GRAS ASSOCIATES, LLC

GRAS Associates, LLC
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June 3, 2020

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

Attention: Dr. Susan Carlson
Re: GRAS Notification – OPTI-BIOME® *Bacillus subtilis* MB40

Dear Dr. Carlson:

GRAS Associates, LLC, acting as the Agent for BIO-CAT Microbials, LLC (U.S.), is submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS Notification for OPTI-BIOME® *Bacillus subtilis* MB40. Along with BIO-CAT Microbials, LLC's determination of safety, an Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use in a wide variety of foods (including baked goods, nonalcoholic beverages, juice, cereal, chewing gum, coffee, tea, condiments, confections, dairy analogs, fats and oils, herbs, frozen dairy products, pasta, candy, milk, processed fruits; processed vegetables and vegetable juices, jams and jellies, sugar and sweet sauces). The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

William J. Rowe

President
Agent for BIO-CAT Microbials, LLC
GRAS Associates, LLC
11810 Grand Park Ave, Suite 500
North Bethesda, MD 20852
wrowe@nutrasource.ca

Enclosure: Form 3667 and GRAS Notification for BIO-CAT Microbials, LLC – OPTI-BIOME® *Bacillus subtilis* MB40

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000955	June 11, 2020
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,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – 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)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 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):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Elaine Cooling	Regulatory Affairs Manager
Organization *(if applicable)*	
BIO-CAT Microbials, LLC	
Mailing Address *(number and street)*	
689 Canterbury Rd	

City	State or Province	Zip Code/Postal Code	Country
Shakopee	Minnesota	55379	United States of America

Telephone Number	Fax Number	E-Mail Address
(434) 591-4661	(434) 591-4507	ecooling@bio-cat.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
William Rowe	President
Organization *(if applicable)*	
GRAS Associates, LLC	
Mailing Address *(number and street)*	
11810 Grand Park Avenue Suite 500	

City	State or Province	Zip Code/Postal Code	Country
North Bethesda	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
519-341-3667	1-888-531-3466	wrowe@nutrasource.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

OPTI-BIOME® Bacillus subtilis MB40

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

☐ Electronic Submission Gateway

☐ Electronic files on physical media

☒ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

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

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

☐ a) GRAS Notice No. GRN _____

☐ 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)* _____

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

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

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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, 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

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The ingredient will be used in a wide variety of foods (including baked goods, nonalcoholic beverages, juice, cereal, chewing gum, coffee, tea, condiments, confections, dairy analogs, fats and oils, herbs, frozen dairy products, pasta, candy, milk, processed fruits; processed vegetables and vegetable juices, jams and jellies, sugar and sweet sauces).

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

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

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that BIO-CAT Microbials, LLC

(name of notifier)

has concluded that the intended use*(s)* of OPTI-BIOME® Bacillus subtilis MB40

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. BIO-CAT Microbials, LLC agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them;

(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; agrees to send these data and information to FDA if FDA asks to do so.

689 Canterbury Rd Shakopee, MN 55379

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

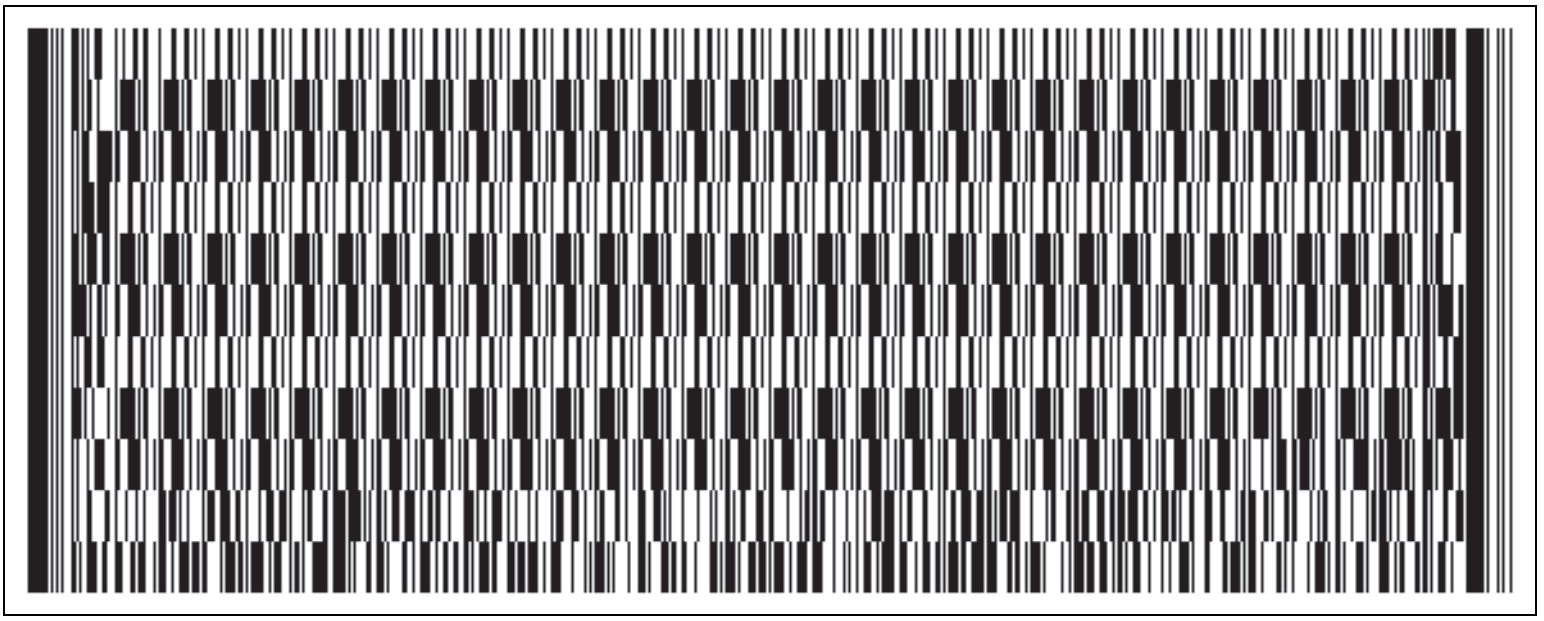
3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Amy Mozingo Digitally signed by Amy Mozingo DN: cn=Amy Mozingo, o=GRAS Associates LLC (a Nutrasource company), ou=GRAS Associates LLC (a Nutrasource company), email=amozingo@gras-associates.com, c=US Date: 2020.06.03 11:02:35 -04'00'	Amy Mozingo on behalf of William J. Rowe, President	06/03/2020

SECTION G – 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)
	Form 3667_BioCat_B. subtilis MB40_03June2020.pdf Transmittal Letter_BioCat_B. subtilis MB40_03June2020.pdf GRAS Notification_BioCat B. subtilis MB40_Revised_03June2020.pdf	

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 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, PRAStaff@fda.hhs.gov. (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.



Safety Evaluation Dossier Supporting the Generally Recognized as Safe (GRAS) Conclusion

of

Bacillus subtilis MB40

6/3/2020

Prepared By:

GRAS Associates
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

Prepared For:

BIO-CAT Microbials, LLC
689 Canterbury Rd
Shakopee, MN 55379

TABLE OF CONTENTS

PART 1. GRAS EXEMPTION CLAIM

A. Claim of Exemption from the Requirement for Premarket approval Pursuant to 21 CFR 170.30

BIO-CAT Microbials, LLC (BIO-CAT) has determined that their *Bacillus subtilis* MB40 (trade name Opti-Biome®) is generally recognized as safe (GRAS), under the intended conditions of use, in accordance with Section 201(s) of the Federal Food Drug and Cosmetics Act. This determination was made in concert with an appropriately convened panel of experts who are qualified by scientific training and experience, coordinated by GRAS Associates, LLC ("GA"). In addition, BIO-CAT has asked that GA act as the Agent for the submission of this GRAS notification.

This GRAS determination is based on scientific procedures as described in the following sections. A search of the scientific and regulatory literature was conducted through June 2, 2020 for information pertinent to the safety of the ingredient. Those references that were deemed pertinent to the objective at hand are listed in Part 7. BIO-CAT based its GRAS assessment on the large body of information that addressed the safety/toxicity/use(s) of *Bacillus subtilis* MB40 and other *Bacillus subtilis* strains, history of use of *Bacillus subtilis*, and compositional details, specifications, and method of preparation of the subject ingredient. Safety/toxicity studies performed with animals and human clinical trials were noted to have value. The totality of information about the composition, safety/toxicity/use(s) and dietary exposure ultimately provide the specific scientific foundation for the GRAS conclusion.

PART 2. SIGNED STATEMENTS AND CERTIFICATION

This signed statement and certification has been prepared in accordance with the requirements of 21 CFR 170.225.

(a) This certification will be signed at a future date by a responsible official of GRAS Associates, LLC acting as agent for BIO-CAT.

(b) This GRAS dossier did not rely on any confidential information;

(c) (1) This Independent GRAS Assessment was conducted in accordance with Subpart E of 21 CFR Part 170;

(c) (2) Names and addresses of organizations;

Sponsoring Party:

BIO-CAT Microbials, LLC
689 Canterbury Rd
Shakopee, MN 55379
U.S.

