeds beyond the traditional feeds in which the species is typically found, or using the strain as a	N/A

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probiotic rather than as a silage starter culture)? (If NO, go to 14b. If YES, go to 13b.)	
13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? If yes, go to 15. If no, go to 14a.)	NO
13b. For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? If yes, go to 15. If no, go to 14b.)	N/A
14a. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.	YES
14b. The strain is deemed to be safe for use in the manufacture of feeds, probiotics, and dietary supplements for animal consumption.	YES
15. The strain is NOT APPROPRIATE for human or animal consumption.	NO

[1] Pariza, M.W., Gillies, K. O., Krack-Ripple, S., Leyer, G., and Smith, A.B. "Determining the safety of microbial cultures for consumption by humans and animals." *Regulatory Toxicology and Pharmacology* 73 (2015): 164-171.

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7.2.2. Allergen Online Search / *lys*A Watermark Insert Query

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v21 (February 14, 2021)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all123D.tmp version2136.fasta
User Query #1 >query

User Query #1

```
     >query
     LAGEKGENAS SVRTYEGTQT AWEGGKAGYR SYSIHYTNGR EHRYGAPTSR TT

# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all123D.tmp version2136.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\all123D.tmp
  1>>>query - 52 aa
Library: version2136.fasta
   540227 residues in  2233 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 5.9061+/-0.00524; mu= 2.0422+/- 0.266
 mean_var=23.3968+/- 6.158, 0's: 0 Z-trim(88.7): 10  B-trim: 0 in 0/38
 Lambda= 0.265153
 statistics sampled from 756 (756) to 756 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.726), E-opt: 0.2 (0.339), width:  16
 Scan time:  0.000

The best scores are:                                      opt bits E(2233) %_id  %_sim  alen
gi|90185688|gid|540|Major strawberry allergen Fra a  ( 159)   56 24.6    0.73 0.364 0.727   22
gi|90185682|gid|540|Major strawberry allergen Fra a  ( 160)   56 24.6    0.73 0.364 0.727   22
gi|88082485|gid|540|Fra a 1-A allergen [Fragaria x a ( 160)   56 24.6    0.73 0.364 0.727   22
gi|90185684|gid|540|Major strawberry allergen Fra a  ( 160)   56 24.6    0.73 0.364 0.727   22

>>>query, 52 aa vs version2136.fasta library

>>gi|90185688|gid|540|Major strawberry allergen Fra a 1-C [Fragaria x ana  (159 aa)
 initn:  65 init1:  56 opt:  56  Z-score: 108.1  bits: 24.6 E(2233): 0.73
Smith-Waterman score: 56; 36.4% identity (72.7% similar) in 22 aa overlap (23-44:61-82)

                              10        20        30        40        50
query                     LAGEKGENASSVRTYEGTQTAWEGGKAGYRSYSIHYTNGREHRYGAPTSRTT
                                              ::.. :: ...::  . ..: :
notag| KAFVLDADNLIPKIAPQAVKCAEILEGDGGPGTIKKITFGEGSHYGYVKHKIHSIDKENHTYSYSLIEGDALSDNIEKID
               30        40        50        60        70        80        90       100
```

7.2.3. Allergen Online Search / *cwI*D Insert Query

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

% id 1 = 100% identity, alen=alignment length

AllergenOnline Database v21 (February 14, 2021)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\allED83.tmp version2136.fasta
User Query #1 >query

User Query #1

```
     >query
     MAGEKGELKN HNRPEGGKAG YRSYSIHYTN GRRGSCHCRL KLAKHRRICA L

# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\allED83.tmp version2136.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\allED83.tmp
  1>>>query - 51 aa
Library: version2136.fasta
   540227 residues in  2233 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 3.9063+/-0.00429; mu= 14.0473+/- 0.218
 mean_var=42.1046+/-11.958, 0's: 0 Z-trim(93.2): 5  B-trim: 12 in 2/39
 Lambda= 0.197656
 statistics sampled from 1006 (1006) to 1006 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.851), E-opt: 0.2 (0.451), width:  16
 Scan time:  0.000
!! No sequences with E() < 1
>>>///

51 residues in 1 query   sequences
540227 residues in 2233 library sequences
 Scomplib [36.3.8g Oct, 2018]
 start: Thu Jan 13 10:52:18 2022 done: Thu Jan 13 10:52:18 2022
 Scan time:  0.000 Display time:  0.000

Function used was FASTA [36.3.8g Oct, 2018]
```

7.2.4 Allergen Online Search / cwlJ Insert Query

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

% id 1 = 100% identity, alen=alignment length

AllergenOnline Database v21 (February 14, 2021)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all1FCB.tmp version2136.fasta
User Query #1 >query

User Query #1

```
     >query
     MAGEKGEPDP KDAHEGGKAG YRSYSIHYTN GRRGSCHCTN TKSQTRRICA L

# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all1FCB.tmp version2136.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\all1FCB.tmp
  1>>>query - 51 aa
Library: version2136.fasta
   540227 residues in  2233 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 5.0560+/-0.0035; mu= 6.9095+/- 0.181
 mean_var=38.4127+/-10.244, 0's: 0 Z-trim(92.1): 11  B-trim: 0 in 0/40
 Lambda= 0.206936
 statistics sampled from 938 (938) to 938 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.819), E-opt: 0.2 (0.42), width:  16
 Scan time:  0.000
!! No sequences with E() < 1
>>>///

51 residues in 1 query   sequences
540227 residues in 2233 library sequences
 Scomplib [36.3.8g Oct, 2018]
 start: Thu Jan 13 10:56:53 2022 done: Thu Jan 13 10:56:53 2022
 Scan time:  0.000 Display time:  0.000

Function used was FASTA [36.3.8g Oct, 2018]
```

7.2.5 Allergen Online Search / *sle*B Insert Query

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v21 (February 14, 2021)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all5BF7.tmp version2136.fasta
User Query #1 >query

User Query #1

```
     >query
     MAGEKGESMP PTVNEGGKAG YRSYSIHYTN GRRGSCHCKI KRQGRRRICA L

# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\all5BF7.tmp version2136.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\all5BF7.tmp
  1>>>query - 51 aa
Library: version2136.fasta
   540227 residues in  2233 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 4.0874+/-0.00439; mu= 14.1806+/- 0.227
 mean_var=52.6619+/-15.146, 0's: 0 Z-trim(92.0): 2  B-trim: 2 in 1/42
 Lambda= 0.176736
 statistics sampled from 934 (934) to 934 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.835), E-opt: 0.2 (0.418), width:  16
 Scan time:  0.000
!! No sequences with E() < 1
>>>///

51 residues in 1 query   sequences
540227 residues in 2233 library sequences
 Scomplib [36.3.8g Oct, 2018]
 start: Thu Jan 13 11:01:31 2022 done: Thu Jan 13 11:01:31 2022
 Scan time:  0.000 Display time:  0.000

Function used was FASTA [36.3.8g Oct, 2018]
```

7.2.6 Allergen Online Search / *ger*D Insert Query

AllergenOnline Search Results

Note: As of August 2015 we have included gid: groupid in the fasta results that provides detailed information on the allergenicity references for the group, type of allergen, other sequences belonging to the same group and more.

%_id 1 = 100% identity, alen=alignment length

AllergenOnline Database v21 (February 14, 2021)

NOTE Addition of Allergenicity* column on the Browse Database page with classification based on Group references was added on 10 May 2018. Please review the "allergenicity" of any matches you find here with the Browse page and look at Group References (gid) if you want to further evaluate relevance of alignments.

fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\allEFE9.tmp version2136.fasta
User Query #1 >query

User Query #1

```
     >query
     MAGEKGETYN NYTTEGGKAG YRSYSIHYTN GRRGSCHCTI QQKDNRRICA L

# fasta36.exe -q -B -m 9i -w 80 -E 1 -d 20 C:\Windows\Temp\allEFE9.tmp version2136.fasta
FASTA searches a protein or DNA sequence data bank
 version 36.3.8g Oct, 2018
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query: C:\Windows\Temp\allEFE9.tmp
  1>>>query - 51 aa
Library: version2136.fasta
   540227 residues in  2233 sequences

Statistics:  Expectation_n fit: rho(ln(x))= 3.3748+/-0.00431; mu= 16.9788+/- 0.226
 mean_var=42.5085+/-10.455, 0's: 1 Z-trim(91.5): 3  B-trim: 0 in 0/40
 Lambda= 0.196714
 statistics sampled from 901 (901) to 901 sequences
Algorithm: FASTA (3.8 Nov 2011) [optimized]
Parameters: BL50 matrix (15:-5), open/ext: -10/-2
 ktup: 2, E-join: 1 (0.816), E-opt: 0.2 (0.403), width:  16
 Scan time:  0.000
!! No sequences with E() < 1
>>>///

51 residues in 1 query   sequences
540227 residues in 2233 library sequences
 Scomplib [36.3.8g Oct, 2018]
 start: Thu Jan 13 11:06:31 2022 done: Thu Jan 13 11:06:31 2022
 Scan time:  0.000 Display time:  0.000

Function used was FASTA [36.3.8g Oct, 2018]
```

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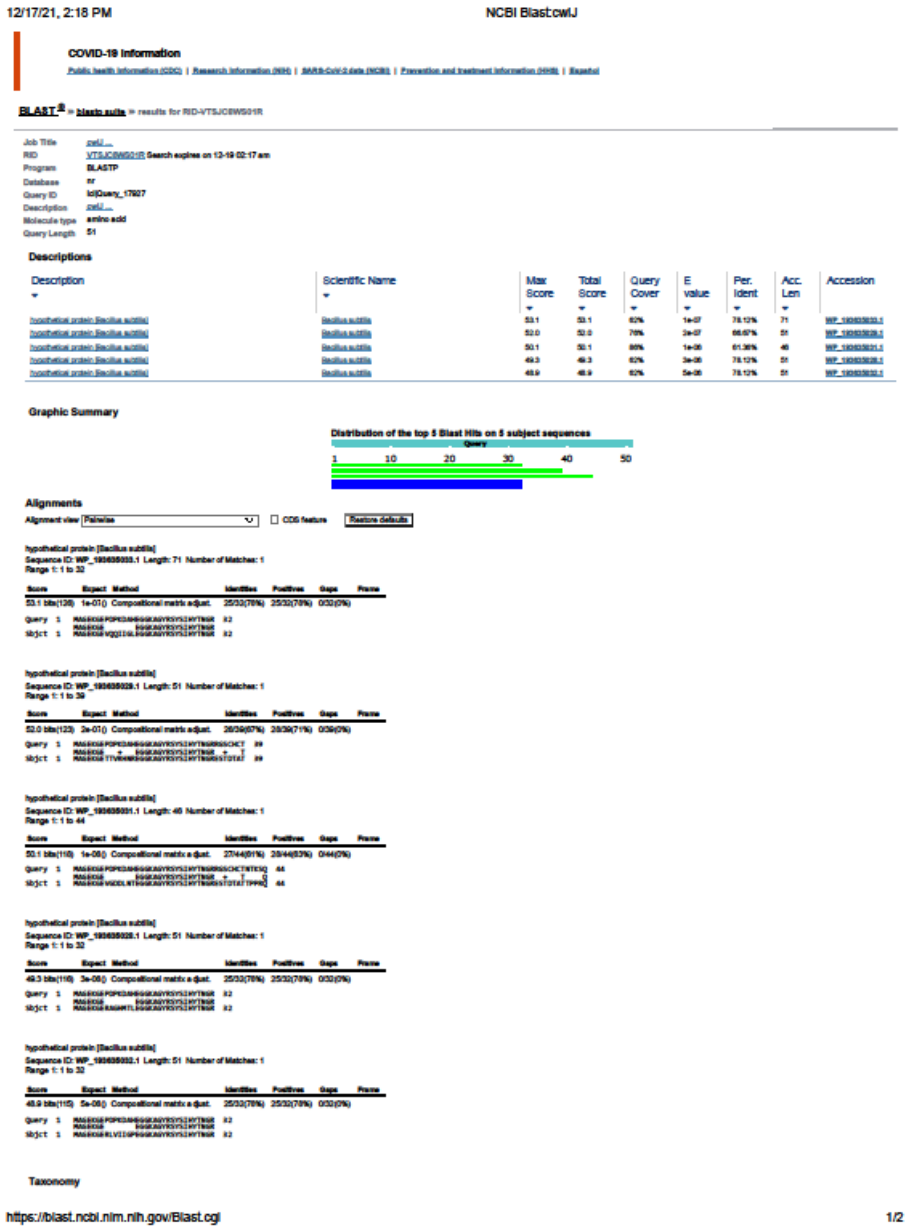
7.2.7 NCBI BLASTP *cwlJ* Insert Query



12/17/21, 2:18 PM NCBI Blast:cwlJ

COVID-19 Information

Public health information (CDC) | Research information (NIH) | SARS-CoV-2 data (NCBI) | Prevention and treatment information (HHS) | Español

BLAST® » blastp suite » results for RID-VTSJC8WS01R

Job Title cwlJ ...
RID VTSJC8WS01R Search expires on 12-19 02:17 am
Program BLASTP
Database nr
Query ID lcl|Query_17927
Description cwlJ ...
Molecule type amino acid
Query Length 51

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	53.1	53.1	62%	1e-07	78.12%	71	WP_190405033.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	52.0	52.0	76%	2e-07	66.67%	51	WP_190405029.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.1	50.1	86%	1e-06	61.36%	46	WP_190405031.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	49.3	49.3	62%	3e-06	78.12%	51	WP_190405028.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	48.9	48.9	62%	5e-06	78.12%	51	WP_190405032.1

Graphic Summary

Distribution of the top 5 Blast Hits on 5 subject sequences

Query
1 10 20 30 40 50

Alignments

Alignment view Pairwise ☐ CDS feature Restore defaults

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_190405033.1 Length: 71 Number of Matches: 1
Range 1: 1 to 32

Score	Expect	Method	Identities	Positives	Gaps	Frame
53.1 bits(126)	1e-01()	Compositional matrix adjust.	25/32(78%)	25/32(78%)	0/32(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_190405029.1 Length: 51 Number of Matches: 1
Range 1: 1 to 39

Score	Expect	Method	Identities	Positives	Gaps	Frame
52.0 bits(123)	2e-01()	Compositional matrix adjust.	26/39(67%)	28/39(71%)	0/39(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_190405031.1 Length: 46 Number of Matches: 1
Range 1: 1 to 44

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.1 bits(118)	1e-06()	Compositional matrix adjust.	27/44(61%)	28/44(63%)	0/44(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_190405028.1 Length: 51 Number of Matches: 1
Range 1: 1 to 32

Score	Expect	Method	Identities	Positives	Gaps	Frame
49.3 bits(116)	3e-06()	Compositional matrix adjust.	25/32(78%)	25/32(78%)	0/32(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_190405032.1 Length: 51 Number of Matches: 1
Range 1: 1 to 32

Score	Expect	Method	Identities	Positives	Gaps	Frame
48.9 bits(115)	5e-06()	Compositional matrix adjust.	25/32(78%)	25/32(78%)	0/32(0%)	

Taxonomy

https://blast.ncbi.nlm.nih.gov/Blast.cgi 1/2

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7.2.8 NCBI BLASTP *cwl*D Insert Query

12/17/21, 2:16 PM NCBI Blast:cwlD Knockout

COVID-19 Information

Public health information (CDC) | Research information (NIH) | SARS-CoV-2 data (NCBI) | Prevention and treatment information (HHS) | Español

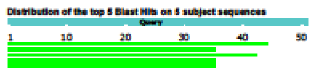
BLAST® » blastp suite » results for RID-VTSBNFPV016

Job Title	cwlD Knockout ...
RID	VTSBNFPV016 Search expires on 12-19 02:13 am
Program	BLASTP
Database	nr
Query ID	lcl|Query_77432
Description	cwlD Knockout ...
Molecule type	amino acid
Query Length	51

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	53.1	53.1	86%	6e-08	61.36%	51	[illegible]
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	53.1	53.1	68%	1e-07	71.43%	46	[illegible]
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	53.5	53.5	82%	1e-07	59.52%	71	[illegible]
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	51.2	51.2	68%	5e-07	74.29%	51	[illegible]
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.8	50.8	68%	6e-07	77.78%	51	[illegible]

Graphic Summary



Alignments

Alignment view: Pairwise ☐ CDS feature [Restore defaults]

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193635028.1 Length: 51 Number of Matches: 1
Range 1: 1 to 44

Score	Expect	Method	Identities	Positives	Gaps	Frame
53.1 bits(126)	9e-08()	Compositional matrix adjust.	27/44(61%)	28/44(63%)	0/44(0%)	

Query 1 [illegible] 44
Sbjct 1 [illegible] 44

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193635031.1 Length: 46 Number of Matches: 1
Range 1: 1 to 35

