ORIGINAL SUBMISSION

INTERTOX

600 Stewart St.
Suite 1101
Seattle, WA 98101
U.S.A.

Tel 206.443.2115
Fax 206.443.2117

October 22, 2014

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: Generally Recognized as Safe Notification for *Bacillus subtilis*

Dear Sir/Madam:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), our client, BiOWiSH Technologies (BiOWiSH), through Intertox, Inc. as its agent, hereby provides notice of a claim that use of *Bacillus subtilis* in wash water for the post-harvest processing of bananas, as described in the enclosed notification document, is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures.

As requested, please find enclosed

1. The full GRAS Notice in the electronic format folder structure stored on the enclosed CD.
2. One full paper copy of the GRAS Notice, including signature pages.

If you have any questions or require additional information, please feel free to contact me at 206.443.2115 or rcpleus@intertox.com.

Sincerely,
INTERTOX, INC.

(b) (6)

Richard C. Pleus, Ph.D.
Managing Director

Enclosures (2)

#62

RECEIVED
DEC 15 2014
OFFICE OF
FOOD ADDITIVE SAFETY

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE (*GRAS*) NOTICE

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000562	
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740-3835.

PART I – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3a. For New Submissions Only: Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: _____

3b. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

PART II – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Richard Carpenter, Ph.D.	Position Chief Technology Officer	
	Company *(if applicable)* BiOWiSH Technologies, Inc.		
	Mailing Address *(number and street)* 2724 Erie Avenue, Suite B		
City Cincinnati	State or Province Ohio	Zip Code/Postal Code 45208	Country United States of America
Telephone Number +1.513.680.8868	Fax Number	E-Mail Address rcarpenter@biowishtech.com	
1b. Agent or Attorney *(if applicable)*	Name of Contact Person Richard C. Pleus, Ph.D.	Position Managing Director	
	Company *(if applicable)* Intertox, Inc.		
	Mailing Address *(number and street)* 600 Stewart St., Suite 1101		
City Seattle	State or Province Washington	Zip Code/Postal Code 98101	Country United States of America
Telephone Number +1.206.443.2115	Fax Number	E-Mail Address rcpleus@intertox.com	

000002

Form Approved: OMB No. 0910-0342; Expiration Date: 02/29/2016
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE

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Name of Contact Person Richard Carpenter, Ph.D.		Position Chief Technology Officer	
Company *(if applicable)* BiOWiSH Technologies, Inc.			
Mailing Address *(number and street)* 2724 Erie Avenue, Suite B			
City Cincinnati	State or Province Ohio	Zip Code/Postal Code 45208	Country United States of America
Telephone Number +1.513.680.8868	Fax Number	E-Mail Address rcarpenter@biowishtech.com	

1b. Agent or Attorney *(if applicable)*			
Name of Contact Person Richard C. Pleus, Ph.D.		Position Managing Director	
Company *(if applicable)* Intertox, Inc.			
Mailing Address *(number and street)* 600 Stewart St., Suite 1101			
City Seattle	State or Province Washington	Zip Code/Postal Code 98101	Country United States of America
Telephone Number +1.206.443.2115	Fax Number	E-Mail Address rcpleus@intertox.com	

PART III – GENERAL ADMINISTRATIVE INFORMATION

1. Name of Substance

Bacillus subtilis

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☐ Paper

☒ Electronic files on physical media with paper signature page

If applicable give number and type of physical media

1 CD, 1 full paper copy of submission

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in FDA's files by reference? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates by reference information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 000020

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRAS Notice No. 000114, 000205, 000274, 000406, and 000476

6. Statutory basis for determination of GRAS status *(Check one)*

☒ Scientific Procedures *(21 CFR 170.30(b))* ☐ Experience based on common use in food *(21 CFR 170.30(c))*

7. Does the submission (including information that you are incorporating by reference) contain information that you view as trade secret or as confidential commercial or financial information?

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Part IV)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, see attached Designation of Confidential Information

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

PART IV – INTENDED USE

1. Describe the intended use of the notified substance including the foods in which the substance will be used, the levels of use in such foods, the purpose for which the substance will be used, and any special population that will consume the substance *(e.g., when a substance would be an ingredient in infant formula, identify infants as a special population).*

Bacillus subtilis is intended for use in post-harvest processing of bananas. *B. subtilis* is added to wash water at 1-3 ppm to improve wash water quality. Use of this product is intended to reduce the amount of chemicals used in the washing process.