Agent:

GRAS Associates, LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

(c) (3) The name of the ingredient is *Bacillus subtilis* MB40.

(c) (4) The ingredient will be used as an ingredient in a wide variety of foods (including baked goods, nonalcoholic beverages, juice, cereal, chewing gum, coffee, tea, condiments, confections, dairy analogs, fats and oils, herbs, frozen dairy products, pasta, candy, milk, processed fruits; processed vegetables and vegetable juices, jams and jellies, sugar and sweet sauces).

(c) (5) The statutory basis for our conclusion of GRAS status is through scientific procedures in accordance with § 170.30(a) and (b).

(c) (6) It is our view that the ingredient is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

(c) (7) If FDA were to ask to see the data and information that are the basis for our conclusion of GRAS status, either during or after FDA evaluation of this notice, we agree to:

(i) make the data and information available to FDA; and

(ii) agree to both of the following procedures for making the data and information available to FDA:

(A) Upon FDA’s request, we will allow FDA to review and copy the data and information during customary business hours at our address specified where these data and information will be available; and

(B) Upon request by FDA, we will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for their evaluation or on paper.

(c) (8) None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552 (e.g., as trade secret or as commercial or financial information that is privileged or confidential).

(c) (9) We certify that, to the best of our knowledge, this Independent GRAS Assessment is a complete, representative, and balanced review that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

(c) (10) BIO-CAT does not intend to add *Bacillus subtilis* MB40 to any meat and/or poultry products that come under FSIS/USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

(c) (11) Signature

William Rowe Date: 6/3/2020
President
GRAS Associates, LLC
11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852

PART 3. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

A. Notified Substance *Bacillus subtilis* MB40 Identification

1. Common or Usual Name

Bacillus subtilis MB40

2. Characterization

Bacillus subtilis originally named *Vibrio subtilis* by Christian Gottfried Ehrenberg was discovered in 1835 and renamed *Bacillus subtilis* by Ferdinand Cohn in 1872 (Ehrenberg, 1835; Cohn, 1872). The BIO-CAT Microbials, LLC (BIO-CAT) produced *Bacillus subtilis* that is the subject of this safety evaluation / GRAS determination is a proprietary preparation of a *Bacillus subtilis* strain derived from *Bacillus subtilis* DSM 10 (DSMZ) and designated as *Bacillus subtilis* MB40. *Bacillus subtilis* MB40 has a faster sporulation time than DSMZ and thus improves turnaround time. It is a non-toxigenic and non- pathogenic organism that has a patent deposit with the ATCC (BS-MB40 PTA-122264). *Bacillus subtilis* MB40 is periodically monitored for genetic drift.

The organism is a gram-positive spore-forming rod that is a facultative aerobe that can also grow under anaerobic conditions. Its size is approximately 4-10 µm long and 0.25-1.0 µm in diameter (Yu et al., 2013). It is not genetically modified in any manner.

3. DNA Ribotyping Analysis and Full Genome Sequence Analysis

Pure DNA was submitted to Beckman Coulter Genomics (Danvers, MA) for full genome sequencing following a series of DNA concentration and purity validations. Illumina® next

generation sequencing was used to sequence the entire *Bacillus subtilis* MB40 genome. This genome is over 1000 pages long and is not provided in this dossier. It is available upon request and would be provided electronically (BIO-CAT Microbials, 2015).

MIDI Labs (Newark, DE) performed 16S ribosomal DNA base pair analysis of *Bacillus subtilis* MB40 and confirmed that *Bacillus subtilis* MB40 was identified to the genus level with 99% similarity to *Bacillus subtilis* when compared to the Applied Biosystems MicroSeq® reference library. The lineage cladogram is shown in Figure 1. Strain MB40 is highlighted, located in the *Bacillus* genus and *subtilis* species library.

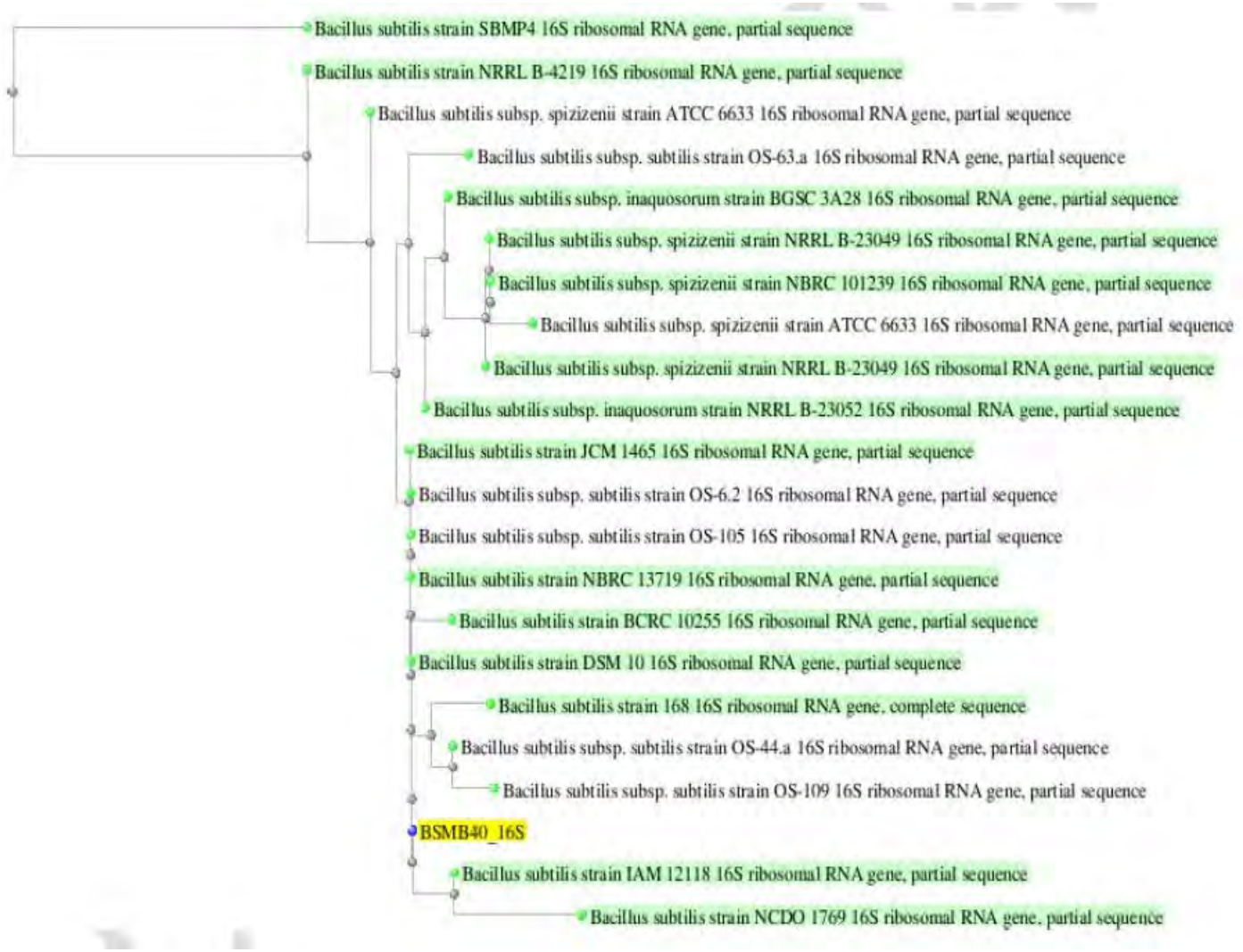


Figure 1. Speciation based on 16S ribosomal DNA sequence

Once the organism was identified to the genus and species level, a whole genome sequence alignment was done to more precisely fit *Bacillus subtilis* MB40 within the species framework. The cladogram for sequence alignment is provided in Figure 2.