Score	Expect	Method	Identities	Positives	Gaps	Frame
53.1 bits(126)	1e-07()	Compositional matrix adjust.	25/35(71%)	28/35(80%)	0/35(0%)	

Query 1 [illegible] 35
Sbjct 1 [illegible] 35

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193635030.1 Length: 71 Number of Matches: 1
Range 1: 1 to 42

Score	Expect	Method	Identities	Positives	Gaps	Frame
53.5 bits(127)	1e-07()	Compositional matrix adjust.	25/42(60%)	30/42(71%)	0/42(0%)	

Query 1 [illegible] 42
Sbjct 1 [illegible] 42

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193635032.1 Length: 51 Number of Matches: 1
Range 1: 1 to 35

Score	Expect	Method	Identities	Positives	Gaps	Frame
51.2 bits(121)	5e-07()	Compositional matrix adjust.	26/35(74%)	27/35(77%)	0/35(0%)	

Query 1 [illegible] 35
Sbjct 1 [illegible] 35

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193635029.1 Length: 51 Number of Matches: 1
Range 1: 1 to 35

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.8 bits(120)	6e-07()	Compositional matrix adjust.	28/36(78%)	29/36(80%)	2/36(5%)	

Query 1 [illegible] 35
Sbjct 1 [illegible] 35

Taxonomy

https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&RID=VTSBNFPV016&OLD_BLAST=false 1/2

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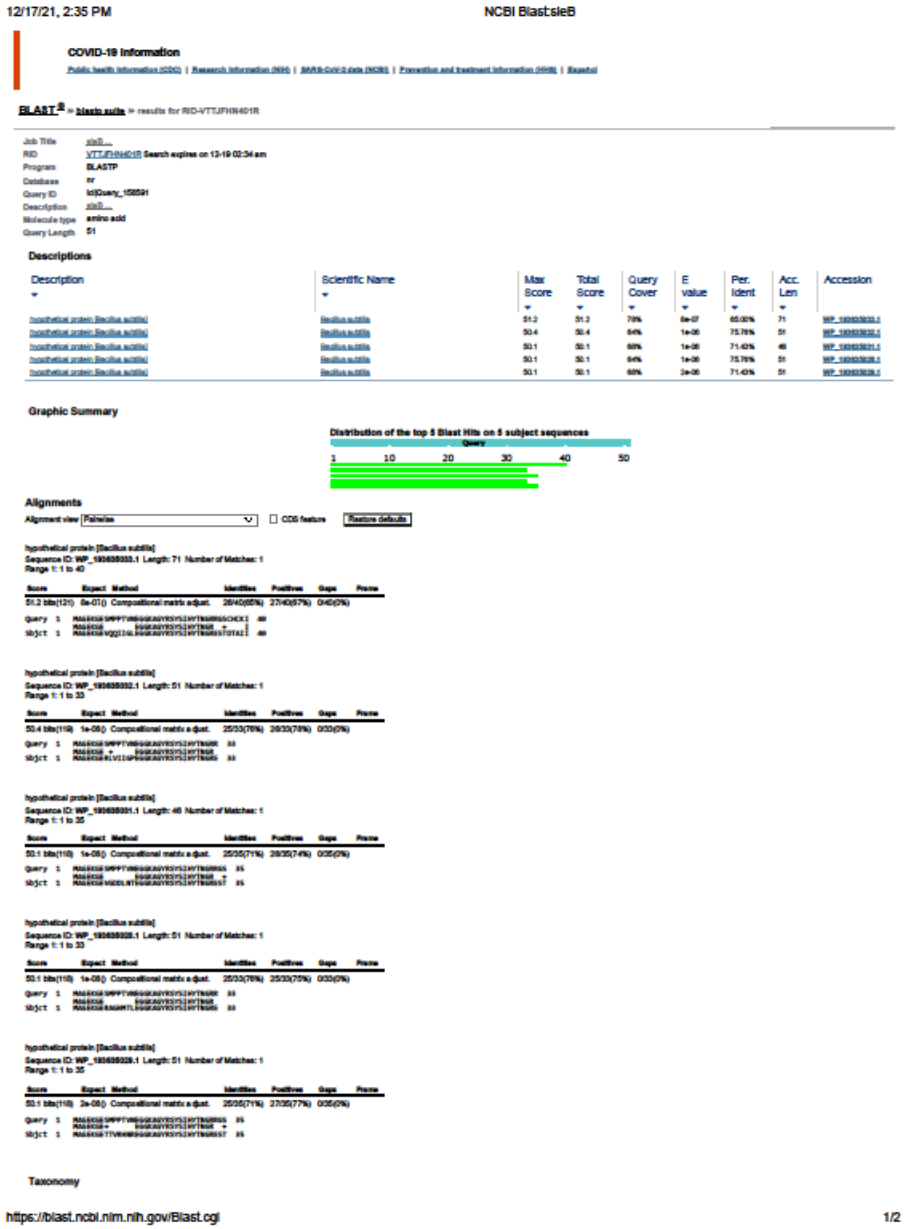
7.2.9 NCBI BLASTP *sle*B Insert Query



12/17/21, 2:35 PM NCBI Blast:sleB

COVID-19 Information

Public health information (CDC) | Research information (NIH) | SARS-CoV-2 data (NCBI) | Prevention and treatment information (HHS) | Español

BLAST® » blastp suite » results for RID-VTTJFHN401R

Job Title sleB_...
RID VTTJFHN401R Search expires on 12-19 02:34 am
Program BLASTP
Database nr
Query ID lcl|Query_158591
Description sleB_...
Molecule type amino acid
Query Length 51

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	51.2	51.2	78%	6e-07	65.00%	71	WP_193935033.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.4	50.4	64%	1e-06	75.76%	51	WP_193935032.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.1	50.1	88%	1e-06	71.43%	46	WP_193935031.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.1	50.1	64%	1e-06	75.76%	51	WP_193935028.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.1	50.1	88%	2e-06	71.43%	51	WP_193935029.1

Graphic Summary

Distribution of the top 5 Blast Hits on 5 subject sequences

Query
1 10 20 30 40 50

Alignments

Alignment view Pairwise ☐ CDS feature Restore defaults

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935033.1 Length: 71 Number of Matches: 1
Range 1: 1 to 40

Score	Expect	Method	Identities	Positives	Gaps	Frame
51.2 bits(121)	6e-07	Compositional matrix adjust.	26/40(65%)	27/40(67%)	0/40(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935032.1 Length: 51 Number of Matches: 1
Range 1: 1 to 33

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.4 bits(119)	1e-06	Compositional matrix adjust.	25/33(76%)	26/33(78%)	0/33(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935031.1 Length: 46 Number of Matches: 1
Range 1: 1 to 35

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.1 bits(118)	1e-06	Compositional matrix adjust.	25/35(71%)	26/35(74%)	0/35(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935028.1 Length: 51 Number of Matches: 1
Range 1: 1 to 33

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.1 bits(118)	1e-06	Compositional matrix adjust.	25/33(76%)	25/33(75%)	0/33(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935029.1 Length: 51 Number of Matches: 1
Range 1: 1 to 35

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.1 bits(118)	2e-06	Compositional matrix adjust.	25/35(71%)	27/35(77%)	0/35(0%)	

Taxonomy

https://blast.ncbi.nlm.nih.gov/Blast.cgi 1/2

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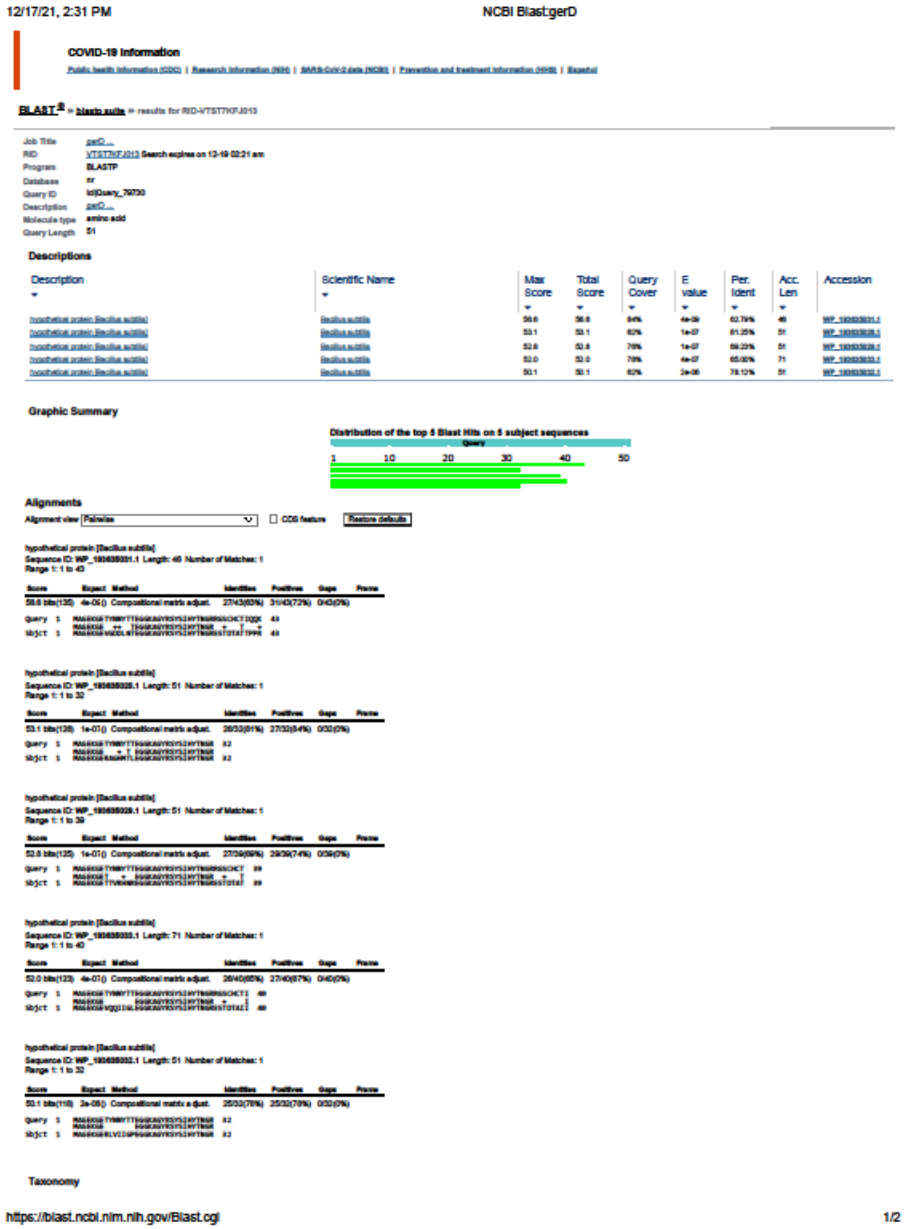
7.2.10 NCBI BLASTP *ger*D Insert Query



12/17/21, 2:31 PM NCBI Blast:gerD

COVID-19 Information

Public health information (CDC) | Research information (NIH) | SARS-CoV-2 data (NCBI) | Prevention and treatment information (HHS) | Español

BLAST® » blastp suite » results for RID-VTST7KFJ013

Job Title gerD ...
RID VTST7KFJ013 Search expires on 12-19 02:21 am
Program BLASTP
Database nr
Query ID lcl|Query_79730
Description gerD ...
Molecule type amino acid
Query Length 51

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	58.6	58.6	84%	4e-09	62.79%	46	WP_193935031.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	53.1	53.1	62%	1e-07	81.25%	51	WP_193935038.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	52.8	52.8	76%	1e-07	69.23%	51	WP_193935039.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	52.0	52.0	78%	4e-07	65.00%	71	WP_193935033.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	50.1	50.1	82%	2e-06	78.12%	51	WP_193935032.1

Graphic Summary

Distribution of the top 5 Blast Hits on 5 subject sequences

Query
1 10 20 30 40 50

Alignments

Alignment view Pairwise ☐ CDS feature Restore defaults

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935031.1 Length: 46 Number of Matches: 1
Range 1: 1 to 43

Score	Expect	Method	Identities	Positives	Gaps	Frame
58.6 bits(138)	4e-09	Compositional matrix adjust.	27/43(63%)	31/43(72%)	0/43(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935038.1 Length: 51 Number of Matches: 1
Range 1: 1 to 32

Score	Expect	Method	Identities	Positives	Gaps	Frame
53.1 bits(126)	1e-07	Compositional matrix adjust.	26/32(81%)	27/32(84%)	0/32(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935039.1 Length: 51 Number of Matches: 1
Range 1: 1 to 39

Score	Expect	Method	Identities	Positives	Gaps	Frame
52.8 bits(125)	1e-07	Compositional matrix adjust.	27/39(69%)	29/39(74%)	0/39(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935033.1 Length: 71 Number of Matches: 1
Range 1: 1 to 40

Score	Expect	Method	Identities	Positives	Gaps	Frame
52.0 bits(123)	4e-07	Compositional matrix adjust.	26/40(65%)	27/40(67%)	0/40(0%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193935032.1 Length: 51 Number of Matches: 1
Range 1: 1 to 32

Score	Expect	Method	Identities	Positives	Gaps	Frame
50.1 bits(118)	2e-06	Compositional matrix adjust.	25/32(78%)	25/32(78%)	0/32(0%)	

Taxonomy

https://blast.ncbi.nlm.nih.gov/Blast.cgi 1/2

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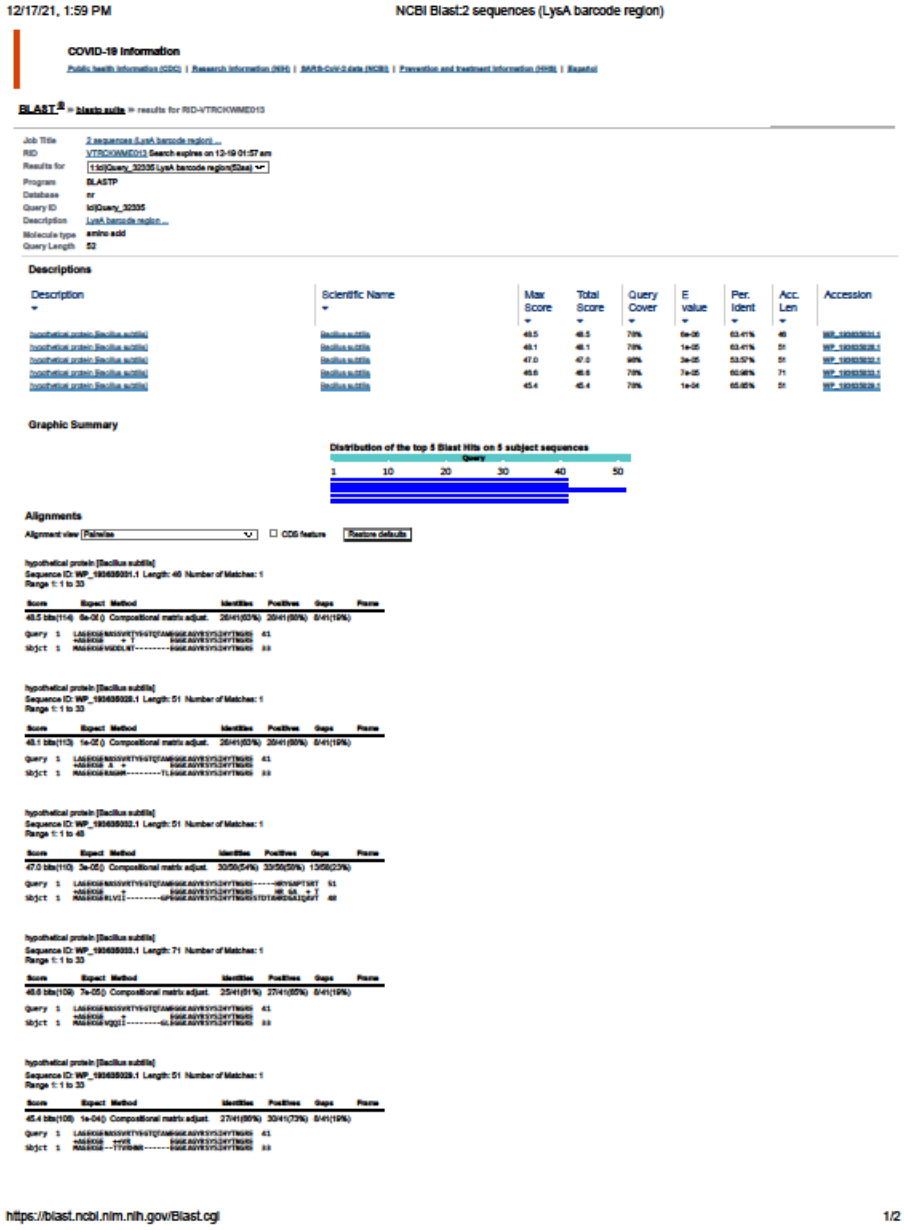
7.2.11 NCBI BLASTP *lysA* Watermark Insert Query