2. Does the intended use of the notified substance include any use in meat, meat food product, poultry product, or egg product? *(Check one)*

☐ Yes ☒ No

PART V – IDENTITY

1. Information about the Identity of the Substance

	Name of Substance[1]	Registry Used *(CAS, EC)*	Registry No.[2]	Biological Source *(if applicable)*	Substance Category *(FOR FDA USE ONLY)*
1	*Bacillus subtilis*	CAS	68038-70-0		
2					
3					

[1] Include chemical name or common name. Put synonyms *(whether chemical name, other scientific name, or common name)* for each respective item (1 - 3) in Item 3 of Part V *(synonyms)*

[2] Registry used e.g., CAS *(Chemical Abstracts Service)* and EC *(Refers to Enzyme Commission of the International Union of Biochemistry (IUB), now carried out by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB))*

2. Description

Provide additional information to identify the notified substance*(s)*, which may include chemical formula*(s)*, empirical formula*(s)*, structural formula*(s)*, quantitative composition, characteristic properties *(such as molecular weight(s))*, and general composition of the substance. For substances from biological sources, you should include scientific information sufficient to identify the source *(e.g., genus, species, variety, strain, part of a plant source (such as roots or leaves), and organ or tissue of an animal source)*, and include any known toxicants that could be in the source.

B. subtilis is a non-toxigenic, non-pathogenic ubiquitous soil microorganism. It is a rod-shaped, Gram positive, aerobic bacterium.

3. Synonyms

Provide as available or relevant:

1	B. subtilis
2	
3	

PART VI – OTHER ELEMENTS IN YOUR GRAS NOTICE

(check list to help ensure your submission is complete – check all that apply)

- ☐ Any additional information about identity not covered in Part V of this form
- ☒ Method of Manufacture
- ☒ Specifications for food-grade material
- ☒ Information about dietary exposure
- ☒ Information about any self-limiting levels of use *(which may include a statement that the intended use of the notified substance is not-self-limiting)*
- ☒ Use in food before 1958 *(which may include a statement that there is no information about use of the notified substance in food prior to 1958)*
- ☒ Comprehensive discussion of the basis for the determination of GRAS status
- ☒ Bibliography

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

PART VII – SIGNATURE

1. The undersigned is informing FDA that Richard Carpenter, Ph.D.
(name of notifier)

has concluded that the intended use*(s)* of Bacillus subtilis
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) exempt from the premarket approval requirements of section 409 of the Federal Food, Drug, and Cosmetic Act because the intended use(s) is (are) generally recognized as safe.

2. ☒ Richard Carpenter, Ph.D. *(name of notifier)* agrees to make the data and information that are the basis for the determination of GRAS status available to FDA if FDA asks to see them.

Richard Carpenter, Ph.D. *(name of notifier)* agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so.

2724 Erie Avenue, Suite B, Cincinnati, OH 45208
(address of notifier or other location)

Richard Carpenter, Ph.D. *(name of notifier)* agrees to send these data and information to FDA if FDA asks to do so.

OR

☐ The complete record that supports the determination of GRAS status is available to FDA in the submitted notice and in GRP No. _____
(GRAS Affirmation Petition No.)

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6) [redacted]	Richard C. Pleus, Ph.D.	12/03/2014

PART VIII – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	CoverLetter_Bsubtilis_2014-10-22.pdf	Administrative
	Form3667_Bsubtilis_2014-12-03.pdf	Administrative
	GRASNotice_Bsubtilis_2014-10-22.pdf	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 150 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, 1350 Piccard Drive, Room 400, Rockville, MD 20850. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

INTERTOX

GRAS NOTIFICATION
BACILLUS SUBTILIS FOR USE IN POST-HARVEST PROCESSING OF BANANAS

Prepared for:

BIOWISH TECHNOLOGIES, INC.
2724 Erie Avenue, Suite B
Cincinnati, OH 45208

October 22, 2014

INTERTOX, INC.
600 Stewart St.
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206.443.2115 phone
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BACILLUS SUBTILIS FOR USE IN POST-HARVEST PROCESSING OF BANANAS

1.0 INTENDED USE

Bacillus subtilis is intended for use in post-harvest processing of bananas. *B. subtilis* is added to wash water to improve wash water quality. Use of this product is intended to reduce the amount of chemicals used in the washing process.

2.0 IDENTITY

B. subtilis (CAS 68038-70-0) is a non-toxigenic, non-pathogenic ubiquitous soil microorganism. It is a rod-shaped, Gram positive, aerobic bacterium.