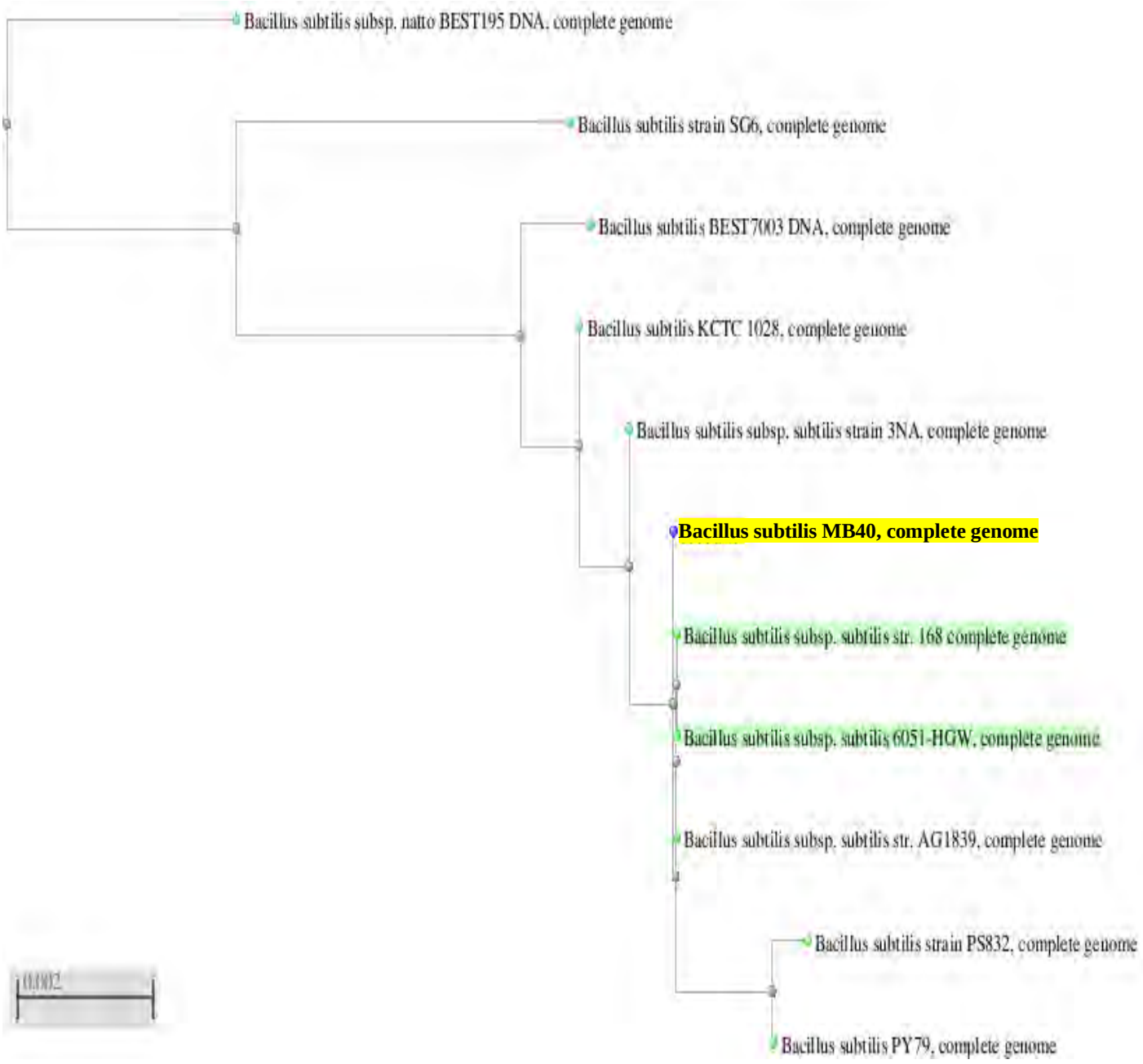


Figure 2. *Bacillus subtilis* MB40, highlighted in yellow, falls within the same in-group as *Bacillus subtilis* subsp. 6051-HGW, synonymous with DSM 10 parent strain.

The resulting sequence data was compared to published *Bacillus* genomes using nBLAST (Nucleotide Basic Local Alignment Search Tool) (National Institutes of Health, 2019a). nBLAST outputs are provided in Table 1. *Bacillus subtilis* MB40 genome sequence alignments showed 99% identity with *Bacillus subtilis subsp. subtilis* 6051- HGW which is deposited as *Bacillus subtilis* DSM-10 at DSMZ (Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures Inhoffenstraße 7B 38124 Braunschweig GERMANY). *Bacillus subtilis* DSM-10 (also known as Marburg strain or Subtilis 6051-HGW, highlighted in bold) is the parent strain of *Bacillus subtilis* MB40.

Table 1. Listing of the nBLAST outputs from the whole genome sequence alignments to *Bacillus subtilis* MB40

DESCRIPTION	TOTAL SCORE	QUERY	E VALUE	IDENT	ACCESSION
Bacillus subtilis subsp. subtilis str. 168, complete genome	7.88E+06	98%	0	99%	CP010052.1
Bacillus subtilis KCTC 1028, complete genome	7.88E+06	98%	0	99%	CP011115.1
Bacillus subtilis subsp. subtilis strain 3NA, complete genome	7.84E+06	97%	0	99%	CP010314.1
Bacillus subtilis subsp. subtilis str. AG1839, complete genome	7.82E+06	97%	0	99%	CP008698.1
Bacillus subtilis subsp. subtilis str. JH642 substr. AG174, complete genome	7.82E+06	97%	0	99%	CP007800.1
Bacillus subtilis strain PS832, complete genome	7.85E+06	98%	0	99%	CP010053.1
Bacillus subtilis subsp. subtilis 6051-HGW, complete genome	**7.88E+06**	**98%**	**0**	**99%**	**CP003329.1**
Bacillus subtilis PY79, complete genome	7.52E+06	94%	0	99%	CP006881.1
Bacillus subtilis QB928, complete genome	7.73E+06	96%	0	99%	CP003783.1
Bacillus subtilis genome assembly BS49Ch, chromosome	7.88E+06	98%	0	99%	LN649259.1
Bacillus subtilis BEST7003 DNA, complete genome	7.46E+06	93%	0	99%	AP012496.1
Bacillus subtilis strain TO-A JPC, complete genome	7.46E+06	93%	0	99%	CP011882.1

4. Phenotype Analysis

Fatty acid profiling via The MIDI Sherlock System Fatty Acid Methyl Ester (FAME) method has confirmed that *Bacillus subtilis* MB40 has a similarity index of 0.907 to the *Bacillus subtilis* species (Appendix 1).

B. Method of Manufacture of *Bacillus subtilis* MB40

Bacillus subtilis MB40 is produced consistent with current Good Manufacturing Practices (cGMP) as a pure spore culture consisting of only fermentation medium and *Bacillus subtilis* MB40 spores. The pure spore culture is concentrated via centrifugation. The concentrated liquid is then blended with enough maltodextrin so the total solids is up to 10% and then spray dried. The preparation is then blended with additional maltodextrin (or other approved diluents including, but not limited to, sodium chloride, calcium carbonate, and sodium bicarbonate) to achieve the finished formulation (OPTI-BIOME®). All stabilizers/additives/media (or diluents) used in the process are food-grade. For the purpose of the toxicological testing described below (see Part 6, for safety evaluations), pure *Bacillus subtilis* MB40 or OPTI-BIOME® (the finished product to be marketed) were used as the test article.

A manufacturing process diagram for OPTI-BIOME® is provided in Figure 3.