12/17/21, 1:59 PM NCBI Blast:2 sequences (LysA barcode region)

COVID-19 Information

Public health information (CDC) | Research information (NIH) | SARS-CoV-2 data (NCBI) | Prevention and treatment information (NIH) | Español

BLAST® » blastn suite » results for RID-VTRCKWME013

Job Title 2 sequences (LysA barcode region) ...
RID VTRCKWME013 Search expires on 12-19 01:57 am
Results for 1-lcl|Query_32335 LysA barcode region(50as)
Program BLASTP
Database nr
Query ID lcl|Query_32335
Description LysA barcode region ...
Molecule type amino acid
Query Length 52

Descriptions

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	48.5	48.5	79%	6e-06	63.41%	48	WP_193455031.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	48.1	48.1	79%	1e-05	63.41%	51	WP_193455028.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	47.0	47.0	69%	3e-05	53.57%	51	WP_193455032.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	46.6	46.6	79%	7e-05	60.98%	71	WP_193455033.1
hypothetical protein [Bacillus subtilis]	Bacillus subtilis	45.4	45.4	79%	1e-04	65.85%	51	WP_193455029.1

Graphic Summary

Distribution of the top 5 Blast Hits on 5 subject sequences
Query
1 10 20 30 40 50

Alignments

Alignment view Pairwise ☐ CDS feature Restore defaults

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193455031.1 Length: 48 Number of Matches: 1
Range 1: 1 to 33

Score	Expect	Method	Identities	Positives	Gaps	Frame
48.5 bits(114)	6e-06	Compositional matrix adjust.	26/41(63%)	26/41(63%)	8/41(19%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193455028.1 Length: 51 Number of Matches: 1
Range 1: 1 to 33

Score	Expect	Method	Identities	Positives	Gaps	Frame
48.1 bits(113)	1e-05	Compositional matrix adjust.	26/41(63%)	26/41(63%)	8/41(19%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193455032.1 Length: 51 Number of Matches: 1
Range 1: 1 to 48

Score	Expect	Method	Identities	Positives	Gaps	Frame
47.0 bits(110)	3e-05	Compositional matrix adjust.	30/56(54%)	33/56(59%)	13/56(23%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193455033.1 Length: 71 Number of Matches: 1
Range 1: 1 to 33

Score	Expect	Method	Identities	Positives	Gaps	Frame
46.6 bits(109)	7e-05	Compositional matrix adjust.	25/41(61%)	27/41(65%)	8/41(19%)	

hypothetical protein [Bacillus subtilis]
Sequence ID: WP_193455029.1 Length: 51 Number of Matches: 1
Range 1: 1 to 33

Score	Expect	Method	Identities	Positives	Gaps	Frame
45.4 bits(108)	1e-04	Compositional matrix adjust.	27/41(66%)	30/41(73%)	8/41(19%)	

https://blast.ncbi.nlm.nih.gov/Blast.cgi 1/2

7.2.12 VFDB Search

***cwl*D Query**

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***cwlJ* Query**

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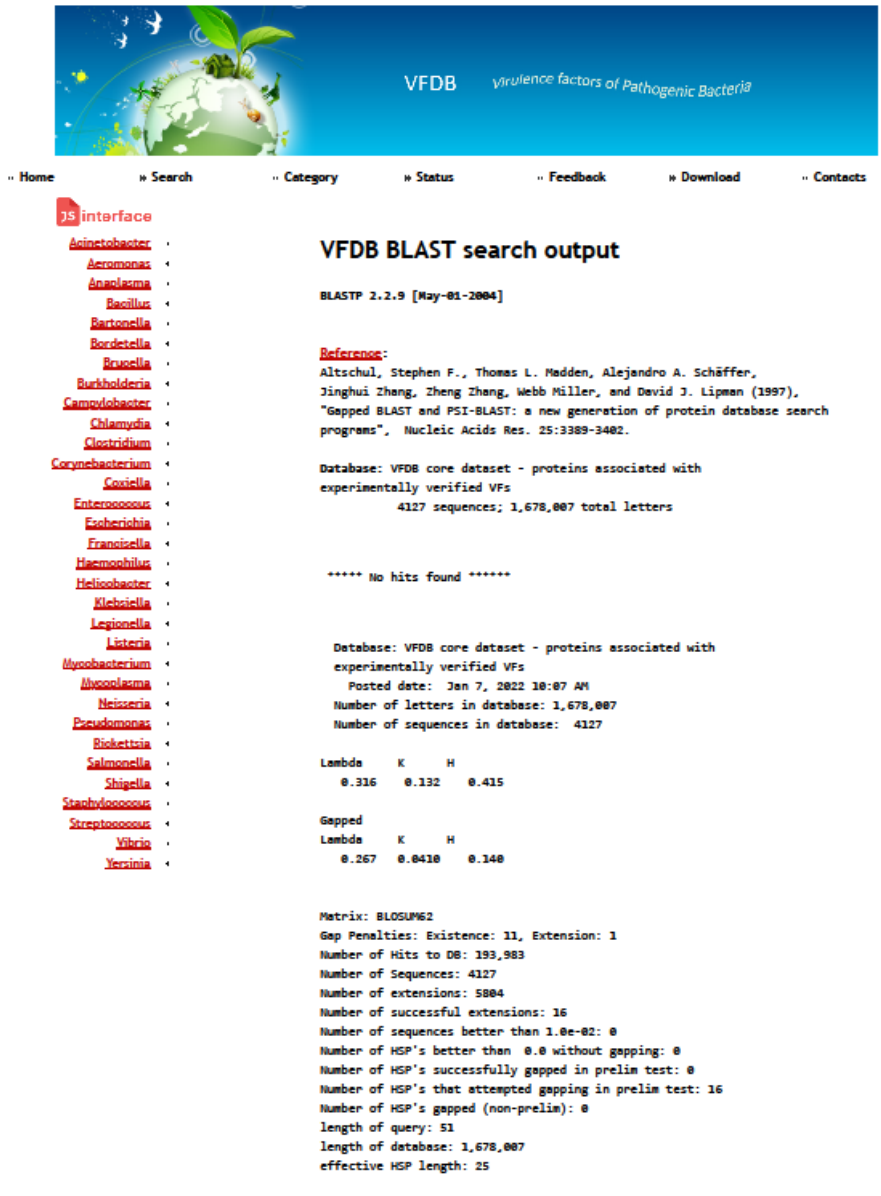

***sle*B VFDB Query**

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gerD VFDB Query

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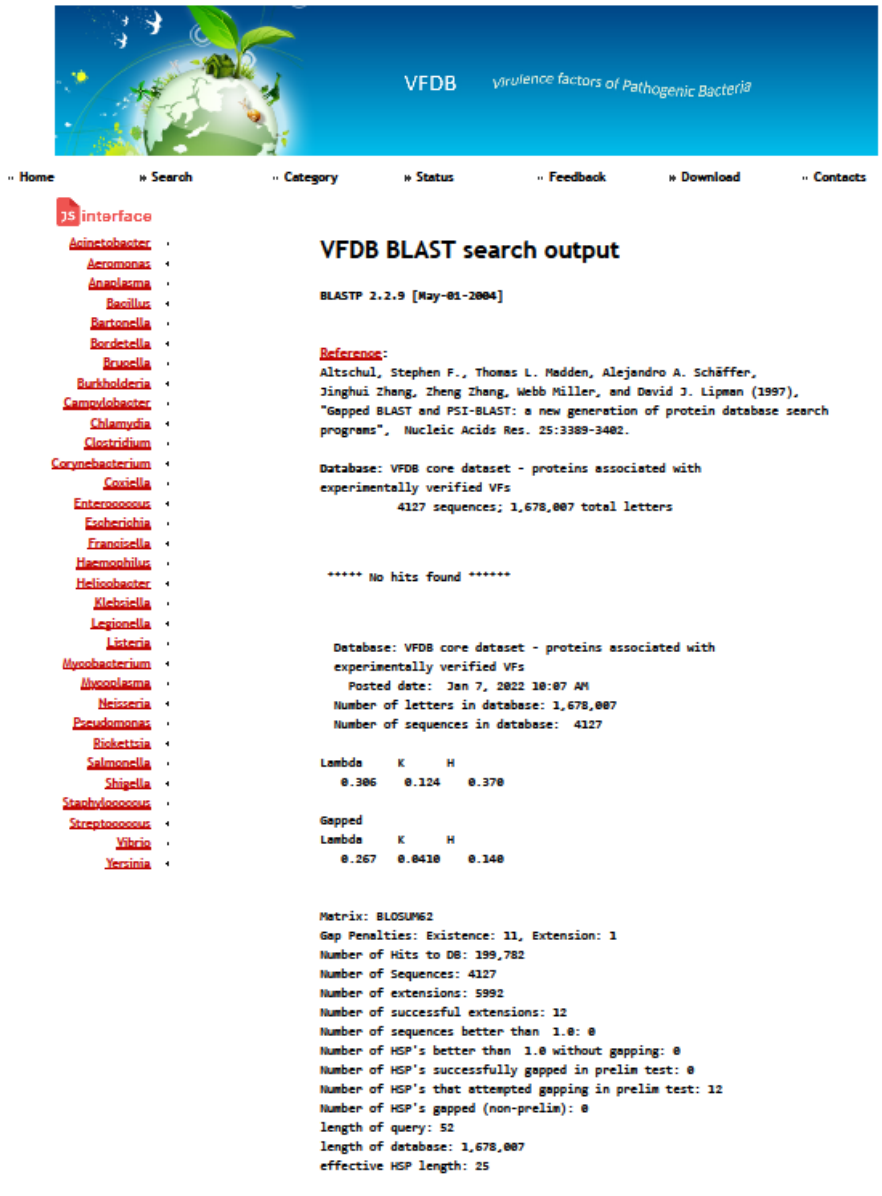

***lys*A VFDB Query**

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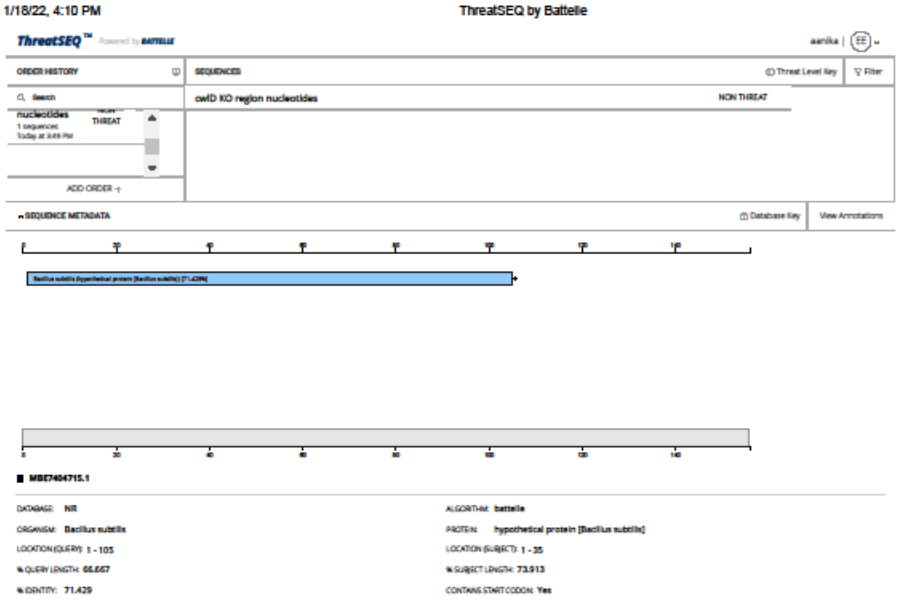
7.2.13 ThreatSEQ Search

cwlD Insert Query



https://app.threatseq.org/dashboard/s/DRkVG8cF2k5jF7c-XEgOfvLVKY9jwDkvX1JTdjwa66o= 1/1

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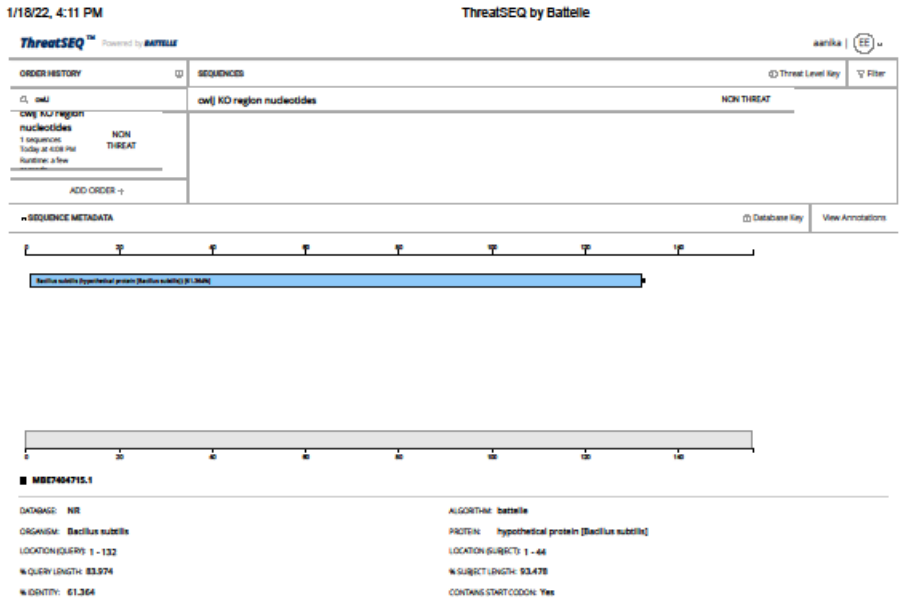
cwlJ Insert Query



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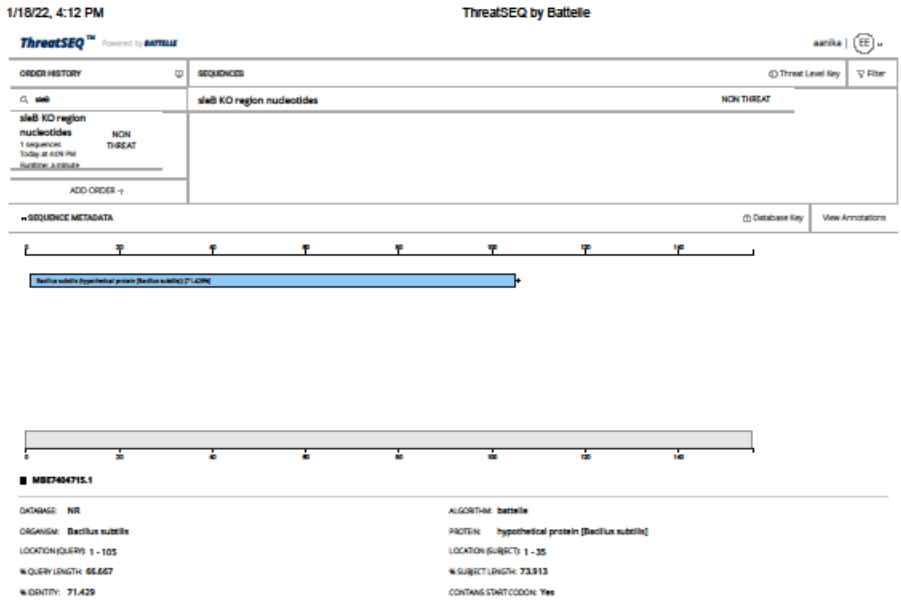
sleB Insert Query



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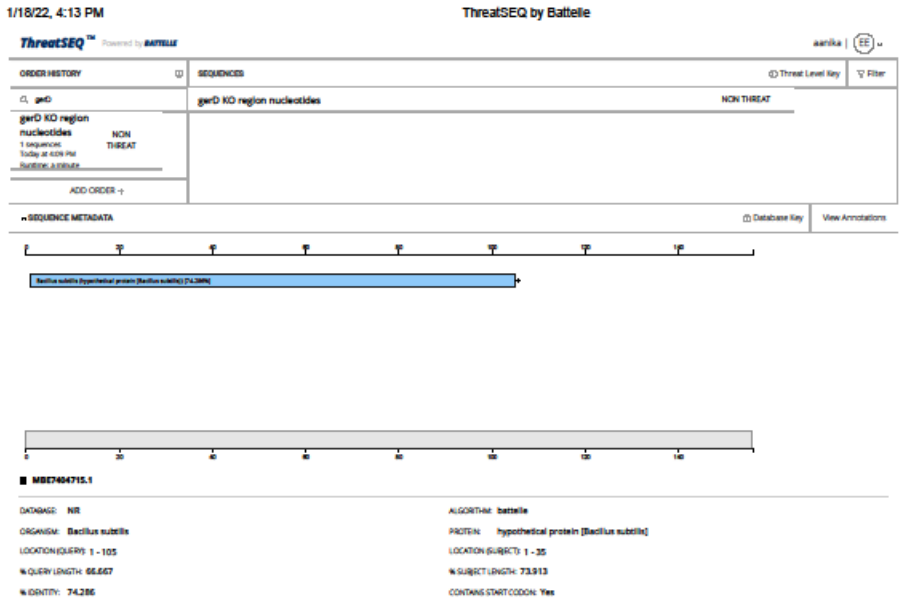
gerD Insert Query