3.0 METHOD OF MANUFACTURE

B. subtilis is obtained from a fermentation culturing and manufacturing process that follows good manufacturing practice (GMP) standards. The organism, composition, and process used have been submitted for Organic Certification with the Washington State Department of Agriculture (WSDA). Sections 3.1 through 3.4 below detail the processes used to manufacture *B. subtilis* and its use in post-harvest process wash water for bananas.

3.1 Microbial Submerged Fermentation

The *Bacillus* species used in the process wash water is grown using a standard deep tank submerged fermentation process, which is a standard process used in this industry.

An individual starter culture of *Bacillus* is grown in a fermentation flask under conditions specific for optimal growth according to a protocol similar to the following: 2 grams (g) Nutrient Broth, 2 g AmberFerm (yeast extract) and 4 g Maltodextrin are added to a 250 milliliter (mL) Erlenmeyer flask. One hundred mL of distilled, deionized water is added and the flask is stirred until all dry ingredients are dissolved. The flask is covered and placed for 30 minutes in an autoclave operating at 121°C and 15 pounds per square inch (psi). After cooling, the flask is inoculated with 1mL of pure *B. subtilis.* The flask is sealed and placed on an orbital shaker at 30°C. The culture is allowed to grow for 3-5 days.

Larger *Bacillus* cultures are prepared according to a process similar to the following: 18 g Nutrient Broth, 18 g AmberFerm, and 36 g Maltodextrin to 1 liter (L) flasks with 900 mL of distilled, deionized water. The flasks are sealed and sterilized as above. After cooling, 100 mL of the microbial media from the 250 mL Erlenmeyer flasks are added. The 1 L flasks are sealed, placed on an orbital shaker, and allowed to grow out for another 3-5 days at 30 - 37°C.

In the final grow-out phase before introduction to the fermenter, the culture from the 1 L flask is transferred under sterile conditions to a sterilized 6 L vessel and fermentation continues at 30 - 37°C with aeration until stationary phase is achieved. The contents of each 6 L culture flask are transferred to individual fermenters which are also charged with a sterilized growth media made from 1 part yeast extract and 2 parts dextrose. The individual fermenters are run under aerobic conditions at the pH and temperature optimal for this species.

3.2 Harvesting and Drying *Bacillus* Cells

After fermentation is complete, the fermentation broth containing the *Bacillus* cells is centrifuged. The filtrate is discarded, and the filtered "wet cake" containing the *Bacillus* cells is lyophilized

(Freeze Dried) for 5-7 days until the moisture is reduced to 3-5% by weight.

3.3 Blending Finished Product

The lyophilized cell powder is ground in a conical grinder and the resulting *Bacillus* powder is blended into dextrose monohydrate using a ribbon or paddle blender. The blended product is then packed directly into labelled foil packages and heat sealed prior to shipment.

3.4 Product Use in Post-Harvest Process Wash Water

Under current post-harvest practices, banana bunches are placed in wash tanks with process wash water containing *B. subtilis*, placed on drain trays, allowed to drain, and then sprayed with a fungicide. The concentration of the finished product in the process wash water is 1-3 mg of finished product per liter of process wash water (mg/L or parts per million (ppm)).

4.0 SPECIFICATIONS

Microbial activity is assayed according to the methods described in Chapter 3 of the US Food and Drug Administration (US FDA) Bacteriological Analytical Manual (BAM). Analysis for heavy metals, including mercury (Hg), selenium (Se), lead (Pb), nickel (Ni), molybdenum (Mo), cobalt (Co), cadmium (Cd), and arsenic (As), utilized US EPA method 6010B. Analysis for yeast and molds follows the methods described in Chapter 18 of the US FDA BAM. Analysis for the pathogens *Salmonella* and *Escherichia coli* (*E.coli*) follow the methods detailed in the Association of Analytical Communities (AOAC) Official Method 2009.03 and Chapter 4 of the US FDA BAM, respectively (see Appendix A).

The estimated stability (i.e. shelf-life) of the processing aid is 3 years if stored closed in a cool, dry location out of direct sunlight. Once in solution, the mixture should be applied within 24 hours.

5.0 DIETARY EXPOSURE

Section 5.1 discusses data from the scientific literature on toxicity of *B. subtilis* and the derivation of an acceptable daily intake (ADI) from these data. Section 5.2 provides an estimated daily intake (EDI) of *B. subtilis* based on the intended use. A comparison between the ADI and EDI is also provided. Section 5.3 discusses additional information relevant to predicted exposure.