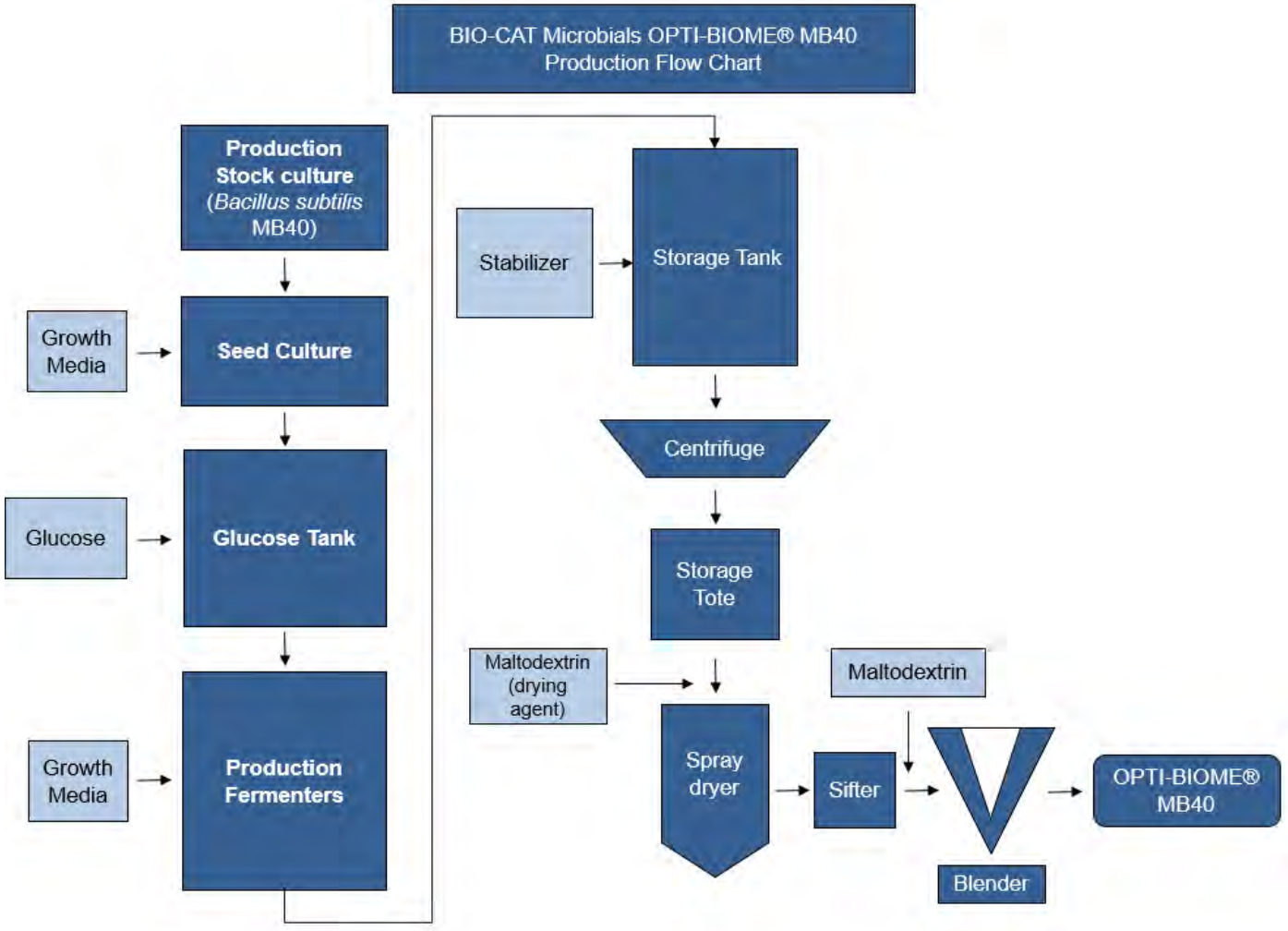


Figure 3. Method of Manufacture of OPTI-BIOME®

All fermentation equipment including tanks, lines, totes, separator, spray dryer, screens, and blenders are cleaned and sterilized prior to starting *Bacillus subtilis* MB40 fermentation batches. All equipment is swabbed (UltraSnap™, Hygiena, LLC) for microbial contamination. If a piece of equipment does not pass the ATP swab, that piece of equipment is not used until it has been recleaned and passes the ATP test. BIO-CAT manufactures other *Bacillus* products that contain soy and milk in the fermentation medium. Although *Bacillus subtilis* MB40 is produced on an allergen-free medium, the label contains the following statement "Although soy and milk are not formulated or used in the manufacture of this product, the product may contain traces of soy and milk."

C. Product Specifications

The food grade specifications for *Bacillus subtilis* MB40 are summarized in Table 2. Conformance to specifications and consistency of *Bacillus subtilis* MB40 manufacturing is demonstrated by the analyses of five non-consecutive lots of commercially representative *Bacillus subtilis* MB40 with results summarized in Table 3 and certificates of analysis provided in Appendix 2.

The collection of these reports demonstrates that the substance is well characterized and meets the established purity criteria.

Table 2. Food Grade Specification for *Bacillus subtilis* MB40

PHYSICAL AND CHEMICAL PARAMETERS	SPECIFICATION (ACCEPTABLE TARGET/RANGE)	TEST METHOD
Color	Light Tan to Tan	Visual
Visual Inspection	Visually free of foreign material	Visual
Texture	Crystalline, free flowing powder	Organoleptic
Odor	Strong fermentation	Organoleptic
Identity*	>98% homology	16S Sequencing
Activity (CFU/g)	NLT 100 Billion	FDA BAM
Moisture Content (%)	<10	Ohaus MB-45
Heavy Metals**		
Lead (ppm)	<0.5	ICP
Mercury (ppm)	<0.5	ICP
Cadmium (ppm)	<0.5	ICP
Arsenic (ppm)	<0.3	ICP
Microbiological Limits		
Yeast and Mold (CFU/g)	≤300	FDA BAM
Salmonella (per 25 g)	Negative	FDA BAM
Coliforms (CFU/g)	≤30	FDA BAM
***E. coli* (per 25 g)**	Negative	AOAC 991.14
Listeria (per 25 g)	Negative	FDA BAM
***S. aureus* (CFU/g)**	Non-detected***	AOAC 2003.07

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing of every lot
*** Detection level for growth of *S. aureus* on product samples is 10 CFU/g. Product with any detectable growth of *S. aureus* is considered a failed test and will be destroyed.
AOAC – Association for Official and Analytical Chemists; BAM – Bacteriological Analytical Manual; CFU – Colony Forming Units; FDA – Food and Drug Administration; g – gram; ICP – Inductively Coupled Plasma; NLT – not less than; ppm – parts per million

Table 3. Analytical Results for *Bacillus subtilis* MB40

Physical and Chemical Parameters	Acceptable Target/Range	*Bacillus subtilis* MB40 Batch Results				
		Lot No. OPTIMB40-MC02	Lot No. OPTIMB40-PC24	Lot No. OPTIMB40-CB13	Lot No. OPTIMB40-SA22	Lot No. OPTIMB40-SC11
Color	Light Tan to Tan	Light Tan	Light Tan	Light Tan	Light Tan	Light Tan
Visual Inspection	Visually free of foreign material	Pass	Pass	Pass	Pass	Pass
Texture	Crystalline, free flowing powder	Pass	Pass	Pass	Pass	Pass
Odor	Strong fermentation	Pass	Pass	Pass	Pass	Pass
Identity*	>98% homology	Pass	Pass	Pass	Pass	Pass
Activity (CFU/g)	NLT 100 Billion	138 Billion	112 Billion	119 Billion	115 Billion	115 Billion
Moisture Content (%)	<10	5.61	3.75	4.95	5.69	4.25
Heavy Metals**						
Lead (ppm)	<0.5	0.03	0.08	0.03	0.02	0.03
Mercury (ppm)	<0.5	<0.01	0.01	0.01	0.01	0.01
Cadmium (ppm)	<0.5	0.01	0.04	0.01	0.01	<0.01
Arsenic (ppm)	<0.3	0.05	0.11	0.07	0.05	<0.03
Microbiological Limits						
Yeast and Mold (CFU/g)	≤300	<300	<300	<300	<300	<300
Salmonella (per 25 g)	Negative	Negative	Negative	Negative	Negative	Negative
Coliforms (CFU/g)	≤30	< 30	< 30	< 30	< 30	< 30
***E. coli* (per 25 g)**	Negative	Negative	Negative	Negative	Negative	Negative
Listeria (per 25 g)	Negative	Negative	Negative	Negative	Negative	Negative
***S. aureus* (CFU/g)**	Non-detected***	<10	<10	<10	<10	<10

*Results determined from testing of Bacillus subtilis raw material
**Results determined from testing of every 5th lot
*** Detection level for growth of *S. aureus* on product samples is 10 CFU/g. Product with any detectable growth of *S. aureus* is considered a failed test and will be destroyed.
CFU – Colony Forming Units; g – gram; NLT– not less than; ppm – parts per million

D. Technical Function

Bacillus subtilis MB40 is intended for use as an ingredient in a wide variety of foods.

E. Stability Data

Shelf-life stability of the manufactured product has been determined for up to 30 months at room temperature (21 ±2°C) storage. Samples from two lots were analyzed at the time of manufacture and at later dates for plate count. Stability for up to 30 months was demonstrated by average plate counts of *Bacillus subtilis* within ±16% of the original value.

Stability data is outlined in Table 4.

Table 4. Shelf-life Stability of *Bacillus subtilis* MB40

SAMPLE ID	DOM	ORIGINAL AVG. PC (BILLIONS)*	RECOUNT DATE	AVG PC ON RECOUNT DATE*	SD	% SD	SURVIVAL (% ±SD)	TIME ELAPSED (MONTHS)
OPTIMB40-CB13	March 13, 2018	119	7/15/2019	118	3.79	3.21	99±3	16
OPTIMB40-SA22	May 22, 2017	115	7/15/2019	131	1.89	1.44	114±2	26
OPTIMB40-SA22	May 22, 2017	115	11/05/2019	133	8.72	6.54	116 ±2	30

*Average of three samples except for SA22 sample at 26 months where value is average from two samples.
DOM – date of manufacture; PC – plate count; SD – standard deviation

F. Intended Food Uses and Anticipated Dietary Exposure

1. Proposed Uses

Bacillus subtilis MB40 is intended for use as an ingredient in a wide variety of foods. *Bacillus subtilis* MB40 will be added to foods at a maximum level of 2 x 10^9 CFU/serving. The food categories, as defined in 21 CFR 170.3(n), to which *Bacillus subtilis* MB40 will be added are listed in Table 5.

Table 5. Proposed Food Uses of *Bacillus subtilis* MB40

FOOD CATEGORY
(1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving.
(3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks.
(4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals.
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(6) Chewing gum, including all forms.
(7) Coffee and tea, including regular, decaffeinated, and instant types.
(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
(9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars.
(10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products.
(12) Fats and oils, including margarine, dressings for salads, butter, salad oils, shortenings and cooking oils.
(16) Fresh fruit juices, including only raw fruits, citrus, melons, and berries, and home prepared "ades" and punches made therefrom.
(20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and

FOOD CATEGORY
specialties.
(21) Fruit and water ices, including all frozen fruit and water ices.
(22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads.
(23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables.
(25) Hard candy and cough drops, including all hard type candies.
(26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors.
(28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads.
(30) Milk, whole and skim, including only whole, lowfat, and skim fluid milks.
(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products*.
(32) Nuts and nut products, including whole or shelled tree nuts, peanuts, coconut, and nut and peanut spreads.
(33) Plant protein products, including the National Academy of Sciences/ National Research Council "reconstituted vegetable protein" category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins.
(35) Processed fruits and fruit juices, including all commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, "ades", and drink substitutes made therefrom.
(36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends.
(37) Snack foods, including chips, pretzels, and other novelty snacks.
(38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies.
(40) Soups and soup mixes, including commercially prepared meat, fish, poultry, vegetable, and combination soups and soup mixes**.
(41) Sugar, white, granulated, including only white granulated sugar.
(42) Sugar substitutes, including granulated, liquid, and tablet sugar substitutes.
(43) Sweet sauces, toppings, and syrups, including chocolate, berry, fruit, corn syrup, and maple sweet sauces and toppings.