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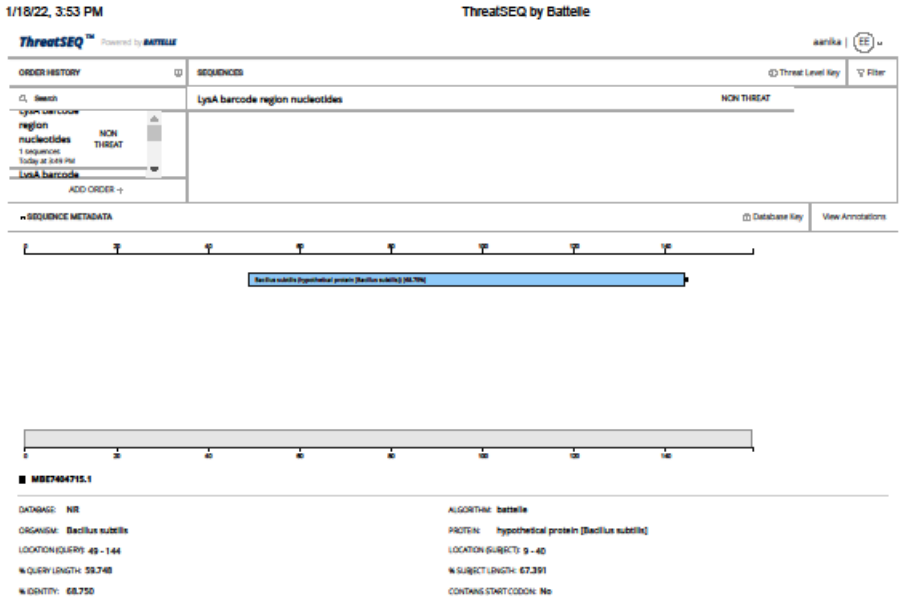
lysA Insert Query



7.2.14Certificates of analysis

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www.balabs.com

Aanika Biosciences
Attn: Ellen Jorgensen
86 34th ST. Suite D605
Brooklyn, NY 11232

Reporting Date: 09/15/2021
Sample Received: 09/09/2021

CERTIFICATE OF ANALYSIS

Laboratory Number: 210143325 **Sample of:** UNKNOWN

Sample Identification: P21-084B **P.O.#:** 09082021A
Date Shipped:

Test	Result	Units	Specification Limits	Method
E. Coli	<10	CFU/g		AOAC 991.14
Staphylococcus Aureus	<10	CFU/g		AOAC 2003.07
Salmonella	Negative	/25g		AOAC 2013.02
Listeria	NEGATIVE	/25g		AOAC RI 081401
Coliforms	<10	CFU/g		AOAC 991.14
Yeast & Mold	<10, <10	CFU/g		AOAC 997.02
Arsenic	<0.5	ppm		AOAC 2011.14, Modified
Cadmium	<0.5	ppm		AOAC 999.10, Modified
Mercury	<0.025	ppm		EPA Method 7471B (SW-846), Modified
Lead	0.965	ppm		AOAC 999.10, Modified

Approved

Greg Crosby
Chief Financial Officer

Questions about this analysis? Please contact customerservice@balabs.com

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Referee Chemists for American Oil Chemists* Society* Official Samplers and Chemists for the Chicago Board of Trade

Certificate of Analysis: V-1 **Page 1 of 1**

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www.balabs.com

Aanika Biosciences
Attn: Ellen Jorgensen
86 34th ST. Suite D605
Brooklyn, NY 11232

Reporting Date: 09/01/2021
Sample Received: 08/26/2021

CERTIFICATE OF ANALYSIS

Laboratory Number: 210135413 **Sample of:** UNKNOWN
Sample Identification: P21-084A **P.O.#:** 08242021A
Date Shipped:

Test	Result	Units	Specification Limits	Method
E. Coli	<10	CFU/g		AOAC 991.14
Staphylococcus Aureus	<10	CFU/g		AOAC 2003.07
Salmonella	Negative	/25g		AOAC 2013.02
Listeria	Negative	/25g		AOAC 997.03
Yeast & Mold	<10, <10	CFU/g		AOAC 997.02
Coliforms	<10	CFU/g		AOAC 991.14
Arsenic	<0.5	ppm		AOAC 2011.14, Modified
Cadmium	<0.5	ppm		AOAC 999.10, Modified
Mercury	<0.025	ppm		EPA Method 7471B (SW-846), Modified
Lead	0.693	ppm		AOAC 999.10, Modified

Approved

Greg Crosby
Chief Financial Officer

Questions about this analysis? Please contact customerservice@balabs.com

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Certificate of Analysis: V-1 **Page 1 of 1**

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Aanika Biosciences
Attn: Ellen Jorgensen
86 34th ST. Suite D605
Brooklyn, NY 11232

Reporting Date: 09/01/2021
Sample Received: 08/26/2021

CERTIFICATE OF ANALYSIS

Laboratory Number: 210135445 **Sample of:** UNKNOWN
Sample Identification: P21-063A **P.O.#:** 08242021B
Date Shipped:

Test	Result	Units	Specification Limits	Method
Arsenic	<0.5	ppm		AOAC 2011.14, Modified
Cadmium	<0.5	ppm		AOAC 999.10, Modified
Mercury	<0.025	ppm		EPA Method 7471B (SW-846), Modified
Lead	<0.5	ppm		AOAC 999.10, Modified
E. Coli	<10	CFU/g		AOAC 991.14
Staphylococcus Aureus	<10	CFU/g		AOAC 2003.07
Salmonella	Negative	/25g		AOAC 2013.02
Coliforms	<10	CFU/g		AOAC 991.14
Yeast & Mold	<10, <10	CFU/g		AOAC 997.02
Listeria	Negative	/25g		AOAC 997.03

Approved

Greg Crosby
Chief Financial Officer

Questions about this analysis? Please contact customerservice@balabs.com

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Referee Chemists for American Oil Chemists* Society* Official Samplers and Chemists for the Chicago Board of Trade

Certificate of Analysis: V-1 **Page 1 of 1**

7.2.15 GRAS panel report

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Report of the GRAS Panel Concerning the *Generally Recognized As Safe* (GRAS) Status of the Proposed Use of Watermarked *Bacillus subtilis* AA07-1 Spores as an Incidental Additive for Tracing Food Products in the Supply Chain

GRAS Panel Members

Michael W. Pariza, Ph.D.

Joseph F. Borzelleca, Ph.D.

April 29, 2022

Introduction

Aanika Biosciences, Inc. (hereinafter Aanika) convened a panel of independent expert scientists (the "GRAS Panel"), qualified by their scientific training and national and international experience to evaluate the safety of ingredients added to foods, to conduct an independent and critical evaluation of the available information on its "watermarked" *Bacillus subtilis* AA07-1 spore preparation, and to determine if, in their opinion, the organism is safe and suitable and *Generally Recognized As Safe* (GRAS) based on scientific procedures for its intended use as an incidental additive for tracing leafy greens such as lettuce, spinach and kale; grains such as rice, wheat and corn; oils such as palm, olive and coconut; dairy products such as milk, cream, butter and cheese; and other food products in the supply chain. The members of the GRAS Panel were Professors Emeriti Michael W. Pariza and Joseph F. Borzelleca.

The GRAS Panel, individually and collectively, critically evaluated a comprehensive package of information and data (the dossier) compiled from the scientific literature and government databases, entitled "Generally Recognized as Safe (GRAS) Determination (for) Watermarked *Bacillus subtilis* AA07-1 Spore Preparation," prepared by Kevin O. Gillies, Head of Regulatory and Scientific Affairs for Aanika Biosciences, Inc. The dossier included the history of the use of *B. subtilis* strains in food fermentations, direct addition to foods intended for human consumption, direct-fed microbials for animal feed, and in the manufacture of enzymes used in food processing; the regulatory history of *B. subtilis* strains with particular focus on *B. subtilis* 168, the parental strain for *B. subtilis* AA07-1; a description of the genetic modifications made to *B. subtilis* 168 to construct watermarked *B. subtilis* AA07-1; the methods and procedures for manufacturing the *B. subtilis* AA07-1 spore preparation; the intended conditions of use of the *B. subtilis* AA07-1 spore preparation; and an assessment of the safety of the *B. subtilis* AA07-1 spore preparation for its intended use. The GRAS Panel also considered other materials that it deemed appropriate or necessary.

The GRAS Panel participated by teleconference with Mr. Gilles on April 25 2022. Following its independent and collective critical evaluation of the available information as part of this call, the GRAS Panel unanimously concluded that the proposed use of the *B. subtilis* AA07-1 spore preparation as an incidental additive for tracing of food products in the supply chain is safe and suitable and *Generally Recognized As Safe* (GRAS) based on scientific procedures.

Summary and Basis for GRAS

The watermarked *B. subtilis* AA07-1 spore preparation (AA07-1) was derived from *B. subtilis* 168. It consists of a water suspension of spores that have been genetically modified by (1) deleting genes required for germination, and (2) adding a non-functional DNA watermark comprised of fewer than 200 nucleotides, which enables the detection of AA07-1 by existing rapid, robust genetic techniques such as real-time polymerase chain reaction (PCR) and Next Gen Sequencing (NGS). The complete genome sequence of AA07-1 has been determined and, as expected, the strain is 99.9% identical to *B. subtilis* 168, differing only in very small DNA

sequence changes related to targeted deletion events and the addition of non-functional watermark DNA. The strain has been deposited with the American Type Culture Collection. (ATCC) as AAN000002.

AA07-1 will have no direct technical or functional effect in the foods to which it is added, but will instead serve solely as a novel mechanism for tracing the food in the event of a foodborne illness outbreak. The intended application level of AA07-1 to foods is approximately 10^6 spores/g, which is equivalent to an Estimated Dietary Intake (EDI) of approximately 4 x10^8 spores per day. Since the spores are very unlikely to germinate, it is expected that they will simply pass through the GI tract of consumers and be excreted in the feces.

Bacillus subtilis 168 has been in laboratory and commercial use for more 40 years without any report of adverse health effects. Its genome has been fully sequenced (F. Kunst *et al*., The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* 390: 249-256, 1997). It is the model microorganism of the Firmicutes phylum (E. Belda *et al*., An updated metabolic view of the *Bacillus subtilis* 168 genome. *Microbiology* 159: 757–770, 2013). *Bacillus subtilis* 168, its derivatives and closely genetically-aligned strains are the subjects of GRAS Notices 714, 905 and 955, all of which carry the decision statement, "FDA has no questions." Accordingly, *B. subtilis* 168 is a safe and suitable parental strain for the creation of AA07-1.

The genetic modification of *B. subtilis* 168, to construct AA07-1, consisted of deleting four genes required for spore germination, and adding a small non-functional sequence of less than 200 nucleotides. The gene deletions were made to limit the potential for the germination and growth of AA07-1 in the foods to which it has been added, so as to optimize its usefulness as a tracing agent while also minimizing the possibility of mutation and/or the transfer of genetic material to other microorganisms. The purpose of inserting the small non-functioning nucleotide sequence into the AA07-1 genome is to enable the detection of AA07-1 by existing rapid, robust genetic techniques such as real-time polymerase chain reaction (PCR) and Next Gen Sequencing (NGS). The inserted sequence is stably integrated in the AA07-1 which also minimizes the possibility that it might be transferred to other microorganisms.

The inserted non-functioning nucleotide sequence was analyzed using a number of different databases for its potential to code for toxins, virulence factors, antibiotic resistance, and allergens. No statistically significant homology to undesirable sequences was found. The methods and procedures used in the construction and manufacture of AA07-1, and the specifications for the final AA07-1 product, are appropriate for an incidental additive intended for use in foods.

AA07-1 complies fully with the elements of the decision tree of Pariza *et al*. (Determining the safety of microbial cultures for consumption by humans and animals. *Regulatory Toxicology and Pharmacology* 73: 164-171, 2015).

Conclusion

We, the members of the GRAS Panel, conclude that Aanika Biosciences, Inc.'s "watermarked" *Bacillus subtilis* AA07-1 spore preparation, manufactured consistent with *current Good Manufacturing Practice* (cGMP) and meeting appropriate food-grade specifications, is safe, suitable, and *Generally Recognized As Safe* (GRAS) based on scientific procedures for use as an incidental additive for tracing leafy greens such as lettuce, spinach and kale; grains such as rice, wheat and corn; oils such as palm, olive and coconut; dairy products such as milk, cream, butter and cheese; and other food products in the supply chain, at an intended application level of approximately 10^6 spores/g, equivalent to an Estimated Dietary Intake (EDI) of approximately 4 x10^8 spores per day.

It is our professional opinion that other qualified experts would concur with these conclusions.

Signature: _____ Date: 29 April 2022

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: 02 May 2022

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

7.3 LIST OF FIGURES

7.4 LIST OF TABLES

From: Kampmeyer, Christopher
To: Kevin Gillies
Cc: Hice, Stephanie; Vishaal Bhuyan
Subject: RE: [EXTERNAL] Re: GPS 000113 - Aanika Biosciences Co.
Date: Monday, December 12, 2022 2:21:01 PM
Attachments: image001.png
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image022.png
image023.png
image024.png
image025.png
image026.png

Hi Kevin,

Thank you for letting us know.

Best regards,
Chris

Chris Kampmeyer, M.S.
Regulatory Review Scientist

Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
christopher.kampmeyer@fda.hhs.gov

From: Kevin Gillies <kgillies@aanikabio.com>
Sent: Monday, December 12, 2022 2:18 PM
To: Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov>
Cc: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>; Vishaal Bhuyan <vb@aanikabio.com>
Subject: Re: [EXTERNAL] Re: GPS 000113 - Aanika Biosciences Co.

Hi Christopher,
I am no longer representing Aanika Biosciences for the GPS 000113 - Aanika Biosciences Co. notice. Please replace my email contact information with vb@aanikabio.com. Thank you.
Best,
Kevin

Kevin O. Gillies
Regulatory and Scientific Affairs
Aanika Biosciences, Inc.

[redacted]
http://www.aanikabio.com

From: Christine Scaduto
To: Hice, Stephanie
Cc: Vishaal Bhuyan; Josh Koch
Subject: Re: [EXTERNAL] RE: GRN 001095 - Questions for Notifier
Date: Friday, June 2, 2023 7:47:14 PM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png
Aanika GRN 001095 - Responses to FDA Questions.pdf

Dear Dr. Hice,

Please find attached our responses to questions for GRN 001095.

Warm Regards,

Christine

Christine Scaduto, Ph.D.
Director of Strategic Execution
Aanika Biosciences Inc.
86 34th St. Suite D-605
Brooklyn, NY 11232
www.aanikabio.com

On Mon, May 22, 2023 at 1:52 PM Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:

> Dear Christine,
>
> Submitting an amendment no later than June 5, 2023, is acceptable.
>
> However, should you find that you are not able to submit responses by the extended deadline of June 5, 2023, then I recommend that you request that we cease our evaluation of GRN 001095.
>
> Sincerely,

Stiffy Hice

Stephanie (Stiffy) Hice, Ph.D. (they/them/their)

Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

From: Christine Scaduto <cscaduto@aanikabio.com>
Sent: Monday, May 22, 2023 12:54 PM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Cc: Vishaal Bhuyan <vb@aanikabio.com>; Josh Koch <jkoch@aanikabio.com>
Subject: Re: [EXTERNAL] RE: GRN 001095 - Questions for Notifier

Hi Stiffy,

Thanks, we very much appreciate the clarification. Most of the clarifications we wanted to make in the text were in regards to the questions raised, so we'll proceed with including these in our response to the questions.

Would it be ok to submit our answers by Monday, June 5 (10 business days from 5/19), instead of this Friday? We would like to make sure our answers are as thorough as possible.