5.1 Exposure Levels Not Expected to Cause Adverse Health Effects

US FDA states (2006b):

> The key determinant in the safety evaluation of a substance found in or added to the diet is the relation of its probable human intake to the level at which adverse effects are observed in toxicological studies.
>
> Generally, animal studies are used to determine an acceptable daily intake (ADI), that has to be greater than the estimated daily intake (EDI) to ensure safety.

A review of the scientific literature was conducted to identify available toxicological animal studies. Then, an ADI was developed based on the study with the best available data for estimating long-term human dietary exposure (i.e., chronic oral studies).

In a study by Sorokulova *et al.* (2008), mice, rabbits, and piglets were orally inoculated with *B. subtilis*. Ten animals of each species were orally inoculated each day over a period of 10 days with *B. subtilis* at doses of: mice, 1.0×10^6 colony forming units per day (cfu/d); rabbits and piglets, $1.0 \times$

10^9 cfu/d. Ten animals of each species in the placebo control group received sterile phosphate-buffered saline. The treatment lasted 10 days, during which time the activity and behavior of each animal were observed. On day 11, all animals were euthanized, and internal organs were observed macroscopically. Samples of different organs and tissues were collected for histological analysis.

There were no adverse effects reported on the general health status of the animals. No changes in the organs and tissues during histopathological study were observed in treated animals. Assuming a body weight of 23 g for mice (Charles River, 2014), 6 kg for rabbits (Charles River, 2013), and 1.5 kg for newborn piglets (AALAS, 1989), the no observable effect level (NOEL) would be 4.35×10^7 colony forming units per kilogram body weight per day (cfu/kg-d), 1.67×10^8 cfu/kg-d, and 6.67×10^8 cfu/kg-d, for mice, rabbits, and piglets respectively.

A study by Hong *et al.* (2008), rabbits were orally dosed by gavage with 1×10^9 cfu/d *B. subtilis* for 30 days. On day 30, blood was withdrawn for hematological analysis and samples of different visceral organs and tissues were collected for histological analysis, including liver, kidneys, spleens, small intestines, and mesenteric lymph nodes. There were no adverse effects reported on the general health status of the animals, and no changes in selected visceral organs and tissues were observed. No significant differences in the hematological indexes were observed in blood from control and treated rabbits.

In another study, 15 rats of each sex were orally dosed by gavage with 2×10^9 cfu/kg-d *B. subtilis* for 28 days (Tompkins *et al.*, 2008). No outward indications of toxicity or oral intolerance were reported, nor were there any significant variations in body mass, food consumption, or mortality with oral exposure to *B. subtilis*. No visible lesions or changes in organ mass were observed for males. The only effect noted was a 10% decrease in heart mass in female rats. However, organ mass/ total animal mass ratios were not affected by treatment for female rats. A microbial examination of the treated animals of both sexes did not detect *B. subtilis* in the liver, kidneys, spleen or heart.

An ADI was estimated by using the lowest NOEL from these studies and applying a 100-fold safety factor as required by 21 CFR 170.22. The lowest NOEL was used to provide the most conservative estimated ADI. Specifically, the lowest NOEL from the Sorokulova *et al.* (2008) study of 4.35×10^7 cfu/kg-d was divided by a 100-fold safety factor to derive an ADI of 4.4×10^5 cfu/kg-d, as calculated below.

$$ADI = \left(\frac{\frac{4.35 \times 10^7\, cfu}{kg - d}}{100} \right) = 4.4 \times 10^5\, cfu/kg - d$$

For the average 10 kg child (6 month to 2 years) and the average 70 kg adult, the ADI of *B. subtilis* would be 4.4×10^6 cfu/day and 3.0×10^7 cfu/day, respectively.

5.2 Estimated Daily Intake and Margin of Exposure

As stated by US FDA (2006b):

> A processing aid is often not expected to remain in the finished food after its technical effect has been accomplished, nor to have any intended effect in the food should residues remain. Therefore, the use level is not pertinent to calculating the EDI unless, as a worst-case scenario, one assumes that all of the additive is carried over to or remains in the treated food. This assumption, however, is rarely needed for processing aids.

In general, *Bacilli* occur at population levels of 10^6 to 10^7 per gram of soil (Alexander, 1977). The

concentration of *B. subtilis* in the process wash water is approximately three orders of magnitude lower than soil population levels and therefore not distinguishable from nominal background microbial levels. Furthermore, any residue would be present on the peel of the banana, not the edible portion of the fruit.