**Bacillus subtilis* MB40 is not intended for use in infant formula.
***Bacillus subtilis* MB40 is not intended for use in any product that would require additional review by USDA.

2. Estimated Dietary Intake (EDI)

Consumer exposure to *Bacillus subtilis* MB40 was estimated using the methods described in GRN 399 (Ganeden, 2011) which utilized data from the USDA Nutrition Insights publication of the USDA Center for Nutrition Policy and Promotion (Basiotis et al., 2000). According to this report, males, aged 51 or older, consume the greatest number of servings of food per day, estimated as 18.2 servings of food/day, from the following categories: grains, fruits, vegetables, milk, meat and other (fats, oils, sweets). Therefore, using this upper intake level of 18.2 servings of food/day and assuming that *Bacillus subtilis* MB40 is added to every category of food outlined above, at the maximum use level of 2×10^9 CFU/ serving, the maximum estimated daily intake (EDI) is calculated as 3.64×10^{10} CFU/day (approximately 36 billion CFU/day or 5.2×10^8 CFU/kg bw/day for a 70 kg human).

3. Acceptable Daily Intake (ADI)

The tolerated level of *Bacillus subtilis* MB40 after repeated administration in human volunteers was determined to be 1 x 10^{10} CFU/day (Spears et al., 2020). This result corroborates the results of published clinical safety studies with other strains of *Bacillus subtilis* in which no adverse effects were reported after repeated administration to human volunteers at up to 1 x 10^{10} CFU/day.

In addition, the acceptable daily intake (ADI) of *Bacillus subtilis* MB40 was calculated using the methodology employed for a similar microbial ingredient with GRAS status as a food ingredient, as described in GRN 399 (Ganeden, 2011) and Endres et al. (2011). Based on the No-Observed-Adverse-Effect-Level (NOAEL) of 2000 mg/kg bw/day (equivalent to 3.7 x 10^{11} CFU/kg bw/day or 8.51 x 10^{10} CFU/day) in the 14-day toxicity study in rats with *Bacillus subtilis* MB40 (Spears et al., 2020) (See Section 6), and conservative 100-fold safety factor for inter- and intra-species differences, the ADI of *Bacillus subtilis* MB40 in humans is calculated as 3.7 x 10^{9} CFU/kg bw/day (or 2.6 x 10^{11} (260 billion) CFU/day for a 70 kg person). Clinical studies with *Bacillus subtilis* MB40, clinical and nonclinical studies with other *Bacillus subtilis* strains and a GRAS Notice for a different *Bacillus subtilis* strain (GRN 831) support the safety and appropriateness of the ADI for *Bacillus subtilis* MB40.

G. Estimated Dietary Exposure to Any Other Substance That is Expected to be Formed in or on Food

Not applicable.

H. Dietary Exposure to Contaminants, Byproducts and Other Bioactives

Potential contaminants of BIO-CAT's *Bacillus subtilis* MB40 include microbes and heavy metals. The specifications set for BIO-CAT's *Bacillus subtilis* MB40 place limits on the maximum permissible levels of these impurities to assure an acceptable final product. The batch data for five different lots document quality control of the final product such that it meets these specifications (Table 3).

PART 4. SELF-LIMITING LEVELS OF USE

There are no inherent self-limiting levels of use for *Bacillus subtilis* MB40.

PART 5. EXPERIENCE BASED ON COMMON FOOD USE IN FOOD BEFORE 1958

The statutory basis for the conclusion of GRAS status of *Bacillus subtilis* MB40 in this document is not based on common use in food before 1958. The GRAS conclusion is based on scientific procedures.

A. Other Information on Dietary Exposure

Humans are inherently exposed to *Bacillus subtilis*, given that the microbe can be isolated from water, soil, air and decomposing plant matter (Lefevre et al., 2017). Bacilli are reported to occur at population levels of 10^6 to 10^7 per gram of soil with 60-100% being in its inactive spore state (EPA, 1997). Bacillus counts of 10^6 CFU/g have been determined in wheat, grain, and whole meal (Sorokulova, 2013). Evidence shows that *Bacillus subtilis* spores have been found in the gastrointestinal tract of humans who have not intentionally consumed *Bacillus subtilis*-containing food or supplements (Tam et al., 2006; Hong et al., 2009; Fakhry et al., 2008).

In addition to indirect consumption, *Bacillus subtilis* has a long history of use in the food industry, specifically in fermented food products marketed in Asian and African regions. Alkaline-fermented foods generated by bacterial cultures containing *Bacillus subtilis* include Thai thua-nao and kinema from cooked soybeans, dawadawa from African locust beans, ugba from African oil beans, and orgiri from melon seeds (Wang and Fung, 1996). The traditional Japanese food “nattō” (fermented soybean) is made from soybeans fermented by *Bacillus subtilis*, and is believed to have been a component of the Japanese diet as early as the year 1450 (Shurtleff, 2012). At least three kinds of commercial nattō starter strains are available in Japan (Nishito et al., 2010). There are up to 1 x 10^9 viable spores of *Bacillus subtilis*/gram of nattō product, the consumption of which has a long history of safe use and is associated with beneficial health effects (Cutting, 2011; Homma et al., 2006). The USDA nutrient databank (USDA, SR-28) states that there are 175 g in a serving of nattō. A person consuming one serving of nattō/day would therefore consume 1.75 x 10^{11} CFU *Bacillus subtilis*/day (175 billion CFU/day) from this source only (USDA, 2019).

It is expected that exposure to *Bacillus subtilis* from foods that have not been supplemented with the bacteria is low relative to the amount of *Bacillus subtilis* that will be added to food per this GRAS determination, with the possible exception of consumers of nattō. It is unlikely, however, that a daily consumer of nattō would also be consuming foods containing *Bacillus subtilis* MB40 at 90th percentile levels of intake.

1. US Regulatory History

a. GRAS Status

The GRAS status of carbohydrase and protease enzyme preparations sourced from nonpathogenic and nontoxigenic strains of *Bacillus subtilis* was affirmed in 1997 (21 CFR 184.1148 and 21 CFR 184.1150). Subsequently, several enzyme preparations sourced from *Bacillus subtilis* have been notified as GRAS for use in foods based on scientific procedures. A GRAS Notice for *Bacillus subtilis* DE111 for use in infant formula and several different foods received a no questions letter from FDA (GRN 831), and one for *Bacillus subtilis* was withdrawn (Table 6). For GRN 831, the estimated daily intake of *Bacillus subtilis* DE111 was 1.3 x 10^{11} CFU/day.

Table 6. Summary of *Bacillus subtilis* in FDA GRAS Inventory

SUBSTANCE	GRN # / CLOSURE DATE	INTENDED USE	USE RATE	COMPANY/ REFERENCE	FDA RESPONSE
Bacillus subtilis **DE111**	GRN 831 Oct 7, 2019	For use in conventional foods and infant formula	Up to 1 x 10^{10} CFU/serving in foods intended for adults Up to 1 x 10^{9} CFU/serving in foods intended for children aged 2-12 Up to 2 X 10^{8} CFU/100 mL infant formula	Deerland Probiotics and Enzymes FDA GRN 831 (2019)	FDA had no questions
Maltogenic alpha-amylase from *Bacillus stearothermophilus* **produced in** *Bacillus subtilis*	GRN 751 July 31, 2018	For use in processing starch in food manufacturing	Minimum levels necessary to achieve the intended technical effect	Novozymes North America, Inc. FDA GRN 751 (2018)	FDA had no questions
Maltogenic amylase from *Geobacillus stearothermophilus* **produced in** *Bacillus subtilis*	GRN 746 June 13, 2018	For use in baking processes	Minimum levels necessary to achieve the intended technical effect	AB Enzymes FDA GRN 746 (2018)	FDA had no questions
Subtilisin from *Bacillus amyloliquefaciens* **produced in** *Bacillus subtilis*	GRN 714 Feb 6, 2018	For use in the processing of protein at to facilitate protein hydrolysis	58-369 mg TOS/kg substrate	Danisco US Inc. (Dupont Industrial Biosciences) FDA GRN 714 (2018)	FDA had no questions
β-galactosidase enzyme preparation from *Bacillus circulans* **produced in** *Bacillus subtilis*	GRN 649 Nov 28, 2016	For use as a processing aid in the production of galacto-oligosaccharides (GOS)	Up to 0.3% of the lactose starting material	GenoFocus, Inc. FDA GRN 649 (2016)	FDA had no questions
β-glucanase from *Bacillus subtilis*	GRN 592 Oct 7, 2015	For use as a processing aid in brewing and potable alcohol production	36.56 mg TOS/kg grist	Danisco US Inc. FDA GRN 592 (2015)	FDA had no questions
Lactase from *Bifidobacterium bifidum* **produced in** *Bacillus subtilis*	GRN 579 Nov 5, 2015	For use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products	1.1 mg TOS/g milk 1.3 mg TOS/g GOS for use in infant formula	Danisco US Inc. FDA GRN 579 (2015)	FDA had no questions
Bacillus subtilis	GRN 562	For use in post-harvest processing of bananas as an ingredient added to wash water	6.3 x 10^{2} CFU/mL to 1.9 x 10^{3} CFU/mL	BiOWiSH Technologies, Inc. FDA GRN 562 (2014)	FDA ceased to evaluate at notifier's request
Asparaginase enzyme preparation produced by genetically modified *Bacillus subtilis*	GRN 476 Feb 3, 2014	As an enzyme in bread, potato, cereals, coffee and chocolate products, at a level of up to 20 milligram	Up to 20 mg TOS/kg food	Novozymes North America, Inc. FDA GRN 476	FDA had no questions