Thanks,

Christine

On Mon, May 22, 2023 at 11:38 AM Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:

> Dear Christine,
>
> It is important to note that we do not accept resubmitted or "corrected" versions of a GRAS notice during our evaluation. Instead, if the clarifying language is in relation to the questions we transmitted to you on May 2, 2023, we suggest that you ensure that your responses to our questions clearly and accurately reflect the information in support of your GRAS conclusion of safe use (e.g., identity, intended use, manufacturing, safety). Please do not submit a "corrected" version of your GRAS notice.
>
> Another option is to request that we cease to evaluate (CTE) the GRAS notice and to resubmit it. If the portions of the submitted GRAS notice that may benefit from clarification (as noted in your email) are large or are unrelated to the questions we transmitted to you, then I recommend that you request that we CTE this GRAS notice, and submit a new GRAS dossier with the revised sections at a later date.
>
> I hope this information was helpful; please let me know if you have any questions.
>
> Sincerely,
>
> Stiffy Hice
>
> **Stephanie (Stiffy) Hice, Ph.D. (they/them/their)**
>
> *Regulatory Review Scientist & Microbiology Reviewer*

Division of Food Ingredients

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

From: Christine Scaduto <cscaduto@aanikabio.com>
Sent: Friday, May 19, 2023 2:42 PM
To: Vishaal Bhuyan <vb@aanikabio.com>
Cc: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>; Josh Koch <jkoch@aanikabio.com>
Subject: Re: [EXTERNAL] RE: GRN 001095 - Questions for Notifier

Hi Stiffy,

Upon a re-read of our application we noted some sections that could benefit from clarifying language. Would it be ok to make clarifying edits to our original application, as long as we made clear which parts were changed? (track changes or similar).

Thanks,

Christine

On Fri, May 19, 2023 at 8:49 AM Vishaal Bhuyan <vb@aanikabio.com> wrote:

> We will have responses by the end of next week.
>
> Regards,
>
> Vishaal
>
> Vishaal B. Bhuyan
>
> Co-Founder & CEO
>
> Aanika Biosciences, Inc.
>
> www.aanikabio.com
>
> Sent via Superhuman iOS
>
> On Fri, May 19 2023 at 8:38 AM, Stephanie Hice <Stephanie.Hice@fda.hhs.gov> wrote:
>
> > Dear Mr. Bhuyan,
> >
> > Thank you for your reply.
> >
> > We understand, and look forward to receiving your response. Are you able to provide an estimated timeframe?
> >
> > Sincerely,

Stiffy Hice

Stephanie (Stiffy) Hice, Ph.D. (they/them/their)

Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

From: Vishaal Bhuyan <vb@aanikabio.com>
Sent: Friday, May 19, 2023 8:14 AM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Cc: Christine Scaduto <cscaduto@aanikabio.com>; Josh Koch <jkoch@aanikabio.com>
Subject: [EXTERNAL] RE: GRN 001095 - Questions for Notifier

Hello Dr. Hice,

My deepest apologies as your previous email was in my spam folder. My team and I will respond shortly.

Best

Vishaal

Vishaal B. Bhuyan

Co-Founder & CEO

Aanika Biosciences, Inc.

www.aanikabio.com

Sent via Superhuman iOS

On Fri, May 19 2023 at 8:08 AM, Stephanie Hice <Stephanie.Hice@fda.hhs.gov> wrote:

> Dear Mr. Bhuyan,
>
> I wanted to follow-up to my May 2, 2023, email to see when you intended to provide responses to our questions for GRN 001095? We typically request from a response within **10 business days**. If you are unable to complete the response within that time frame, you may contact me to discuss further options.
>
> Thank you for your attention to our comments.
>
> Sincerely,
>
> Stiffy Hice

Stephanie (Stiffy) Hice, Ph.D. (they/them/their)

Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

From: Hice, Stephanie
Sent: Tuesday, May 2, 2023 8:50 AM
To: Vishaal Bhuyan <vb@aanikabio.com>
Subject: GRN 001095 - Questions for Notifier

Dear Mr. Bhuyan,

During our evaluation of GRAS Notice No. 001095, we noted questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your response.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stiffy Hice

Stephanie (Stiffy) Hice, Ph.D. (they/them/their)

Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

--

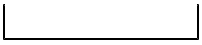
Christine Scaduto, Ph.D.

Director of Strategic Execution

Aanika Biosciences Inc.

86 34th St. Suite D-605

Brooklyn, NY 11232



www.aanikabio.com

--

Christine Scaduto, Ph.D.

Director of Strategic Execution

Aanika Biosciences Inc.

86 34th St. Suite D-605

Brooklyn, NY 11232

www.aanikabio.com

AANIKA

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

June 2, 2023

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

5001 Campus Drive

College Park, MD 20740

Attention: Stephanie A. Hice, Ph.D.

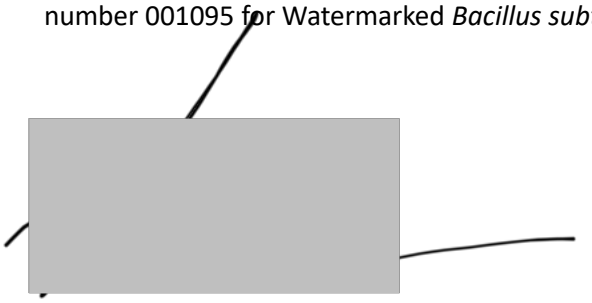
Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients

stephanie.hice@fda.hhs.gov

Dear Dr. Hice,

Aanika Biosciences submits this Addendum to answer questions/comments regarding GRAS Notification number 001095 for Watermarked *Bacillus subtilis* AA07-1 Spore Preparation.



Vishaal Bhuyan

CEO

Aanika Biosciences Inc.

86 34th St. Suite D-605

Brooklyn, NY 11232

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

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

FDA Questions/Comments Regarding GRN 001095:

1. The notifier states, "Because Aanika envisions the generation of many watermarked strains to be used in or on foods to provide unique identifiers of the food, it will employ the above safety evaluation for all newly created strains" (page 32).

a. Please clarify whether future "watermarks" will be subject to the same manufacturing process and specifications described in the submission, and whether the intended use and use level will remain unchanged as new "watermarks" are developed.

As watermarked *Bacillus subtilis* strains are generated they will be subject to the same manufacturing process and specifications described in the submission. The intended use and level of use will remain the same.

b. Further, the notifier states, "By extension, the AA07 strain may be used as a "chassis" strain for future modifications for various functionalities" (page 38, emphasis added). Please clarify whether the "chassis" strain will be used in the production of future "watermarks."

The germination-deficient strain AA07 will be used as the chassis strain for future watermarks.

2. The notifier states, "The spore preparation is then heated to kill any remaining vegetative cells. The multi-step recovery process is designed to recover spores from the culture broth and separate them from the vegetive cells. Centrifugation or filtration is used to recover spores and vegetative cells. This is followed by washes to remove nutrient broth components" (page 28). Please confirm whether the final preparation is comprised of 100% Bacillus subtilis strain ATCC AAN000002 spores.

To ensure that the final preparation contains only AAN000002 spores, the following steps are taken.

Pure initial seed stocks of the AAN000002 watermarked strain are generated for the production process by streaking the master stock of the strain for single colonies on an agar plate, overnight growth at 37°C, and then visual analysis to confirm the purity of the starting culture. A single colony from the plate is picked into liquid media, grown overnight, seed stocks are prepared, and the stocks are stored at -80°C. In parallel to this overnight culture, a sterility control containing media without inoculum is performed. If this were to show growth after overnight incubation, then the AAN000002 seed stock culture would be discarded. Seed stock cultures that pass these tests are then confirmed as the AAN000002 watermarked strain by sequencing. Production batches produced using these seed stocks are analyzed by microscopy to confirm the preparation only contains *B. subtilis* spores, and by sequencing to confirm it is the AAN000002 watermarked strain. Additionally, this final spore suspension is tested to confirm it only consists of AAN000002 by plating on rich microbiological media to show the absence of other viable

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Brooklyn, NY 11232

bacterial species (Tables 1 and 2); at this point the AAN000002 preparation is only present in its germination-deficient spore state, and so is not able to generate colonies on agar plates. The preparation is also tested by an external laboratory for the presence of coliforms, *E. coli*, *Listeria* species, *Salmonella* spp., *S. aureus*, yeasts, and molds (Tables 1 and 2).

3. Please describe whether B. subtilis strain ATCC AAN000002 produces any antibiotics, and whether this poses a safety concern.

Many *B. subtilis* strains are known to produce a range of antimicrobial agents such as non-ribosomally synthesized lipopeptides (Stein, 2005), however as a derivative of *B. subtilis* 168, AAN000002 does not produce these due to the inactive form of the *sfp* gene that it possesses (Tsuge *et al*., 2005). The Sfp gene product performs an essential step in the production of antimicrobial lipopeptides. As well as lipopeptides, many *B. subtilis* strains can also produce lantibiotic peptides such as subtilin, sublancin, and subtilosin. In the case of each of these, as a derivative of *B. subtilis* 168, their production by AAN000002 is very low or entirely absent (Liu *et al*., 1991; Ji *et al*., 2015; Babasaki *et al*., 1985). Only one antimicrobial produced by *B. subtilis*, bacitracin, is considered to be a medically important antimicrobial (World Health Organization, Critically important antimicrobials for human medicine). However, the genes required for synthesis of bacitracin are absent from the genome of *B. subtilis* AAN000002 and *B. subtilis* 168 (Ohki *et al*., 2003). Furthermore, any antimicrobial agents produced by *B. subtilis* are secreted into the media during vegetative growth, as AAN000002 is supplied in a spore form that is unable to germinate, and after the growth medium has been removed through successive washes with sterile water, these will be absent from the final product. Overall, antibiotic production by AAN000002 does not pose a safety concern.

4. The notifier states, "Whole genome sequence comparison of strain 168, AA07 and AA071 demonstrates that the strains exhibit antibiotic resistance traits similar to other Bacillus subtilis that have been reviewed by FDA that are currently sold in the US market and do not raise additional concerns related to the traits" (page 24). As it relates to B. subtilis strain ATCC AAN000002, for the administrative record, please clarify what "exhibit antibiotic resistance traits" means in this context.

The complete genome of AAN000002 was sequenced at over 200x depth. Sequencing confirmed that the intended changes to the genome were present and that the remainder of the genome matched that of the parental strain, *B. subtilis* 168. The sequence reads were aligned against the Ion AmpliSeqTM Antimicrobial Resistance (AMR) Research Panel to determine if any of 478 antimicrobial resistance genes across 25 antibiotic classes are present in the genome (Appendix A). Two genes from the AMR panel were found to be encoded by the genome of AAN000002, *aadK* and *tetL*. These gene sequences are also present in the genomes of *B. subtilis* derived food products such as those described in GRNs 000905 and 000955, both of which received "no questions" letters.

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AadK

The *aadK* gene encodes aminoglycoside 6-adenylyltransferase, a streptomycin-modifying enzyme. This gene is present in the parental strain of AAN000002, *B. subtilis* 168, and contributes to a low-level streptomycin resistance (Noguchi *et al*., 1993). Homology searches show that this gene is common to several *Bacillus* species and is thought to be an ancient and intrinsic part of the genome rather than being acquired by horizontal gene transfer (Agersø *et al*., 2019). Resistance to streptomycin is a known characteristic of both laboratory strains of *B. subtilis* and environmental isolates (Adimpong *et al*., 2012), the presence of the *aadK* gene in the *B. subtilis* AAN000002 genome is not a safety concern.

TetL

The *tetL* gene encodes a membrane transporter protein that facilitates efflux of tetracycline from the cell (Safferling *et al*., 2003) and confers low level resistance to tetracycline (Wei and Bechhofer, 2002). BLASTP analysis has shown that the gene sequence is shared among many *Bacillus* and other closely related species. As a widely conserved chromosomally encoded gene that is not associated with mobile genetic elements it is an intrinsic part of the *B. subtilis* genome, and its presence in the genome of *B. subtilis* AAN000002 is not a safety concern.

5. Please state whether all raw materials and processing aids used in the manufacture of B. subtilis strain ATCC AAN000002 are food grade.

All raw materials used in the manufacture of *B. subtilis* AAN000002 are food grade.

6. The notifier states, "No major food allergens are used in the process or formulation," and provides a reference to the Food Allergen Labeling and Consumer Protection Act (page 28).

a. Per the Food Allergy Safety, Treatment, Education, and Research Act, sesame has been added as one of the major food allergens. Please state whether any of the raw materials used in the manufacturing process are sesame or are derived from sesame. If any of the raw materials used are sesame or are derived from sesame, please discuss why these materials do not pose a safety concern.

None of the raw materials used in the manufacturing process are sesame or derived from sesame.

AANIKA

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b. The notifier states, "B. subtilis strain 168, from which the chassis strain AA07 is derived, is a tryptophan auxotroph (trpC2) and therefore requires the addition of tryptophan to the growth media, including media containing acid-hydrolyzed protein components such as casein" (page 37). Please state whether casein is used in the fermentation media.

Casein is not used in the growth medium.

7. When describing the results of the stability of the spore preparation when stored at room temperature (Figure 8), the notifier states "… there is essentially no change in spore count;" however, the data presented in Figure 8 suggest that there is a nearly 2-log increase in the amount of "tags" detected between T3 and T5 when stored at room temperature (less variation when stored at 4 °C). For the administrative record, please explain these results in further detail.

The observed increase in the amount of tags between T3 and T5 in the GRN 001095 Figure 8 stability data is a result of variability in the testing method, rather than actual changes in the stability of the AAN000002 spore preparation. The quantitative PCR (qPCR) method used to quantify the tags did show day-to-day variability, however throughout the time course the Ct value has never exceeded 25; values below this threshold indicate a strongly positive tag signal. Figure 1 shows the qPCR Ct values for the data presented in GRN 001095 Figure 8 extended to 12 months together with a line fit to the data using linear regression. From this, neither an upward nor downward trend in the stability data for AAN000002 can be seen over 12 months, confirming that the spore preparation has been stable over the measured period.

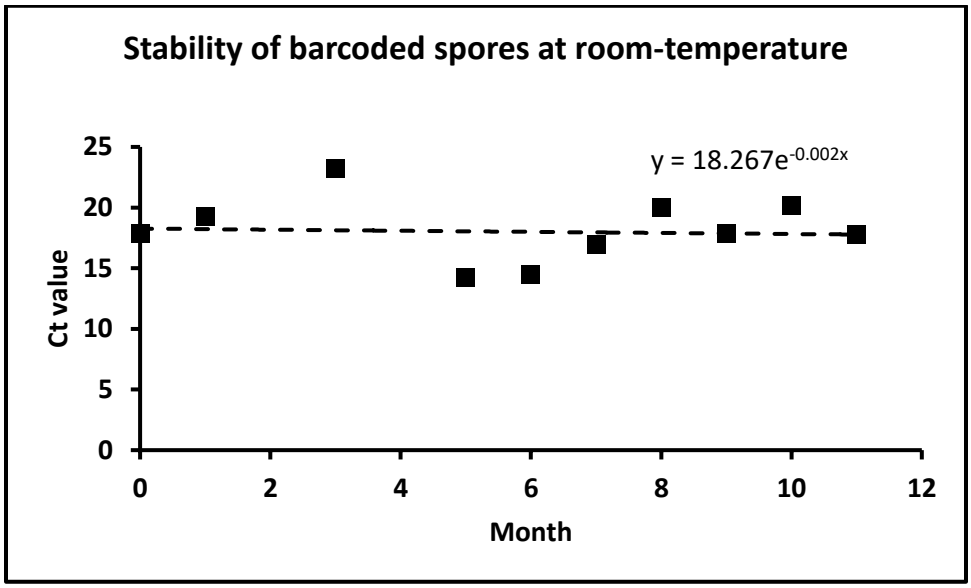
8. The notifier provides a list of specifications in Table 5 (pages 30-31):

a. The specification for E. coli is listed as negative in 25 g; however, in Table 6 (page 31) the specification for E. coli is listed as <10 CFU/g. The results of the batch analyses, and corresponding certificates of analyses (COAs), do not conform to the listed specification in Table 5. For the administrative record, please clarify this discrepancy.

This is a typographical error. The correct specification for *E. coli* is <10 CFU/g. See Table 1.