Here, we utilized a worst-case scenario approach to derive an EDI for *B. subtilis* used in process wash water for bananas. An EDI was estimated as follows:

- According to the Certificate of Analysis, the estimated final concentration of *B. subtilis* in the finished product is 6.3×10^8 cfu/g.
- Per the manufacturer, the concentration of the finished product in the wash water is 1-3 mg/L (or ppm). This equates to a concentration range in the process wash water of $6.3 \times 10^2 - 1.9 \times 10^3$ cfu/mL, as calculated below.

  $$\textit{Maximum concentration in process wash water} = \frac{3\,mg}{L} \times \frac{1\,g}{1000\,mg} \times \frac{6.3 \times 10^8\,cfu}{g} \times \frac{1\,L}{1000\,mL} = 1.9 \times 10^3\,cfu/mL$$

- A worst-case estimate yields an average residue of 3.8×10^3 cfu/banana. This assumes the following: (1) a typical banana bunch is 5 bananas, (2) the amount of wet residue on the bananas once removed from the wash bin and drained is 10 mL (their surfaces do not retain much water) (Carpenter, 2014), (3) the entire 10 mL wet residue (at 1.9×10^3 cfu/mL; for a total of 1.9×10^4 cfu/ 5 bananas) dried on the surface with no activity loss and no reduction from subsequent processing steps (Carpenter, 2014).
- US Department of Agriculture's (USDA) MyPlate.gov recommends that women ages 19 to 30 and men ages 19 and over get 2 cups of fruit per day, and women ages 31 and over 1 ½ cups per day (USDA, 2014a). One banana provides about 1 cup of fruit (USDA, 2014b).

  Data from US EPA suggests that most people do not consume more than 2 cups of fruit per day (US EPA, 2011). The 95th percentile of fruit intake for consumers only is 25.8 grams of fruit per kilogram body weight per day (g/kg-d) and 3.8 g/kg-d for infants (birth to 1 year) and adults, respectively. Using a body weight of 10 kg for a child and 70 kg for the average adult the 95th percentile intake for consumers is 258 g/d for children and 266 g/d for adults. Two cups of mixed fruit is approximately 350g.

Using the assumptions provided above, the EDI would be 7.6×10^3 cfu/day and assumes ingestion of two whole bananas plus their peels.

A comparison of the EDI of 7.6×10^3 cfu/ day to the ADI of 4.4×10^6 cfu/day for children and 3.0×10^7 cfu/day for adults yields a margin of exposure of 575 and over 3,900, respectively. To equal the ADI of 4.4×10^6 cfu/day, a child would have to consume over 1,000 bananas per day with the peel.

5.3 Other Information Relevant for EDI

While difficult to estimate, microbiologist researchers for BiOWiSH indicated that wet residue would lose another 10-fold level of activity of the when air dried on a surface (Carpenter, 2014). This would yield the residual concentration at approximately 10^2 cfu/mL. As a result all comparisons presented above would be greater.

Under current post-harvest practices, banana bunches are washed with process wash water containing *B. subtilis*, placed on drain trays, allowed to drain, and then sprayed with a fungicide. This would

inherently rinse off most of the process wash water and leave behind the fungicide as the primary residue on the banana peel.

6.0 SELF-LIMITING LEVELS OF USE

Some food additives may have a technologically self-limiting use level; that is, the food additive has a maximum concentration in food above which the food becomes unpalatable, unappealing, or otherwise unfit for human consumption.

There are no known self-limiting levels of use for *B. subtilis* under this intended use.

7.0 COMMON USE IN FOOD BEFORE 1958

B. subtilis is found in traditional Asian and African fermented foods (Ouoba *et al.*, 2008; Phromraksa *et al.*, 2008; Feng *et al.*, 2013). *B. subtilis* is used in the preparation of Natto, which has a long history of use as a fermented soybean food in Japan and contains more than 10^8 viable *B. subtilis* per gram of food products (Hong *et al.*, 2008). While documentation for use in food before 1958 was not retrieved, these foods are part of a traditional diet and likely to be used prior 1958.

8.0 BASIS FOR GRAS DETERMINATION

Section 8.1 details the current uses of *B. subtilis* and the scientific literature supporting the GRAS determination of *B. subtilis* for human exposure. Section 8.2 discusses reports of investigations that may appear to be inconsistent with the GRAS determination. Section 8.3 summarized the evidence in support of the GRAS determination.