SUBSTANCE	GRN # / CLOSURE DATE	INTENDED USE	USE RATE	COMPANY/ REFERENCE	FDA RESPONSE
		Total Organic Solids per kilogram of food		(2014)	
1,4-α-glucan branching enzyme preparation from *Bacillus subtilis* strain 168 expressing the glucan branching enzyme gene from *Aquifex aeolicus* strain VF5	GRN 406 Sep 11, 2012	As an enzyme in the production of cyclic dextran and enzymatically-synthesized glycogen	0.07 mg TOS/ g substrate for cyclic dextran production 0.67 mg TOS/g substrate for glycogen production	Ezaki Glico Co., Ltd. FDA GRN 406 (2012)	FDA had no questions
Branching glycosyltransferase enzyme preparation from *Bacillus subtilis* expressing a branching glycosyltransferase gene from *Rhodothermus obamensis*	GRN 274 Jun 25, 2009	As an enzyme in the starch industry to obtain dextrins with improved physical properties, such as higher solubility, lower viscosity, and reduced retrogradation	Up to 4%	Novozymes North America, Inc. FDA GRN 274 (2009)	FDA had no questions
Pullulanase enzyme preparation from *Bacillus subtilis* expressing the pullulanase gene from *B. acidopullulyticus*	GRN 205 Dec 4, 2006	As an enzyme in the brewing industry (to hydrolyze 1-6-alpha-D-glucosidic linkages in pullulan, amylopectin, and glycogen	Up to 25 L/ton of starch dry substance	Novozymes North America, Inc. FDA GRN 205 (2006)	FDA had no questions
Pectate lyase enzyme preparation from *Bacillus subtilis*	GRN 114 Jan 27, 2003	Use in fruit and vegetable purees and concentrates as an enzyme	0.5-1.0 % by weight	Japan Cellfoods Co., Ltd. FDA GRN 114 (2003)	FDA had no questions
Pullulanase derived from *Bacillus subtilis* carrying a gene encoding pullulanase from *Bacillus naganoensis*	GRN 20 Sep 30, 1999	Use in hydrolyzing starch and starch-related compounds in the production of corn sweeteners, baked goods, and alcoholic beverages at minimum levels necessary to accomplish the intended effect in accordance with current good manufacturing practices	Minimum levels necessary to accomplish the intended effect in accordance with cGMP	Enzyme Bio-Systems Ltd. FDA GRN 20 (1999)	FDA had no questions

CFU – colony forming units; cGMP – current Good Manufacturing Practices; GOS – galacto-oligosaccharides; kg – kilogram; mg – milligram; mL – milliliter; TOS – total organic solids

b. New Dietary Ingredient Notifications

Four New Dietary Ingredient Notifications (NDINs) for various *Bacillus subtilis* strains have been submitted to FDA, none of which have been accepted (Table 7). In all cases. FDA was unable to establish the safety of the ingredient. Reasons cited for lack of approval include lack of information about identity, consumption, antibiotic resistance, colonization in the gastrointestinal tract, effect on normal gut flora, metabolites known to be produced by the particular strain, or potential for allergy.

Despite the fact that no NDINs for *Bacillus subtilis* have been accepted, a total of 95 dietary supplement products containing *Bacillus subtilis* are mentioned on the National Institutes of Health (NIH) Dietary Supplement Label Database (National Institutes of Health, 2019b). The majority of these products did not mention the recommended use levels; however, two products mentioned daily use of 1 x 10^9 CFU/day and 2.0 x 10^9 CFU/day. A number of dietary supplements containing *Bacillus subtilis* in combination with other live microbials are available for sale on the internet, and a few contained only *Bacillus subtilis*. Recommended usage rates of two additional supplements containing only *Bacillus subtilis* that were found on websites are 3.1 x 10^9 CFU/day and 1 x 10^{10} CFU/day (Amazon.com, 2019; Acupuncture Atlanta, 2019).

Table 7. Summary of *Bacillus subtilis* in FDA NDI Inventory

SUBSTANCE	NDIN # / DATE OF FDA'S RESPONSE	RECOMMENDED DAILY DOSE	COMPANY/ REFERENCE	FDA RESPONSE
Bacillus Subtilis Strain PB6 ATCC PTA-673	NDI 741 Jan.30, 2012	95 billion CFU /serving/day	Kemin Pharma FDA NDIN 741(2012)	FDA was unable to establish the safety of the ingredient
Bacillus subtilis PB6	NDI 477 July 31, 2008	1 x 10^9 to 1 x 10^{10} CFU/serving/day	Kemin Industries, L.C. FDA NDIN 477 (2008)	FDA was unable to establish the safety of the ingredient
Bacillus Subtilis Strain DB9001	NDI 324 March 3, 2006	7.5 x 10^8 CFU/serving/day	BAU Inc. FDA NDIN 324 (2006)	FDA was unable to establish the safety of the ingredient
Bacillus subtilis **DB9011**	NDI 277 June 15, 2005	16.5 mg/capsule 1-3 capsules/day	BAU Inc. FDA NDIN 277 (2005)	FDA was unable to establish the safety of the ingredient

CFU – colony forming unit; NDI – New Dietary Ingredient; NDIN – New Dietary Ingredient Notification

c. Animal Feed

Under section 36.14 of the 2019 Association of American Feed Control Officials (AAFCO) Official Publication, *Bacillus subtilis* is listed as a microorganism that was reviewed by the Food and Drug Administration, Center for Veterinary Medicine and found to present no safety concerns when used in direct-fed microbial products (AAFCO, 2019).

d. Pesticides

Several *Bacillus subtilis* strains have been approved for use as biocides by the Environmental Protection Agency (EPA), and have been exempted from tolerances in food crops, including GB03; FMCH002, BU1814; MBI 600; CX-9060, QST 713, and QST 713 variant soil (EPA, 2008; EPA, 2017; EPA, 2018; EPA, 2009; 2012a; EPA, 2012b). In the Federal Register notice for the QST 713

variant soil exemption, the EPA stated that *Bacillus subtilis* is not considered to be toxic or pathogenic to humans, animals or plants (EPA, 2012b).

2. European Regulatory History

The European Food Safety Authority (EFSA) confirmed a Qualified Presumption of Safety Determination for the use of *Bacillus subtilis* as an animal feed additive based on the absence of toxigenic potential (EFSA, 2013).

3. Canadian Regulatory History

Bacillus subtilis is recognized by the Natural and Non-Prescription Health Products Directorate (NNHPD) of Health Canada as a Natural Health Product (NHP) ingredient under Schedule 1, Item 1 (bacterium) of the *Natural Health Product Regulations*. In order to sell NHPs like OPTI-BIOME® in Canada, a Product Licence in the form of an eight digit Natural Product Number (NPN) must be issued by Health Canada. Thus, submission of a Product Licence Application (PLA) to the NNHPD is required. Only once Health Canada has reviewed and approved a PLA for safety, efficacy, and quality, is an NPN granted. This unique identifier (i.e. 8000XXXX) must appear on the label's Principal Display Panel (PDP). The Master File pathway precedes the PLA process, and is a mechanism which enables manufacturers of raw materials or finished products to protect safety, efficacy, manufacturing, packaging, processing, and/or quality data. This proprietary information is held on file with the Government, preventing direct disclosure to the customer/Clinical Trial or Product Licence Applicant, while still permitting efficient investigation, approval, and registration. The OPTI-BIOME® Master File and associated mock PLA protects BIO-CAT's unique manufacturing process and allows their customers to efficiently register finished products containing *B. subtilis* MB40 – several of which have already done so. Although the Master File is specific to NHP use, it should be noted that food enzymes produced by various strains of *B. subtilis* are also recognized as food additives in Canada (Government of Canada, 2019).

PART 6. NARRATIVE

A. *Bacillus subtilis* Safety Evaluation (Other Strains)

Bacillus subtilis is not considered pathogenic or toxigenic to humans, animals, or plants (EPA, 1997). Based on a review of literature citing human infections with *Bacillus subtilis* ((de Boer and Diderichsen, 1991), almost all cases of *Bacillus subtilis* infection were related to drug abusers or debilitated patients. In general, there was no evidence of any pathogenic potential of *Bacillus subtilis* to humans and very few examples of *Bacillus subtilis* strains as confirmed causes of food poisoning.