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

A



B

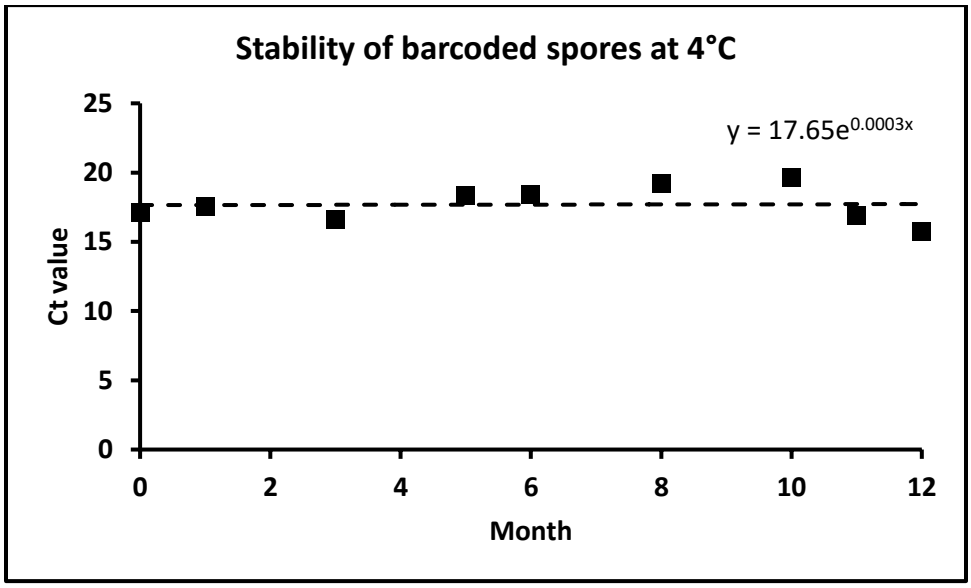


Figure 1. Stability of the AAN000002 spore preparation over a 12-month period. A qPCR analysis of spores stored at room-temperature. **B** qPCR analysis of spores stored at 4°C.

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86 34th St. Suite D-605
Brooklyn, NY 11232

Table 1. Food Grade Specifications for *B. subtilis* strain AA07-1. Amendment to GRN 001095 Table 5.

Physical and Chemical Parameters	Specification (Acceptable Target/Range)	Test Method
Appearance	Beige Slurry	Visual
Identity	Barcode Present	DNA Sequencing
Spore Count	> 10^9 spores/ml	Microscopy
Aerobic colonies	No colonies	LB-agar plate
Heavy Metals Limits	PPM	
Lead	<0.5	AOAC 2011.19, 993.14 and 2015.01 (modified)
Mercury	<0.1	
Cadmium	<0.5	
Arsenic	<0.3	
Microbiological Limits		
Yeast and Mold	≤300 CFU/g	FDA BAM Chapter 18 mod.
Salmonella species	Negative in 25 g	AOAC-RI 121501
Coliforms	< 30 CFU/g	AOAC 991.14
Escherichia coli	< 10 CFU/g	AOAC 991.14
Listeria species	Negative in 25 g	AOAC-RI 061702
Staphylococcus aureus	< 10 CFU/g	AOAC 2003.07

b. The corresponding method cited for the analysis of Listeria is AOAC 997.03; however, in one of the COAs, the referenced method is AOAC RI 081401 (page 71). For the administrative record, please confirm which method is used for the analysis of Listeria.

An external lab performs testing for Listeria spp. on our behalf using an accredited AOAC methodology. For two of the reported batches (P21-063A and P21-084B) method AOAC 997.03 was used. For the third batch (P21-084A), method AOAC RI 081401 was used. We are also providing analysis for an additional four non-consecutive batches (Table 2), and for those (Batch numbers 230227, 230308, 230315, and 230417), a third method, AOAC-RI 061702, was used. We now list the current method, AOAC-RI 061702, in Table 1.

c. Please specify whether Listeria refers to Listeria spp. or Listeria monocytogenes.

Listeria refers to Listeria spp. Test method AOAC 997.03 detects both *Listeria monocytogenes* and all other Listeria spp., AOAC RI 081401 detects all Listeria spp., and AOAC-RI 061702 detects both *Listeria monocytogenes* and all other Listeria spp.

d. The corresponding method cited for the analysis of coliforms is AOAC 997.02, which corresponds to the analysis of yeast and mold counts in foods; however, in the COAs, the referenced method is AOAC 991.14,

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which corresponds to analysis of coliform and E. coli counts in foods. For the administrative record, please clarify this discrepancy.

This is a typographical error. The correct method for analyzing coliforms is AOAC 991.14. See Table 1.

e. The specification limit for arsenic is listed as 0.3 mg/kg; however, the results of the batch analyses, and the corresponding COAs, do not conform to the listed specification limit. For the administrative record, please clarify this discrepancy.

For measuring arsenic levels, we are now using tests (AOAC 2011.19, 993.14, and 2015.01 (modified)) where the Limit of Quantification for arsenic is <10 ppb, or <0.01 mg/kg, thirty times lower than the specification limit. In addition, we are reporting results for an additional four non-consecutive batches (230227, 230308, 230315, and 230417; Table 2 and Appendix B) and the levels of arsenic in each is <0.01 mg/kg.

Table 2. Quality Control test results for four non-consecutive lots of AA07-01 spore preparations.

Physical and Chemical Parameters	Specification (Acceptable Target/Range)	Lot 230227	Lot 230308	Lot 230315	Lot 230417
Appearance	Beige Slurry	Conforms	Conforms	Conforms	Conforms
Identity	Barcode Present	Conforms	Conforms	Conforms	Conforms
Spore Count	> 10^9 spores/ml	Conforms	Conforms	Conforms	Conforms
Aerobic Colonies	No colonies	Conforms	Conforms	Conforms	Conforms
Heavy Metals Limits	PPM				
Lead	<0.5	<5.00 ppb[1]	<5.00 ppb	<5.00 ppb	<5.00 ppb
Mercury	<0.1	<5.00 ppb	<5.00 ppb	<5.00 ppb	<5.00 ppb
Cadmium	<0.5	<5.00 ppb	<5.00 ppb	<5.00 ppb	<5.00 ppb
Arsenic	<0.3	<10.0 ppb	<10.0 ppb	<10.0 ppb	<10.0 ppb
Microbiological Limits					
Yeast and Mold	≤300 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Salmonella species	Negative in 25 g	Negative in 25 g	Negative in 25 g	Negative in 25 g	Negative in 25 g
Coliforms	< 30 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Escherichia coli	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Listeria species	Negative in 25 g	Negative in 25 g	Negative in 25 g	Negative in 25 g	Negative in 25 g
Staphylococcus aureus	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g

[1]parts per billion, as reported in the analytical report for each lot tested for heavy metals

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9. Please state whether all analytical methods used to analyze the batches for conformance with the stated specifications have been validated for that particular purpose.

All analytical methods used to analyze batches conform with the stated specifications and have been validated for that purpose.

10. On pages 30-31, the notifier states, "Food grade specifications for QC release of B. subtilis strain AA07-1 (Table 3) conform to food industry norms and contained in GRAS notices reviewed by FDA," and "Quality Control test results summarized in Table 4 for 3 lots of AA07-1 demonstrating that the production process described in Section 2.3 is capable of producing product to the established food grade specifications in Table 4;" however, the tables presented on pages 30-31 are labeled Tables 5 and 6, and there does not appear to be a Table 4 in the notice. For the administrative record, please provide a statement affirming that the above quoted passages are meant to refer to Tables 5 and 6.

The quoted passages are in reference to Tables 5 and 6.

11. The notifier provides the results from the batch analyses in the table on page 31:

a. We typically request the results of analyses from a minimum three nonconsecutive batches. Please confirm that Lots P21-084A, P21-084B, and P21063A are non-consecutive batches or provide the results from the analyses of additional non-consecutive batches, if needed.

The three batches analyzed (P21-084A, P21-084B, and P21063A) are non-consecutive, in addition we are providing new analyses from four non-consecutive batches (Batches 230227, 230308, 230315, and 230417). See Table 2 and Appendix B.

b. The levels of lead in the batch analyses of B. subtilis strain ATCC AAN000002 range from <0.5 mg/kg to 0.965 mg/kg. We typically do not see levels of lead this high in an ingredient that is produced by controlled fermentation from a pure bacterial culture in accordance with current good manufacturing practices. Please discuss the possible sources of the lead in the batches of the ingredient.

Our SOPs now require that all raw materials are sent for heavy metal analysis, and that the amount of heavy metals present conforms to the criteria set in Table 1, prior to their use in production. Under these new protocols, including the use of more sensitive testing methods, our new batches are shown to contain very low amounts of lead (see Table 2).

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c. Please explain the significant difference (20 times) in the level of mercury in Lot P21-063A (<0.5 mg/kg) compared to the levels in Lots P21-084A and P21-084B (<0.025 mg/kg).

There is a typographical error in GRN 001095 Table 6, listing the level of mercury in Lot P21-063A as <0.5 ppm. The COA for that lot reports the level of mercury to be <0.025 ppm, meaning the levels of mercury for all three lots are the same (<0.025 mg/kg).

d. We note that most of the results for heavy metals are reported as "<" values. Please provide the limit of quantitation (LOQ) and limit of detection (LOD) for the methods used to test for lead, mercury, cadmium, and arsenic. In addition, please provide the results representing the measured levels of heavy metals if those levels are ≥ LOQ, or state that the levels are < LOQ (or LOD). If the results reported as "<" value represent LOD or LOQ of the method. We note that there are more sensitive analytical methods that the notifier may consider using for testing batches of the ingredient. We also recommend that the notifier consider lowering the specification limits for heavy metals to be reflective of batch analyses and to be as low as possible. As the notifier is probably aware, FDA has released a "Closer to Zero" initiative that focuses on reducing dietary exposure to heavy metals to as low as possible.

For heavy metal analysis, our external testing partner uses inductively coupled plasma with mass spectrometric detection (ICP-MS). As stated above, for heavy metal testing, we are now using tests AOAC 2011.19, 993.14, and 2015.01 (modified). For these, the LOQ is <10 ppb (parts per billion) for As (<0.01 mg/kg) and <5 ppb for Pb, Cd, Hg (<0.005 mg/kg). For the newly analyzed batches, the reported value for each heavy metal corresponds to the LOQ (Table 2.)

In order to keep the heavy metal content of our product as low as possible, we have lowered our specification limits for lead (from <1 ppm to <0.5 ppm) and mercury (from <0.5 ppm to <0.1 ppm) (Table 1).

12. Please confirm that B. subtilis strain ATCC AAN000002 is not intended for use in foods where standards of identity preclude its use.

B. subtilis ATCC AAN000002 is not intended for use in foods where standards of identity preclude its use.

13. The notifier states that the intended uses of B. subtilis strain ATCC AAN000002 include but are not limited to food categories listed on page 32. If the intended use is in food categories that are in addition to those listed on page 32, please specify these additional food categories and provide the revised dietary exposure estimate based on all the intended uses of the ingredient.

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The intended uses of *B. subtilis* ATCC AAN000002 are the food categories listed on page 32, however we have widened these categories to include all foods present in each category. These categories are vegetables, grains, oils, and dairy. Three additional categories have also been added, nuts and seeds, fruit, and seafood. Revised dietary exposure estimates for these are included in the response to question 14.

14. The notifier provides the dietary exposure estimate based on food consumption data from the 2007-2008 National Health and Nutrition Examination Survey (NHANES). Please provide the dietary exposure estimate based on available recent U.S. food consumption data.

The most recent food consumption dataset available is the 2017-March 2020 pre-pandemic NHANES. However, the Food Commodity Intake Database (FCID) (https://fcid.foodrisk.org/percentiles), which disaggregates the gram weights of the individual ingredients in the foods listed in the NHANES dataset, has not been updated with current NHANES data since 2010. Because of this, Conrad *et al*. (2022) have published an updated consumption analysis of food commodity groups using the 2011-2018 NHANES data. We have calculated the dietary exposure for *B. subtilis* ATCC AAN000002 using this analysis of the 2017-2018 NHANES and have also widened the exposure categories to cover the entire category, rather than a subset of commodities (Table 3). From this data, in an extreme case where the entirety of the food consumed in each of these groups were tagged with AAN000002 at 1×10^6 spores per gram, the Estimated Dietary Intake would be 8.17×10^8 spores/day. This level of dietary exposure is several times lower than the *Bacillus* derived products described in GRNs 000831, 000905, 000955, and 000956.

Table 3. Estimated daily intake of AAN000002. Average daily consumption of complete food categories using analysis of the 2017-2018 NHANES dataset compiled by Conrad *et al*. (2022). Vegetables: Dark green vegetables, Red and orange vegetables, Starchy vegetables, Other vegetables, and Legumes. Grains: Grains. Oils: Vegetable and seed oils, and Tropical oils. Dairy: Dairy. Seeds and nuts: Seeds and nuts. Fruit: Citrus fruit, melons, and berries, Other fruit. Seafood: Seafood.

Food category	Average daily intake (g)	AAN000002 applied (spores/g)	Total number of spores
Vegetables	219.73	1.00E+06	2.20E+08
Grains	279.10	1.00E+06	2.79E+08
Oils	36.27	1.00E+06	3.63E+07
Dairy	171.04	1.00E+06	1.71E+08
Seeds and nuts	9.50	1.00E+06	9.50E+06
Fruit	84.70	1.00E+06	8.47E+07
Seafood	16.60	1.00E+06	1.66E+07
		Total	8.17E+08

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15.) On page 10, the notifier states that GRNs 000831, 000905, 000955, and 000969 are "… incorporated herein by reference," but does not identify or summarize the relevant information from each GRAS notice that is incorporated. As each GRAS notice stands on its own, for the administrative record, please briefly summarize the information incorporated by reference from the GRAS notices listed on page 10.

Please find the information relevant from each notification summarized below:

- **GRN000831** describes a *Bacillus subtilis* spore preparation of strain DE111. The authors report that the genome of this strain has 95% identity to *Bacillus subtilis* 168. The product is intended to be added to a wide range of foods, including baked goods, breakfast cereals, chewing gum, tea/coffee, gelatins, and grain products. The estimated daily intake in adults is 1.3×10^{11} CFU/day. The study includes four unpublished clinical trials performed by the notifiers, all of which conclude that consumption of DE111 is safe for humans.

- **GRN000905** concerns a spore preparation of *Bacillus subtilis* SG188 (DSM 32444), for use in beverages such as milk drinks and juices, as well as dry and shelf-stable food. The intended consumption level is 10^9 CFU per serving. Through both sequencing of the *gyrA* gene and whole genome sequencing, the authors concluded that strain SG188 is closely related to *B. subtilis* 168. Through analysis of all coding ORFs (open reading frames), the authors determined that strain SG1888 did not contain any medically important antibiotic resistance genes. Consistent with this, SG188 did not carry any MIC values above those recommended by EFSA for antibiotics of human and veterinary importance. This notice also describes numerous published clinical studies documenting the safety of consumption of several *Bacillus subtilis* strains (R0179, 3H, CU1) by both healthy and immunocompromised individuals (Hanifi *et al.*, 2015; Pushkarev *et al*., 2007; Lefevre *et al*., 2017).

- **GRN000955** describes *Bacillus subtilis* strain MB40, a proprietary strain derived from *Bacillus subtilis* DSM 10 (DSMZ) and deposited with the ATCC (BS-MB40-PTA122264). Whole genome sequencing of this strain demonstrated 99% identity with *Bacillus subtilis* strain 168. MB40 is intended as an ingredient in a wide variety of foods including but not limited to baked goods, beverages, cheeses, coffee/tea, juices, processed vegetables, and sweet sauces. The maximum EDI is 3.64×10^{10} CFU/day. The authors performed a safety evaluation using a bioinformatics approach (virtual PCR and nBLAST) and concluded that the MB40 had no in-frame matches for genes encoding major enterotoxins (Hbl, Nhe, CytK, entFM, BceT) produced by illness-related *Bacillus* species. They found no evidence that MB40 produces antibiotics used in clinical or veterinary medicine or that could disrupt the intestinal microbiome. The authors describe a published short-term toxicity study performed in rats (Spears *et al*., 2020), which involved 14-day oral gavage at a range of doses, with a maximum of 8.51×10^{10} CFU/day. No mortality or treatment-related effects were found, and a NOAEL (No Observed Adverse Effect Level) of 2000 mg/kg bw/day, or 8.51×10^{10} CFU/day was reported. The authors also described two studies testing the safety of MB40 in human clinical trials. In the first, (Spears *et al*., 2020), twenty-seven normal, healthy adult volunteers received a dosage of 10 billion CFU/day for 21 days. No

adverse effects were reported, and the authors concluded that administration of MB40 within this dosage and time was well tolerated. In the second (Penet *et al.*, 2019), participants (99 total, 50 in treatment group and 49 in placebo group) received a single dose of OPTI-BIOME (MB40) containing 5 x 10^9 CFU of MB40 or placebo once daily for 28 days. No serious adverse events were reported, and the authors concluded that the product was well tolerated.