8.1 Evidence in Support of GRAS Determination

The section below includes a history of human exposure and other scientific and evidence-based research that corroborates the safety of human consumption of *B. subtilis*.

1. *B. subtilis* is used in the probiotic Biosporin in Russia and Ukraine and is intended for human use (Sorokulova *et al.*, 2008).
2. *B. subtilis* is used in probiotics for veterinary and aquaculture uses (Cutting, 2011).
3. US FDA has determined that carbohydrase and protease enzyme preparations derived from *B. subtilis* are GRAS (21 CFR 184.1148 and 21 CFR 184.1150).
4. US FDA had no questions in response to six GRAS Notices for enzyme preparations obtained from *B. subtilis* for use in a variety of foods (US FDA, 1999; 2003; 2006a; 2009; 2012; 2014).
5. US FDA allows for use of *B. subtilis* as a GRAS ingredients (e.g., direct-fed microbes) in animal feed (AAFCO, 2014).
6. Food additives and animal feed ingredients are regulated in Canada by Health Canada. Eight enzymes derived from *B. subtilis* are permitted for use by Health Canada in a variety of foods (Health Canada, 2014b).
7. Drugs and health products (including probiotics) sold in Canada must be licensed by Health Canada. Per the Licensed Natural Products Database provided by Health Canada, *B. subtilis* is listed as ingredient in two natural health products licensed for use in Canada, Fermalac Sb (Lallemand Health Solutions Inc.) and Prox10 (Bio Trust Nutrition LLC.) (Health Canada, 2014a).

8. Dehydrated *Bacillus* culture may be used in animal feed per Canadian Feed Regulations Schedule IV-Part 2-Class 8.6 and assigned the International Feed Ingredient number (IFN) 8-19-119.
9. Dehydrated *B. subtilis* fermentation extract (IFN 5-06-147), products (IFN 5-06-150 and 5-06-156), and solubles (IFN 5-29-779) are approved for use in animal feed per Canadian Feed Regulations Schedule IV.
10. *B. subtilis* is permitted for use as a "Food for Specified Health Use" by the Japanese Ministry of Health, Labor, and Welfare (Shimizu, 2003).
11. In 2007, the European Food Safety Authority (EFSA), proposed that *B. subtilis* receive a qualified presumption of safety (QPS) (EFSA, 2007). The QPS status of *B. subtilis* was reaffirmed in the 2013 EFSA QPS update, with the qualification of the absence of toxigenic activity (EFSA, 2013).
12. US EPA states that *B. subtilis* is "not considered pathogenic or toxigenic to humans, animals, or plants" (US EPA, 1997).
13. Ten human studies using *B. subtilis* have been published in peer-reviewed academic journals between 1996 and 2012. While the studies were designed to detect efficacy as a medical food or dietary supplement, rather than safety, the ten publications are included in this safety assessment because they demonstrate a lack of adverse events when *B. subtilis* is administered to human study subjects. These studies are summarized in Table 1.
14. There are no adverse events reported on the US FDA Safety Information and Adverse Event Reporting Program (Medwatch) from consumption of *B. subtilis* as of this writing.
15. Ten-day repeated dose oral toxicity of *B. subtilis* was studied in mice, rabbits, and pigs (Sorokulova *et al.*, 2008). There were no adverse effects on the general health status of the animals. No changes in the organs and tissues during histopathological study were observed in treated animals. There were no differences in the hematological indexes measured in the blood from control and treated rabbits. Two additional oral toxicity studies in rabbits (30-day repeated dose) and rats (28-day repeated dose) also showed no adverse effects from treatment (Hong *et al.*, 2008; Tompkins *et al.*, 2008).
16. Over 150 stains of *B. subtilis* are available from the American Type Culture Collection (ATCC), all of which are classified as Biosafety Level 1 (BSL-1) (ATCC, 2014). The Centers for Disease Control and Prevention (CDC) defines BSL-1 organisms as those "not known to consistently cause disease in immunocompetent adult humans, and present minimal potential hazard to laboratory personnel and the environment" (CDC, 2009).
17. With regards to the allergenicty of *B. subtilis*, only one study was retrieved. In this study, enzymes from *B. subtilis* were able to hydrolyze allergenic gliadin fragments and the major allergenic epitope of beta-lactoglobulin (Phromraksa *et al.*, 2008). Based on these findings, the authors suggest that *B. subtilis* could be applied in the production of hypoallergenic wheat flour or milk food products.