In a case report of two patients presenting with cholestatic hepatitis, pruritus, and/or cirrhosis after consumption of Herbalife® preparations, samples of the Herbalife® products ingested by both patients showed growth of *Bacillus subtilis* (identified via sequencing of 16S rRNA and *gyrB*

genes), likely from contamination by an environmental source (Stickel et al., 2009). Although causality between consumption of Herbalife® products and disease was scored ‘‘probable” in both cases, Gram-positive bacteria are extremely rare causes for liver injury. Further, the NIH has examined 50 cases of liver injury attributed to Herbalife® products and opined that the mechanism is unexplained (NIH, 2018). The clinical safety of preparations containing *Bacillus subtilis* (discussed below) also supports the conclusion that the isolated case reports of hepatoxicity from *Bacillus subtilis*-contaminated Herbalife® preparations do not give rise to safety concerns regarding the intended use of *Bacillus subtilis* MB40.

1. Toxicology Data on *Bacillus subtilis* (Other Strains)

a. Safety studies in experimental animals

Oral toxicity studies with *Bacillus subtilis* in rats, mice, rabbits, and piglets confirm the lack of adverse effects associated with repeated exposures to *Bacillus subtilis* and are consistent with the results of the oral toxicity study with *Bacillus subtilis* MB40 (Spears et al., 2020). Although these published repeated exposure studies were generally conducted at a single dose level that did not permit evaluation of a dose-response relationship, the results of these studies support the safety assessment of several *Bacillus subtilis* species, some of which have documented histories of commercial applications (Sorokulova et al., 2008), at anticipated consumer exposure levels from use as an ingredient in foods. As discussed by Tompkins et al. (2008), the 28-day study with *Bacillus subtilis* R0179 (summarized below) was conducted “to ensure safety at high doses” of a product for which the therapeutic efficacy has been documented in a number of clinical trials.

A 10-day oral (gavage) toxicity study of *Bacillus subtilis* VKPM B2335 (BS3) was conducted in male BALB/c mice, male New Zealand white rabbits, newborn piglets (strain and sex not reported) (n=10/species) and a separate 30-day study was performed with rabbits (n=20) (Sorokulova et al., 2008). *Bacillus subtilis* VKPM B2335 (BS3) was administered at a single dose (1.0×10^6 CFU/day for mice; 1.0×10^9 CFU/day for rabbits and piglets) in sterile phosphate buffered saline. An additional 10 animals/species received the vehicle alone in each study. The animals were observed for activity and behavior and histopathological evaluation of select tissues and organs was conducted after euthanasia. Blood samples were collected from rabbits by cardiac puncture on days 10 and 30 and evaluated for hematology parameters. Leukocytes were counted to determine the differential percentages of white blood cells (lymphocyte, monocytes, eosinophils, and heterophils). Total red blood cells, sedimentation rate and hemoglobin concentration were determined. Hematology parameters were not evaluated in mice or piglets.

There were no adverse effects on the general health status of the animals, and no changes in the organs and tissues of treated animals were reported. There were no differences in the hematological indexes measured in the blood from control and treated rabbits. The authors concluded that the test strain of *Bacillus subtilis* (VKPM B2335; BS3) “may therefore be considered as non-pathogenic and safe for human consumption” (Sorokulova et al., 2008).

Hong et al. (2008) conducted a 30-day gavage study of *Bacillus subtilis* Nattō administered to six male New Zealand White rabbits at a single dose of 1.0 x 10^9 CFU/day. A naïve control group received the vehicle (saline) at the same volume (1 mL/day). Blood samples for hematological evaluation (total red blood cells, leucocytes, hemoglobin concentration, and differential percentages of white blood cells) were collected by cardiac puncture from anaesthetized animals on day 30 and select tissues and organs were collected for histopathological examination after euthanasia, including liver, kidneys, spleens, small intestines, and mesenteric lymph nodes. In a separate acute single-dose study conducted by the same authors, groups of 5 male and female Harley Dunkin guinea pigs were administered a 1 ml dose of *Bacillus subtilis* Nattō at 1.0 x 10^{12} CFU or the vehicle (saline) and observed for 14 days. Animals were observed daily for behavior, appearance, activity and feces. Body weights were recorded on days 0, 7, 14, and 17. On day 17, blood was drawn (by cardiac puncture from anaesthetized animals) for hematological analysis (same parameters as 30-day study). Select tissues and organs were collected for histopathological examination after euthanasia including liver, kidneys, spleens, small intestines, and mesenteric lymph nodes.

There were no reported adverse effects on the general health status or feed intake of rabbits administered *Bacillus subtilis* Nattō at 1.0 x 10^9 CFU/day for 30 days. No changes in selected visceral organs and tissues were reported and no significant differences in the hematological indexes were reported in treated rabbits compared to controls. In the acute toxicity study, a statistically significant higher weight gain in female guinea pigs administered 1.0 x 10^{12} CFU *Bacillus subtilis* Nattō was reported on day 14 (but not days 7 or 17), while feed intake was unaffected in both males and females. Histological analysis of organs and tissues revealed no signs of inflammation or pathological changes and no differences in the hematological indices between control and treated groups. The authors concluded that "*Bacillus subtilis* appeared to show no sign of toxicity or virulence using *in vivo* assessments" (Hong et al., 2008).

A 28-day oral (gavage) toxicity study of *Bacillus subtilis* R0179 in rats was reported by Tompkins et al. (2008). *Bacillus subtilis* R0179 was administered to 15 male and 15 female Sprague- Dawley albino rats at a single dose of 2 x 10^9 CFU/kg bw/day (vehicle not reported). A control group received an equal volume of the vehicle. Animals were monitored daily for mortality, morbidity, and clinical signs of toxicity. Body mass, food consumption, anatomic pathology, intestinal colonization, and infection were evaluated. The sensory reactivity to auditory, visual and proprioceptive stimuli, grip strength, and motor activity were also assessed. At the end of the treatment period, all animals were sacrificed and select organs (liver, kidneys, spleen, heart, and lungs) were subjected to histopathological and microbiological examination. Terminal portions of the small and large intestine from 4 animals/sex/group were removed for microbial examination of intestinal contents.

No clinical signs of toxicity or oral intolerance were reported in the study. There were no variations in body mass, food consumption, or mortality compared to the vehicle control group. There were no gross lesions at necropsy or changes in organ weights with the exception of lower absolute heart weights reported for test article-treated females only; heart weights relative to body weight were not

affected. The intestinal contents collected from treated animals were found to contain high levels of *Bacillus subtilis*. The authors concluded that the results of this study in combination with the observations of clinical studies in both infants and adults indicate that these microbes are safe for use and pose low risk to the consumer(Tompkins et al., 2008).

The effect of *Bacillus subtilis* 18 (BS-18) on intestinal health of 15-day old mice was studied by Li et al. (2019). Groups of 10 KM mice (5/sex) were necropsied after treatment with 0 or 1×10^9 CFU/day BS-18 for 18 days and the intestine (duodenum, jejunum, ileum, and cecum), liver, spleen, and kidney were analyzed macroscopically and microscopically. The diversity of bacteria in the intestine was also examined. The mice exhibited no abnormal behavior during the treatment period and no pathological lesions were observed in tissues that were examined after necropsy. There also were no adverse effects on the microbiome of the intestine or on body weight.

b. Feeding studies in livestock

Several studies have been conducted in pigs and rabbits to assess the effect of *Bacillus subtilis* on performance. The results show that up to 1.3 x 10^8 CFU/day *Bacillus subtilis* has no effect on performance of rabbits, that up to 1.2 x 10^9 CFU/day during gestation and 6.2 x 10^9 CFU/day during lactation has no effect on reproduction or development of pigs, and that up to 3.1 x 10^8 CFU/day has no effect on the performance of piglets. Results of these studies are summarized in Table 8.

Table 8. Results of *Bacillus subtilis* studies in livestock

SPECIES	CONCENTRATION/ DOSE/DURATION	ENDPOINTS MEASURED	RESULTS	REFERENCE
Pigs (sucking)	0 or 2 × 10^9 CFU/kg formula powder[3] (3.1×10^8 CFU/day)[1] 21 days	BW, ADG, ADMI, FCR, intestinal morphology, weight of heart, liver, spleen, kidney, pancreas and intestine, differential white blood cell count, plasma immunoglobulins and cytokines, digestive enzyme activities, bacteria in colonic digesta, expression of genes associated with innate immunity in ileal tissue	No adverse effect on any parameter measured	Hu et al. (2017)
Pigs (pregnant sows and offspring)	0 or 3 x 10^5 CFU/g feed (8.4×10^8 CFU/day)[2]	Reproductive performance for two generations, body condition, feed consumption, BW, fecal bacteria	No adverse effect on any parameter measured	Kritas et al. (2015)
Pigs (pregnant sows and offspring)	0 or 5 x 10^5 CFU/g gestation feed plus 1 x 10^6 CFU/g lactation feed 0 or 5 x 10^5 CFU/g nursery feed	ADG, ADFI, BW, fecal consistency, fecal microbes, litter size and weight, number of piglets total born, born alive, stillborn, and mummies, pre-wean mortality	No adverse effect on any parameter measured with the exception of ↓ ADG and ADFI in late nursery period	Menegat et al. (2019)