- **GRN000969** concerns a spore preparation of *Bacillus subtilis* strain Bss-19 (ATCC SD-7780) for use in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary, snacks, and other foods at a level of 1 x 10^{10} CFU per serving. The estimated dietary exposure for Bss-19 is 9.1 x 10^{10}-1.4 x 10^{11} CFU/day if 50% of food servings contain the maximum intended use level of Bss-19, and 1.82-2.78 x 10^{11} CFU/day if 100% of food servings contain Bss-19 at the maximum intended use level. The authors reported an unpublished acute toxicity study performed in three female rats each given a dose of 5000 mg/kg of Bss-19. No adverse effects were reported and the LD50 (lethal dose) was determined to be greater than 5000 mg/kg body weight in female rats. The authors did not perform clinical studies of the safety of Bss-19 but cited published human studies regarding the safety of DE111, a closely related *B. subtilis* strain. In one such study (Cuentas *et al*., 2017), 1 x 10^9 CFU DE111 or placebo was administered daily for 105 days in 50 adults. Safety was monitored by measuring C-reactive protein lipid panels and complete metabolic panels. Both reports stayed within normal range for both the treatment and placebo groups. Similarly, a study by Maher *et al*. (2019) concluded that daily administration of 5 x 10^9 CFU DE111 was well tolerated in healthy young adults.

16.) In Section 2.2.2, the notifier lists several GRAS notices, where the subject of the notice was a strain of B. subtilis for use as a spore preparation or in the production of an enzyme, that have been submitted to FDA and have received "no questions" letters (page 20). We evaluated GRNs 000746, 000751, 000861, 000956, 000974, and 001011, and responded in letters respectively dated June 13, 2018, July 31, 2018, July 21, 2020, August 18, 2021, February 2, 2022, and July 18, 2022 (correction letter dated January 11, 2023), stating that we had no questions at the time regarding the notifiers' GRAS conclusions. We note these GRAS notices are not included in the notifier's list. For the administrative record, please briefly discuss these GRNs in the context of the notifier's safety conclusion.

- GRAS notice **GRN000746** describes the production of the enzyme maltogenic amylase from *Bacillus subtilis* strain RF12029. Strain RF12029 is derived from a parental strain which was modified by conventional mutagenesis to prevent sporulation. Strain RF12029 has been genetically modified to contain a synthetic maltogenic amylase gene derived from *Geobacillus stearothermophilus*. The enzyme is produced by submerged-batch fermentation. Quality control measures are similar to our own, in that purity specifications of the final product require *Salmonella* and *E. coli* species to be absent in 25g of sample, consistent with food industry standards. Overall, this notice provides additional support to the first bullet of Section 2.2 – a "no question" letter issued for GRNs relating to the use of *Bacillus subtilis* as a production

organism for food enzymes.

- GRAS notice **GRN000751** describes production of the enzyme maltogenic alpha amylase, a processing aid used in baking and brewing. The production strain is BRG-1, which is a *Bacillus subtilis* strain expressing maltogenic alpha-amylase derived from *Bacillus stearothermophilus* and additionally containing modifications to inactivate certain proteases and prevent sporulation. Fermentation was conducted in accordance with Good Manufacturing Practices, and enzyme was isolated from the microbial biomass, purified, and concentrated. Three enzyme batches were analyzed for potential contaminants, including lead (limit of < 5mg/kg), *Salmonella* species (absent in 25g of sample) total coliforms (no more than 30 per g), and *E. coli* (absent in 25g of sample). We note that we use similar safety standards and that our upper threshold for the presence of lead is 10-fold lower. Additionally, the general safety of *Bacillus subtilis* for human consumption is described. Overall, this application provides additional evidence of the safety of food-grade enzyme production using *Bacillus subtilis*, as discussed in Section 2.2 of our application.

- GRAS notice **GRN000861**, "Pullulanase from *Bacillus deramificans* Expressed in *Bacillus subtilis* as a Food Processing Aid" describes the production of the enzyme pullulanase, an enzyme used for hydrolysis of carbohydrates during processing of starch-containing foods, from genetically modified *B. subtilis*. The production strain contained genetic modifications including the inactivation of proteins that play a role in spore formation and the integration of a truncated version of the *B. deramificans* pullulanase gene at the *amyE* locus. Pullulanase is produced by submerged fermentation and secreted into the supernatant by *B. subtilis*. The enzyme is manufactured by cGMP standards. Once purified, the final enzyme preparation conforms to purity described in *Food Chemicals Codex*. The authors cite several scientific studies supporting the safety of *B. subtilis* as a production organism, including that the species meets the criteria for a safe production organism as described by Pariza and Foster (1983), that it is well characterized in the production of food enzymes (Kunst *et al*., 1997), and that it has been shown not to contain any genes with those that encode known *B. cereus* enterotoxin (de Boer and Diderichsen, 1991; Olempska-Beer *et al*., 2006). Overall, this notification supports our safety conclusion.

- GRAS notice **GRN000956** pertains to a spore preparation of *B. subtilis* strain ATCC SD-7280. SD-7280 is a proprietary strain of Advanced Enzymes Technologies Ltd that was originally isolated from the soil. The spores are intended for consumption in baked goods, breakfast cereals, coffee/tea, process grains, processed fruits, processed vegetables, and additional food items at 1×10^6 to 6×10^9 CFU per serving. The Estimated Dietary Intake for SD-7290 is 1.1×10^{11} CFU/day, which we note is significantly greater than the EDI provided in our application, 8.17×10^8 spores/day. The spores are produced by fed batch fermentation in occurrence with cGMP practices, and all food grade materials are used in spore preparation. The spores are separated from the medium by centrifugation and subsequently washed and spray dried. The authors performed an acute oral toxicity study and a repeat-dose toxicity analysis in rats, both of which demonstrated no mortality or clinical abnormalities. This is consistent with six additional studies

cited in this proposal which demonstrated that consumption of *B. subtilis* is generally safe in animals across six *B. subtilis* strains. Additionally, the authors cite over ten human studies with numerous *B. subtilis* strains demonstrating that no adverse effects are observed when *B. subtilis* is administered to humans. These findings are consistent with our safety conclusion that *B. subtilis* is safe to consume at an EDI of 8.17×10^8 spores/day.

- In GRAS notice **GRN000974** the notifier (AB Enzymes Inc) describes a maltogenic amylase enzyme preparation from genetically modified *Bacillus subtilis*. The intended use of maltogenic amylase is as a processing aid in food manufacturing. The production strain is RF130138, which contains a synthetic gene encoding maltogenic amylase derived from *Bacillus stearothermophilus* as well as a hydrolase gene derived from *B. amyloliquefaciens* in order to aid in the fermentation process. The parental strain of RF130138 was also genetically modified to introduce an intended auxotrophy and to prevent sporulation. The enzyme preparation is produced by fed-batch fermentation. Production is done in accordance with current Good Manufacturing Practices for food and following the principles of Hazard Analysis of Critical Control Points. The raw materials conform to specifications set out in the Food Chemical Codex. During fermentation, the enzyme is secreted into the media, isolated by filtration, and concentrated. The final enzyme preparation is compliant with JECFA specifications. The authors further describe a toxicity assay using the supernatant of RF130138 and found that it was non-toxic to Vero cells. The authors note that *B. subtilis* has been used as an enzyme producer for many years without safety problems, and that the EPA published an extensive risk assessment of *B. subtilis*, including its history of commercial use (1997) and concluded it is not a human pathogen and is non-toxic.

- GRAS notice **GRN00010111** discusses the production of alpha-amylase from genetically modified *B. subtilis*. The use of alpha-amylase is as a processing aid in baking. The production strain is AR-651, which contains a plasmid expressing alpha-amylase derived from *Thermoactinomyces vulgaris*, a *Bacillus spp.* hydrolase gene, additional regulatory elements, and a complementary auxotrophic gene to monitor for transformation. Specific genomic deletions were made in the parental strain of AR-651, rendering the strain sporulation deficient and introducing a specific auxotrophy. No antibiotic resistance genes were present in the final AR-651 strain. Genetic stability of AR-651 was assayed by activity of the alpha-amylase preparation. Preparation of alpha-amylase was done by microbial fermentation followed by filtration and concentration. Food grade materials were used, and the preparation followed cGMP and HACCP standards. The final enzyme preparation was JECFA compliant. The general safety of *B. subtilis* as a production strain is discussed, and the authors note that *B. subtilis* has been used in enzyme food production for decades without report of adverse effects to humans or the environment (de Boer and Diderichsen, 1991).

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17. Please provide an updated literature search that discusses the safety of B. subtilis, including the date (month and year) the literature search was performed. Please discuss how these studies pertain to the safety of the intended uses of the ingredient. Examples include, but are not limited to, the following:

a. La Jeon, Y., Yang, J., Kim, M., Lim, G., Cho, S., Park, T., Suh, J., … Lee, H. (2012). Combined Bacillus licheniformis and Bacillus subtilis infection in a patient with oesophageal perforation. Journal of Medical Microbiology, 61, 1766-1769. doi: 10.1099/jmm.0.042275-0

b. Tanaka, I., Kutsuna, S., Ohkusu, M., Kato, T., Miyashita, M., Moriya, A., and Ohkusu, K. (2022), Bacillus subtilis variant natto bacteremia of gastrointestinal origin, Japan. Emerging Infectious Diseases, 28(8), 1718-1719. doi: 10.3201/eid2808.211567

c. Harwood, C. R., Mouillon, J., Pohl, S., and Arnau, J. (2018). Secondary metabolite production and the safety of industrially important members of the Bacillus subtilis group. FEMS Microbiology Reviews, 42, 721-738. doi: 10.1093/femsre/fuy028

An updated literature search was performed in May of 2023, which was inclusive of new literature published since submission of our GRAS notification (2022-2023). We found three additional studies that address the safety of *B. subtilis* in human trials or in industrial applications, summarized below:

Garvey, S.M., Mah, E., Blonquist, T.M. *et al.* (2022) The probiotic *Bacillus subtilis* BS50 decreases gastrointestinal symptoms in healthy adults: a randomized, double-blind, placebo-controlled trial. *Gut Microbes,* (14):1, 2122668.

- The authors investigated the safety and efficacy of consuming *B. subtilis* BS50 for the treatment of gastrointestinal symptoms. The study was a randomized, double-blind placebo-controlled clinical trial in 76 healthy adults, with 38 participants consuming placebo and 38 consuming BS50 at a dosage of 2 x 10^9 CFU/day (in capsule form) for six weeks. The authors reported that there were no clinically meaningful changes in safety laboratory values, and no serious adverse effects were reported. It was determined that consumption of 2 x 10^9 CFU/day of BS50 was well tolerated in healthy adults.

Piewngam, P., Khongthong, S. Roekngam, N., *et al*. (2023) Probiotic for pathogen-specific *Staphylococcus aureus* decolonization in Thailand: a phase 2, double-blind, randomized placebo-controlled trial. *Lancet Microbe*, 4: e75-83.

- In this study the authors tested the effect of consumption of *B. subtilis* strain MB40 on controlling *Staphylococcus aureus* colonization in humans. Fifty-five participants were assigned to the placebo group and sixty received a dosage of 10 x 10^9 CFU of strain MB40 once a day for thirty days. No severe adverse effects were reported, and no changes to the overall composition of the intestinal microbiome were detected. The study met the primary outcome of reducing *S. aureus* colonization in the intestine and nares. The authors concluded that *B. subtilis* MB40 was a safe and effective product for use in reducing *S. aureus* colonization.

Kim, S.H., Yehuala, G.A., *et al.* (2002) Safety Evaluation of *Bacillus subtilis* IDCC1101, Newly Isolated from Cheonggukjang, for Industrial Applications. *Microorganisms*, (10): 2494.

- This paper examines the safety of a newly isolated *B. subtilis* strain, IDCC1101. Using whole genome sequencing, the authors found genes encoding secondary metabolites such as fengycin, bacillibactin, and bacilysin. The genome did not encode enterotoxin genes associated with pathogenicity in *B. cereus*. IDCC1101 did not exhibit hemolytic activity on blood agar. The authors noted that antibiotic resistance and virulence genes were unlikely to be transferred to other organisms as they were not proximal to mobile elements in the genome. The strain was susceptible to medically relevant antibiotics (as recommended by EFSA) with the exception of streptomycin. The strain was non-toxic in HaCaT cells and rats. The authors concluded that EDCC1101 was safe for use in industrial applications.

In addition, below we address studies requested by the FDA:

a. La Jeon, Y., Yang, J., Kim, M., Lim, G., Cho, S., Park, T., Suh, J., … Lee, H. (2012). Combined Bacillus licheniformis and Bacillus subtilis infection in a patient with oesophageal perforation. Journal of Medical Microbiology, 61, 1766-1769. doi: 10.1099/jmm.0.042275-0

- This article describes a case of bacteremia and mediastinitis which the authors state is caused by a co-infection of *B. subtilis* and *B. licheniformis*. The 71-year-old male patient had a history of pulmonary tuberculosis, a mild drinking habit, and was taking medicine for chronic COPD. The authors describe that 6 colonies were isolated from the blood and pleural fluid on days 1-7. One colony was identified as *B. subtilis* and the other five as *B. licheniformis*. The authors concluded that these microbes were the causative agents in the disease but noted that in the case of *B. licheniformis* "the possibility of contamination cannot be ruled out completely." The authors report that "pre-disposing conditions to non-anthracis *Bacillus* infections include alcoholism and diabetes." They further note that cases of *Bacillus* bacteremia have previously been reported in immunocompromised patients.

- We note that some authors have stated that *Bacillus* infections "tend to be circumstantial rather than unambiguously proven." (Harwood *et al*., 2018). In line with this assertion, in a discussion of *Bacillus* safety within GRN000905, the notifiers mention that experiments to fulfill Koch's postulates, considered the "gold standard" for identifying microbes as the causative agents of disease (Segre, 2014), have "never been reported" for cases of *Bacillus* infection that were not caused by *B. cereus* and *B. anthracis*.

- A large body of clinical trials indicate that human consumption of *Bacillus subtilis* is safe (Hanifi *et al*., 2015; Lefevre *et al*., 2017; Pushkarev *et al*., 2007; Spears *et al*., 2020). Additionally, the EPA has stated that *Bacillus subtilis* "is not considered pathogenic or toxigenic to humans, animals, or plants." (Environmental Protection Agency, 1997).

- Our spore preparations are genetically "locked" in the metabolically inactive spore state, and *B. subtilis* 168 is not known to encode any enterotoxins associated with pathogenic *Bacillus* species.

- Given the above points, this article does not contradict our statement that our product is GRAS under its intended use.

b. Tanaka, I., Kutsuna, S., Ohkusu, M., Kato, T., Miyashita, M., Moriya, A., and Ohkusu, K. (2022), Bacillus subtilis variant natto bacteremia of gastrointestinal origin, Japan. Emerging Infectious Diseases, 28(8), 1718-1719. doi: 10.3201/eid2808.211567

- This research articles presents a single case of bacteremia caused by *Bacillus subtilis* var. *natto* after gastrointestinal perforation in a 56-year-old woman in Japan. The patient had a history of hypertension and consumed natto every day. *B. subtilis* was detected in blood culture samples on day 11 of treatment, and its identity was confirmed by its inability to grow on medium lacking biotin and the presence of an early stop codon in the *bioW* gene, which is described as being essential for biotin production and specific to *B. subtilis* var. natto.

- We would note that this infection appears to have occurred after gastrointestinal perforation, suggesting that an initial separate injury may have been required for infection. Additionally, fermented soy foods have been commonly consumed in Asia for centuries (Cao *et al*., 2017), with the discovery of natto suggested to have occurred thousands of years ago in Japan (Afzaal *et al*., 2022). A discussion of fermented soy products can also be found in GRN000956, which cites commonly consumed products such as natto, ogiri, dawadwa, and cheonggukjang, among others. It is further asserted by Harwood *et al*. (2018) that 7 billion servings of natto are consumed annually in Japan.

- Our spore preparation does not germinate which we would expect would minimize its ability to cause infection after gastrointestinal injury, as described above.