Table 1. Human Studies Using *B. subtilis*

Study	Patients	Treatment groups	Adverse events
(Liu *et al.*, 2012)	Elderly patients with functional constipation (n=97)	(A) Lactulose (B) *B. subtilis* (C) Lactulose + *B. subtilis*	No statistical difference in total adverse reaction rates among groups
(Li *et al.*, 2012)	Patients with functional constipation (n=216)	(A) Lactulose + placebo (B) Lactulose + *B. subtilis* and *Enterococcus faecium*	None reported in abstract (article in Chinese)
(Sohn *et al.*, 2012)	Patients with irritable bowel syndrome (IBS) (n=228)	(A)Tianeptine + *B. subtilis* and *Streptococcus faecium* (B) Amitriptyline + *B. subtilis* and *S. faecium*	Serious adverse events did not occur in either group. Adverse events such as dry mouth and constipation were significantly lower in group A than group B. "...probiotics are commonly used in IBS, with few adverse effects."
(Lee *et al.*, 2010)	Patients with constipation undergoing colonoscopy (n=211)	(A) Placebo + Sodium phosphate (B) *B. subtilis and S. faecium* + Sodium phosphate	Preparation-related adverse events and postendoscopic symptoms were more frequent in group A than group B.
(Park *et al.*, 2007)	*Helicobacter pylori* infected patients (n=352)	(A) Proton pump inhibitor-based triple therapy only (B) Proton pump inhibitor-based triple therapy + *B. subtilis* and *S. faecium*	Diarrhea and overall side effects were more common in group A than group B. "Supplementation with probiotic strains were shown to...reduce side effects..."
(Kim *et al.*, 2006)	Patients with IBS (n=40)	(A) Placebo (B) *B. subtilis* and *S. faecium*	*B. subtilis* and *S. faecium* well tolerated with no adverse events
(Pushkarev, 2005)	Patients with intrahospital urinary tract infection (n=36)	(A) Conventional therapy (B) *B. subtilis*	No adverse events reported in abstract (article in Russian)
(Zhao *et al.*, 2004)	Patients with liver cirrhosis (n=50)	(A) *Bifidobacterium*, *Lactobacillus acidophilus* and *Enterococcus* (B) *Bacillus subtilis* and *Enterococcus faecium*	No adverse events reported
(Vukovic, 2001)	Patients with acute non-typhoid salmonella enteritis (n=63)	(A) Placebo (B) *B. subtilis*	No adverse events reported in abstract (article in Croatian)
(Gracheva *et al.*, 1996)	Patients with acute enteric infections	*B. subtilis and B. licheniformis*	Well tolerated by patients, no side effects observed.

8.2 Potentially Conflicting Data Regarding GRAS Determination

Some species of *Bacillus* bacteria are known to possess toxigenic activity, particularly *B. cereus* and *B. anthracis*. However, bacillus strains other than *B. cereus* and *B. anthracis* are rarely cytotoxic and in almost all cases do not carry genes encoding enterotoxins and emetic toxins. Enterotoxin and emetic genes of concern include hemolysin BL (Hbl A, Hbl C, Hbl D), non-hemolytic enterotoxin (NheA, NheB, NheC), cytotoxin K (cytK), and emetic toxin (EM1 or cereulide).

A study by From *et al.* (2005) collected 333 *Bacillus* spp. outside the *B. cereus* group from different environments and tested the strains for production of cytotoxins. Of these only eight strains demonstrated cytotoxic activity, two of which were *B. subtilis*. One *B. subtilis* strain showed putative emetic toxin activity as determined by the boar sperm motility test and one *B. subtilis* strain demonstrated cytotoxicity on Vero cells. However, enterotoxin genes (HblC, NheA, NheB, NheC, cytK) were not detected in either strain.

One study tested a *B. subtilis* strain used in the preparation of Natto, which has a long history of use as a fermented soybean food in Japan and contains more than 10^8 viable *B. subtilis* per gram of food products (Hong *et al.*, 2008). This strain produced biosurfactant and its culture supernatant was toxic to Caco-2, Hep-2 and HT29 cells. In regards to this study, EFSA (2008) stated "This shows that cell culture tests for *Bacillus* spp. not belonging to the *B. cereus* group, which are rarely implicated in human diseases and which virulence mechanisms are poorly understood, should be interpreted with caution." EFSA also noted that while these peptides can exhibit toxic activities on cell lines and sperm cells similar to that of the emetic toxin of *B. cereus*, their ability to cause emesis has not been proven (EFSA, 2008).