- Given the long history of safe consumption of natto and the inability of our spore product to germinate, we conclude that the findings described in this paper do not contradict our assertion that our product is GRAS under its intended use.

c. Harwood, C. R., Mouillon, J., Pohl, S., and Arnau, J. (2018). Secondary metabolite production and the safety of industrially important members of the Bacillus subtilis group. FEMS Microbiology Reviews, 42, 721-738. doi: 10.1093/femsre/fuy028

- This review article discusses the production of secondary metabolites from industrially relevant *Bacillus* species. Secondary metabolites are defined as “small organic molecules that are normally non-essential for the growth and development of the producing organism, but which contribute to their fitness over an evolutionary time.” The review focuses on two categories of secondary metabolites, polyketide synthases (PKs) and non-ribosomal peptide synthetases (NRPS). The authors also mention ribosomally synthesized and post-translationally modified peptides (RiPPs) that are structurally and functionally similar to PKs and NRPS. The authors take a computational approach, using a program known as antiSMASH3.0 (Weber *et al.* 2015) in

order to identify genes encoding these secondary metabolites within the genomes of completely sequenced *Bacillus* species (accessed through the National Center for Biotechnology Information). Within the *B. subtilis* 168 genome, the authors identify the following metabolites: Bacillibactin, Plipastatin/Fengycin, Surfactin, Bacilysin, Bacillaene, and Sublancin 168. The authors note that *B. subtilis* 168 is unable to produce surfactin, plipstatin, or bacillaene due to harboring an inactive form of the *sfp* gene which encodes 4-phosphopatenthinyl transferase. Thus, these metabolites will not be discussed further. Bacilysin is an antimicrobial peptide which causes cell lysis in bacteria and fungi (Islam *et al.*, 2022). Sublancin 168 is an antimicrobial peptide active against gram-positive bacteria. Neither Bacilysin nor Sublancin 168 are listed in the sixth edition of the World Health Organization Critically Important Compounds (WHO, 2019), which ranks medically important compounds to monitor in order to manage antimicrobial resistance. Bacillibactin is a siderophore that obtains iron in the environment (Hider and Kong, 2018, as cited in Harwood *et al*., 2018) and transports it to the cytoplasm. The authors note that there are "no direct reports" of toxicity associated with bacillibactin.

- Moreover, in addition to considering the toxicity and/or antibiotic activity of individual secondary metaboblites, we note that there are at least two main characteristics of our product that render threats of toxicity unlikely. First, our spore preparations are carefully washed three times, and all excess media is removed. The product is also diluted at the time of application, usually on the order of a 1/1000 dilution, meaning that any secondary metabolite residue that may have been secreted during fermentation would be negligible. Perhaps more importantly, our product is genetically engineered to remain "locked" in the spore state, via the deletion of key germination genes, as described in our notification. Given that spores are notoriously dormant with little to no metabolic activity (Setlow, 2014), we see it as highly unlikely that our product would be capable of producing secondary metabolites on food products.

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

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

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

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

ONE CODEX

Ion AmpliSeq™ Antimicrobial Resistance Research Panel

This report summarizes the results of the Ion AmpliSeq™ Antimicrobial Resistance (AMR) Research Panel run on sample **Quadruple/Bc1.1 (3834db6da42a4bff)**. The Ion AmpliSeq™ Antimicrobial Resistance (AMR) Research Panel is comprised of a total of 814 amplicons to assess the presence of 478 antimicrobial resistance genes across 25 antibiotic classes. Additional information is available in our documentation portal.

The sequences in this sample are predicted to be resistant to **Aminoglycosides and Tetracyclines** class antibiotics. 2 genes have a status of "present" and are predictive of resistance. The table below summarizes the predicted status of each drug class analyzed.

DRUG CLASS	GENE	ACCESSION	STATUS	COVERAGE / DEPTH / ID
Aminoglycosides	**aadK-1**	M26879	Present	100.00% / 286.43x / 99.80%
Bacitracin			Absent	
Beta-lactams			Absent	
Bleomycin			Absent	
Chloramphenicol			Absent	
Fosfomycin			Absent	
Fusaric acid			Absent	
Fusidic acid			Absent	
Integrase			Absent	
Lincosamides			Absent	
Mls (macrolides, lincosamides, streptogramins)			Absent	
Macrolides			Absent	
Multidrug efflux			Absent	
Mupirocin			Absent	
Nitroimidazole			Absent	
Platensimycin			Absent	
Polymyxin			Absent	
Quarternary ammonium compounds			Absent	
Quinolones			Absent	
Streptogramins			Absent	
Streptothricins			Absent	
Sulfonamides			Absent	
Tetracyclines	**tetLc**	X08034	Present	100.00% / 323.81x / 99.78%
Trimethoprim			Absent	
Vancomycin			Absent	

For each gene, the status is called as 'Present' if coverage is >=85% and identity is >=95%, and 'Probable' if coverage is >80% and identity is >90%. The coverage, depth, and identity statistics for each resistance gene are calculated as a weighted average of the corresponding markers with the highest detection status. These statistics are calculated from a sequence alignment to a curated reference database. The full amplicon-level results are available from https://app.onecodex.com/panel/9c1f2ca3585e4769.

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

ANALYTICAL REPORT

AR-23-UC-009531-01

Client Code: UC0000401

Received On: 23May2023
Reported On: 31May2023

Eurofins Sample Code:	126-2023-05230082	**Sample Registration Date:**	23May2023
Client Sample Code:	230227	**Condition Upon Receipt:**	acceptable, 18.5°C
Sample Description:	Bacillus subtilis spores	**Sample Reference:**	

FS001 - Heavy Metals (As, Cd, Hg, and Pb)	Reference	Accreditation	Completed	Sub
	AOAC 2011.19, 993.14 and 2015.01 (modified)		30May2023	1

Parameter	Result
Arsenic	<10.0 ppb
Cadmium	<5.00 ppb
Lead	<5.00 ppb
Mercury	<5.00 ppb

Subcontracting partners:
1 - Eurofins Food Chemistry Testing US Madison, WI

Respectfully Submitted,

Jordan Ramsby
Microbiologist

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-009532-01

Received On: 23May2023
Reported On: 31May2023

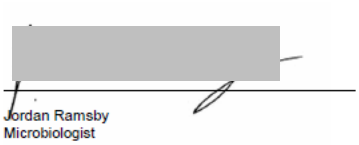
Eurofins Sample Code:	126-2023-05230083	**Sample Registration Date:**	23May2023
Client Sample Code:	230308	**Condition Upon Receipt:**	acceptable, 18.5°C
Sample Description:	Bacillus subtilis spores	**Sample Reference:**	

FS001 - Heavy Metals (As, Cd, Hg, and Pb)	Reference	Accreditation	Completed	Sub
	AOAC 2011.19, 993.14 and 2015.01 (modified)		30May2023	1

Parameter	Result
Arsenic	<10.0 ppb
Cadmium	<5.00 ppb
Lead	<5.00 ppb
Mercury	<5.00 ppb

Subcontracting partners:
1 - Eurofins Food Chemistry Testing US Madison, WI

Respectfully Submitted,



Jordan Ramsby
Microbiologist

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-009336-01

Received On: 23May2023
Reported On: 26May2023

Eurofins Sample Code:	126-2023-05230085	**Sample Registration Date:**	23May2023
Client Sample Code:	230417	**Condition Upon Receipt:**	acceptable, 18.5°C
Sample Description:	Bacillus subtilis spores	**Sample Reference:**	

FS001 - Heavy Metals (As, Cd, Hg, and Pb)	Reference	Accreditation	Completed	Sub
	AOAC 2011.19, 993.14 and 2015.01 (modified)		26May2023	1

Parameter	Result
Arsenic	<10.0 ppb
Cadmium	<5.00 ppb
Lead	<5.00 ppb
Mercury	<5.00 ppb

Subcontracting partners:
1 - Eurofins Food Chemistry Testing US Madison, WI

Respectfully Submitted,

Jordan Ramsby
Microbiologist

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-009533-01

Received On: 23May2023
Reported On: 31May2023

Eurofins Sample Code:	126-2023-05230084	**Sample Registration Date:**	23May2023
Client Sample Code:	230315	**Condition Upon Receipt:**	acceptable, 18.5°C
Sample Description:	Bacillus subtilis spores	**Sample Reference:**	

FS001 - Heavy Metals (As, Cd, Hg, and Pb)	Reference	Accreditation	Completed	Sub
	AOAC 2011.19, 993.14 and 2015.01 (modified)		30May2023	1

Parameter	Result
Arsenic	<10.0 ppb
Cadmium	<5.00 ppb
Lead	<5.00 ppb
Mercury	<5.00 ppb

Subcontracting partners:
1 - Eurofins Food Chemistry Testing US Madison, WI

Respectfully Submitted,

Jordan Ramsby
Microbiologist

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

ANALYTICAL REPORT

AR-23-UC-004514-01

Client Code: UC0000401

Received On: 15Mar2023
Reported On: 21Mar2023

Eurofins Sample Code: 126-2023-03150103
Client Sample Code: 230308
Sample Description: Bacillus subtilis spores
Sample Registration Date: 15Mar2023
Condition Upon Receipt: atypical, 13.9°C
Sample Reference:

UM4BV - Yeast - FDA BAM Chapter 18 mod.	**Reference** FDA BAM Chapter 18 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 21Mar2023
Parameter Yeast	**Result** < 10 cfu/g		
Parameter Moulds	**Result** < 10 cfu/g		
UM73J - Total Coliforms - AOAC 991.14	**Reference** AOAC 991.14	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 18Mar2023
Parameter Coliforms	**Result** < 10 cfu/g		
Parameter Escherichia coli	**Result** < 10 cfu/g		
UMDTC - Salmonella spp. - AOAC-RI 121501	**Reference** AOAC-RI 121501	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 18Mar2023
Parameter Salmonella	**Result** Not Detected per 25 g		
UMIJ7 - Staphylococcus aureus - AOAC 2003.07	**Reference** AOAC 2003.07	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 16Mar2023
Parameter Staphylococcus aureus	**Result** < 10 cfu/g		
UMQDX - Listeria species - AOAC-RI 061702	**Reference** AOAC-RI 061702	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 16Mar2023

AANIKA

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

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

ANALYTICAL REPORT

AR-23-UC-004514-01

Client Code: UC0000401

Received On: 15Mar2023
Reported On: 21Mar2023

Eurofins Sample Code:	126-2023-03150103	**Sample Registration Date:**	15Mar2023
Client Sample Code:	230308	**Condition Upon Receipt:**	atypical, 13.9°C
Sample Description:	Bacillus subtilis spores	**Sample Reference:**	

UMQDX - Listeria species - AOAC-RI 061702	Reference	Accreditation	Completed
	AOAC-RI 061702	ISO/IEC 17025:2017 A2LA 3329.08	16Mar2023

Parameter	Result
Listeria Species	Not Detected per 25 g

Respectfully Submitted,

[redacted]

Charishma M
Executive Registration (US Reporting)

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

ANALYTICAL REPORT

AR-23-UC-007216-01

Client Code: UC0000401

Received On: 21Apr2023
Reported On: 27Apr2023

Eurofins Sample Code: 126-2023-04210127
Client Sample Code: 230417
Sample Description: Bacillus subtilis spores
Sample Registration Date: 21Apr2023
Condition Upon Receipt: acceptable, 18.3°C
Sample Reference:

UM4BV - Yeast - FDA BAM Chapter 18 mod.	**Reference** FDA BAM Chapter 18 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 27Apr2023
Parameter	**Result**		
Yeast	< 10 cfu/g		
Parameter	**Result**		
Moulds	< 10 cfu/g		
UM63K - Salmonella species - AOAC-RI 121501	**Reference** AOAC-RI 121501	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 22Apr2023
Parameter	**Result**		
Salmonella	Not Detected per sample		
UM73J - Total Coliforms - AOAC 991.14	**Reference** AOAC 991.14	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 24Apr2023
Parameter	**Result**		
Coliforms	< 10 cfu/g		
Parameter	**Result**		
Escherichia coli	< 10 cfu/g		
UMIJ7 - Staphylococcus aureus - AOAC 2003.07	**Reference** AOAC 2003.07	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 22Apr2023
Parameter	**Result**		
Staphylococcus aureus	< 10 cfu/g		
UMQDX - Listeria spp. - AOAC-RI 061702	**Reference** AOAC-RI 061702	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 22Apr2023

AANIKA

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

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-007216-01

Received On: 21Apr2023
Reported On: 27Apr2023

Eurofins Sample Code:	126-2023-04210127	**Sample Registration Date:**	21Apr2023
Client Sample Code:	230417	**Condition Upon Receipt:**	acceptable, 18.3°C
Sample Description:	Bacillus subtilis spores	**Sample Reference:**	

UMQDX - Listeria spp. - AOAC-RI 061702	Reference	Accreditation	Completed
	AOAC-RI 061702	ISO/IEC 17025:2017 A2LA 3329.08	22Apr2023

Parameter	Result
Listeria Species	Not Detected per 25 g

Respectfully Submitted,

[redacted]

Charishma M
Executive Registration (US Reporting)

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

ANALYTICAL REPORT

AR-23-UC-004980-01

Client Code: UC0000401

Received On: 21Mar2023
Reported On: 27Mar2023

Eurofins Sample Code: 126-2023-03210154
Client Sample Code: 230315
Sample Description: Bacillus Subtilis Spores
Sample Registration Date: 21Mar2023
Condition Upon Receipt: acceptable, 16.4°C
Sample Reference:

UM4BV - Yeast - FDA BAM Chapter 18 mod.	**Reference** FDA BAM Chapter 18 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 27Mar2023
Parameter	**Result**		
Yeast	< 10 cfu/g		
Parameter	**Result**		
Moulds	< 10 cfu/g		
UM63K - Salmonella species - AOAC-RI 121501	**Reference** AOAC-RI 121501	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 23Mar2023
Parameter	**Result**		
Salmonella	Not Detected per sample		
UM73J - Total Coliforms - AOAC 991.14	**Reference** AOAC 991.14	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 24Mar2023
Parameter	**Result**		
Coliforms	< 10 cfu/g		
Parameter	**Result**		
Escherichia coli	< 10 cfu/g		
UMIJ7 - Staphylococcus aureus - AOAC 2003.07	**Reference** AOAC 2003.07	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 23Mar2023
Parameter	**Result**		
Staphylococcus aureus	< 10 cfu/g		
UMQDX - Listeria species - AOAC-RI 061702	**Reference** AOAC-RI 061702	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 23Mar2023

3/27/23 8:22 am

AANIKA

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

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-004980-01

Received On: 21Mar2023
Reported On: 27Mar2023

Eurofins Sample Code:	126-2023-03210154	**Sample Registration Date:**	21Mar2023
Client Sample Code:	230315	**Condition Upon Receipt:**	acceptable, 16.4°C
Sample Description:	Bacillus Subtilis Spores	**Sample Reference:**	

UMQDX - Listeria species - AOAC-RI 061702	Reference	Accreditation	Completed
	AOAC-RI 061702	ISO/IEC 17025:2017 A2LA 3329.08	23Mar2023

Parameter	Result
Listeria Species	Not Detected per 25 g

Respectfully Submitted,

[redacted]

Charishma M
Executive Registration (US Reporting)

AANIKA

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

eurofins | Eurofins Microbiology Laboratories (New England)

Eurofins Microbiology Laboratories (New England)

646 Camp Ave.
North Kingstown, RI 02852
+1 401 352 6950
Micro-NewEngland@EurofinsUS.com

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-004124-01

Received On: 08Mar2023
Reported On: 14Mar2023

Eurofins Sample Code: 126-2023-03080131
Client Sample Code: 230227
Sample Description: Bacillus Subtilis Spores
Sample Registration Date: 08Mar2023
Condition Upon Receipt: acceptable, 17.7°C
Sample Reference:

UM4BV - Yeast - FDA BAM Chapter 18 mod.	**Reference** FDA BAM Chapter 18 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 14Mar2023
Parameter Yeast	**Result** < 10 cfu/g		
Parameter Moulds	**Result** < 10 cfu/g		
UM63K - Salmonella species - AOAC-RI 121501	**Reference** AOAC-RI 121501	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 10Mar2023
Parameter Salmonella	**Result** Not Detected per sample		
UM73J - Total Coliforms - AOAC 991.14	**Reference** AOAC 991.14	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 12Mar2023
Parameter Coliforms	**Result** < 10 cfu/g		
Parameter Escherichia coli	**Result** < 10 cfu/g		
UMIJ7 - Staphylococcus aureus - AOAC 2003.07	**Reference** AOAC 2003.07	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 10Mar2023
Parameter Staphylococcus aureus	**Result** < 10 cfu/g		
UMQDX - Listeria species - AOAC-RI 061702	**Reference** AOAC-RI 061702	**Accreditation** ISO/IEC 17025:2017 A2LA 3329.08	**Completed** 10Mar2023

AANIKA

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

Aanika Bio Sciences

Jamie Richards
86 34th St
Brooklyn, NY 11232

Client Code: UC0000401

ANALYTICAL REPORT

AR-23-UC-004124-01

Received On: 08Mar2023
Reported On: 14Mar2023

Eurofins Sample Code: 126-2023-03080131
Client Sample Code: 230227
Sample Description: Bacillus Subtilis Spores
Sample Registration Date: 08Mar2023
Condition Upon Receipt: acceptable, 17.7°C
Sample Reference:

UMQDX - Listeria species - AOAC-RI 061702	Reference	Accreditation	Completed
	AOAC-RI 061702	ISO/IEC 17025:2017 A2LA 3329.08	10Mar2023

Parameter	Result
Listeria Species	Not Detected per 25 g

Respectfully Submitted,

[redacted]

Charishma M
Executive Registration (US Reporting)