In addition, some Bacillus species are known to be pathogenic. *B. cereus* is commonly identified as a cause of food-borne illness. However, *B. subtilis* has been rarely identified as pathogenic. A comprehensive review of the literature by EFSA from 2008 to 2012 identified only two studies in which *B. subtilis* was implicated in human disease.

In 2011, EFSA identified the following study (and responses to the study) regarding pathogenicity of a single case of *B. subtilis* associated (not causative) with an infectious disease (EFSA, 2011). EFSA stated:

> An acute hepatitis of a young adult was linked to the consumption of a dietary supplement (Krones *et al.*, 2010) and the authors suspected toxigenic Bacillus isolated from the dietary supplement to have caused the disease. The two toxigenic strains were identified as one Bacillus cereus and one from the Bacillus subtilis group. The method used in this work to identify the strains (16S rDNA sequencing) is not able to distinguish all the strains within the Bacillus subtilis group. However, the species from the Bacillus subtilis group are on the QPS list, so it is possible that this hepatitis was at least partly caused by a Bacillus included in the QPS list. This paper refers to a previous report of several similar severe hepatotoxicity associated to the consumption of another dietary supplement contaminated with toxigenic Bacillus subtilis (Stickel *et al.*, 2009). In the latter work, Bacillus cereus was not isolated from the dietary supplement and the B. subtilis strains were adequately identified using both 16S rDNA and gyrB sequences. In both works, the cytotoxins were detected in the supernatant of the strains grown in the laboratory, but not in the dietary supplements, indicating a likely infection of the patient by the Bacillus and production of the toxins in the patient, instead of an intoxination (i.e. production of the toxin in the food). However the works did not quantified the numbers of Bacillus present in the dietary supplements and Stickel *et al.* (2009) and Seff (2009) discuss a possible contribution of the supplement itself to the hepatotoxicity.

In the 2013 update, EFSA noted "*Bacillus subtilis* and *Bacillus licheniformis* were identified as the cause of a bacteraemia in a patient with an oesophageal perforation (Jeon *et al.*, 2012)." EFSA then stated that "These cases of infections in human are linked to specific predisposing factors and do not suggest a risk for the consumer via exposure through the food and feed chain."

8.3 Summary of Evidence Supporting GRAS Determination

In summary, a review of the scientific literature, including human clinical studies and animal toxicity studies, demonstrates the lack of adverse effects from dietary exposure to *B. subtilis*.

According to US FDA (2006), an ADI (determined from animal studies) has to be greater than the EDI to ensure safety. For *B. subtilis*, we determined that there is a large margin of exposure between the ADI and the EDI using conservative assumptions.

On the basis of the evaluation conducted for this GRAS Notification, *B. subtilis* can be used in food for its intended use without any expected adverse health effects.

9.0 CLAIM OF EXEMPTION FROM THE REQUIREMENT FOR PREMARKET APPROVAL PURSUANT TO PROPOSED 21 CFR § 170.36 (C)(1) [62 FR 18938 (17 APRIL 1997)]

Intertox, Inc. hereby claims that the use of *Bacillus subtilis* in food, as described in this GRAS Notice, is exempt from the requirement of premarket approval of the Federal Food, Drug, and Cosmetic Act because we have determined that such use is GRAS.

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[redacted]

22 October 2014

Richard C. Pleus, Ph.D.
Managing Director

Date

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APPENDIX A.
CERTIFICATE OF ANALYSIS OF *BACILLUS SUBTILIS*

Bacillus subtilis

Certificate of Analysis*

Measurement	Analysis	Assayed	Unit	Method
Microbial Activity				
Total Aerobic Plate Count	$\geq 1.0 \times 10^8$	6.3×10^8	CFU/g	FDA BAM Chapt. 3
Heavy Metals				
Hg, Se, Pb, Ni, Mo, Co, Cd, As	< 1		mg/kg	EPA 6010
Yeasts and Molds	< 100		CFU/g	FDA BAM Chapt. 18
Pathogens				
Salmonella	negative		org/25g	AOAC 2003.09
E. coli	n.d.		MPN/g	FDA BAM 8th ed. Chpt. 4
Appearance				
Off-white, fine powder				

*Analyses reported are from an outside 3rd party analytical laboratory and are the property of BiOWISH™ Technologies.

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SUBMISSION END