SPECIES	CONCENTRATION/ DOSE/DURATION	ENDPOINTS MEASURED	RESULTS	REFERENCE
	(1.2 x 10^9 CFU/day during gestation, 6.2 x 10^9 CFU/day during lactation and 3 x 10^8 CFU/day during the nursery period)[3]		in piglets born from treated sows. There was, however, no effect of sow dietary treatment on piglet gain: feed during this period.	
Rabbits (8 weeks old)	4 x 10^9 CFU/g 0, 200, 400 g /ton feed (5 x 10^7 or 1.3 x 10^8 CFU/day)[4] 56 days	FC, BW, BW gain, FCR, carcass characteristics, serum cholesterol, hemoglobin, RBC, platelets, cell-mediated immunity	No adverse effect on any parameter measured	Fathi et al. (2017)
Rabbits (28 days old)	0 or 1×10^6 CFU/g feed (5 x 10^7 CFU/day)[5] 42 days	ADFI, BWG, FCR, performance index, fecal score, intestinal bacteria and VFA, feed digestibility	No adverse effect on any parameter measured	Phuoc and Jamikorn (2017)

[1] Calculated using stated CFU/kg powder, average initial body weight (2.69 kg) and average daily dry matter intake from Days 1-7 (154 g/day)

[2] Calculated using stated CFU/kg feed and feed consumption of 2.8 kg/day from 65th day of gestation to farrowing

[3] Calculated using stated CFU/kg feed and ADFI in sows of 2.4 kg/day during weaning and 6.2 kg/day during lactation and overall ADFI in offspring of 600 g/day during nursery period

[4] Calculated using 907 kg/ton feed, stated CFU/g microbial, g feed consumed over study (3193.1 and 3987.1 g feed consumed in low and high dose groups, and 56 study days

[5] Calculated using stated CFU/g feed and ADFI of 48.42 g/day from Days 28-42.

ADFI – average gaily feed intake; ADG – average daily gain, ADMI – average daily dry matter intake; BW – body weight, CFU – colony forming units; FC – feed consumption; FCR – feed conversion ratio; RBC – red blood cell count; VFA – volatile fatty acids

2. Clinical Safety Data on *Bacillus subtilis* (other strains)

A number of clinical studies have been performed with *Bacillus subtilis*, and for the purpose of this dossier, we have focused on any discussion of potential adverse effects associated with their intake.

Tompkins et al. (2010) published a review of 24 clinical investigations and 3 case studies with Medilac® formulations containing *Bacillus subtilis* R0179 and *E. faecium* R0026. Male and female study participants with ulcerative colitis, diarrhea, irritable bowel syndrome, and other gastrointestinal conditions were included in these studies. Although the clinical trials were predominantly designed to assess efficacy, several reported adverse event details. No adverse reactions were directly linked to the use of Medilac® formulations.

Total enrollment ranged from 34 to 352 subjects in each study with an overall median enrollment of 56 subjects. The median age in treatment groups ranged from 27 to 65 years. The dose regimen in nearly all of the reviewed studies was two capsules three times/day, resulting in approximately 3.0 x 10^9 CFU/day for 5 days to 12 weeks, with the exception of one study in which subjects

received 1.5 x 10^9 CFU/day for 2 weeks. The basis for the selection of doses administered in the 27 studies reviewed was not described in this publication; however, all studies were reported to be investigator or institution-initiated, post-market clinical trials evaluating efficacy of supplementation (Tompkins et al., 2010).

A study in critically ill patients performed after publication of the Tompkins review reported no adverse effects of three times/day treatment with one capsule of Medilac-S® (total dose of *Bacillus subtilis* and *E. faecium*,1.35 x10^{10} and 1.5 x10^9 CFU/day, respectively) for up to 14 days (Zeng et al., 2016).

In a randomized, double-blind, placebo-controlled trial, healthy adults (n=81; 18-50 years old) received *Bacillus subtilis* R0179 at doses of 0.1, 1.0 or 10 x 10^9 CFU/capsule/day for four weeks (Hanifi et al., 2015). The test article was comprised of 75% *Bacillus subtilis* R0179 in spore form and 25% in vegetative form. Participants were instructed to consume one capsule/day at the end of a meal. General wellness was assessed using a daily questionnaire evaluating gastrointestinal (GI), cephalic, ear-nose-throat, behavioral, emetic, and epidermal symptoms. GI symptoms were further evaluated using a weekly gastrointestinal symptom rating scale (GSRS). GI transit viability of *Bacillus subtilis* R0179 was assessed by plating and microbiota analysis by 16S rRNA at baseline, week 4 of the intervention and washout.

There were no reported Adverse Events related to consumption of the study product. General wellness and GI function were not affected by oral consumption of *Bacillus subtilis* R0179 at any dose. Daily questionnaire syndrome scores were not different from baseline and did not exceed a clinically significant score of 1. GSRS syndrome scores were not different from baseline and ranged from 1.1±0.1 to 1.9±0.2. Fecal viable counts of *Bacillus subtilis* R0179 were statistically significantly higher compared to the placebo group and demonstrated a dose response. The authors concluded that "*Bacillus subtilis* R0179 survives passage through the human GI tract and is well tolerated by healthy adults at intakes from 0.1 to 10 x 10^9 CFU/day".

A double-blind, randomized, placebo-controlled trial was conducted to assess the effect of *Bacillus subtilis* C-3102 on chronic diarrhea in healthy volunteers with loose stools (Hatanaka et al., 2018). The subjects (n=44/group) received three tablets/day of a placebo or *Bacillus subtilis* C-3102 spores (total of 2.2×10^9 CFU/day) for a total of eight weeks. Evaluations included Bristol stool scale, a physician-conducted GSRS, a subject's perception of general health questionnaire, and water and microbial analyses of feces. Two subjects in the placebo group and four in the *Bacillus subtilis* C-3102 group dropped out of the study – none for intolerance to their designated treatment. Compliance was good – 99.4% in the placebo group and 99.7% in the *Bacillus subtilis* C-3102 group. There were no adverse effects of treatment on any parameter measured in the study, and there was no mention of any adverse events.

Hatanaka et al. (2020) recently performed a double-blind, randomized, placebo-controlled trial to determine whether ingestion of 4.8×10^{10} CFU/day *Bacillus subtilis* C-3102 for 28 days was safe for healthy adults. The subjects (n=44) were equally divided into the treatment and placebo groups.

Safety parameters, including physical examination, urinalysis, hematology, clinical chemistry, and bone mineral density (BMD) were measured at baseline, 2 and 4 weeks. Adverse events were recorded in a medical questionnaire administered by a clinical trial physician and daily reports written by the subjects. All subjects completed the study without violating the protocol and their rates of consumption were >90 %. There were no statistically significant differences in urinalysis, BMD or adverse events between groups. Statistically significant differences were noted in values of some parameters between the *Bacillus subtilis* C-3102 and placebo groups; however, they were not considered toxicologically relevant because they were transient and/or within stated reference ranges. These include increases in systolic blood pressure and mean corpuscular hemoglobin level and decreases in body fat percentage, cholinesterase, total cholesterol, and triglyceride levels at two weeks and an increase in direct bilirubin and a decrease in total cholesterol at 4 weeks. It is altogether possible that the statistically significant differences in blood pressure denoted at 2 weeks and direct bilirubin at 4 weeks are erroneous, because the values for systolic blood pressure in the two groups differed by less than 1 mm Hg (117.1 ± 14.8 mm Hg in the treatment group versus 116.4 ± 18.0 mm Hg in the placebo group) and the values for direct bilirubin were equal (0.1 ± 0.0 mg/dL in both groups).

The effect of *Bacillus subtilis* CU1 on immune stimulation and resistance to common infectious disease episodes was tested in healthy, free-living seniors (age 60-74) in a randomized, double-blind, placebo-controlled, parallel-arm study (Lefevre et al., 2015). Results of safety tests are reported in a different publication (Lefevre et al., 2017). Subjects (50/group) consumed either the placebo or the test material (2.1×10^9 *Bacillus subtilis* CU1 spores daily) for 10 days, followed by 18 days without consumption of the study products (break period). This scheme was repeated four times during the 16-week study. Blood was collected at baseline (1-2 weeks before the start of the study) and at week 16 for hematology and evaluation of liver and kidney markers. Hemodynamic parameters, including arterial pressure and heart rate, were evaluated on the first day of the study (prior to test material consumption), halfway through the study (Day 56), and at the end of the study. Symptoms of gastrointestinal and upper/lower respiratory tract infections were recorded daily by the subjects. Blood, saliva and stool samples were collected in a predefined subset of the first forty-four subjects enrolled in the study (22/group) for analysis of Immunoglobulin A (all samples) and cytokines (blood only). *Bacillus subtilis* CU1 was found in stool of treated, but not control subjects. None of the subjects withdrew from the study after treatment start. There was no difference between groups in the number of subjects experiencing at least one adverse event or the likelihood of the adverse events being associated with study participation. Three events in the treatment group were possibly associated with participation in the study (2 incidents of nasal obstruction episodes in the same subject and one report of headache in another subject), and one event was likely related (mild pain for about 10 min after test capsule consumption) but remained an isolated event. In the placebo group, one event (a headache that appeared minutes after taking the test product and disappeared over the course of the day) was possibly related to study participation. All adverse events related to treatment in both groups were mild in severity. There was no effect of treatment with the test material on hematology, markers of liver or kidney toxicity or hemodynamics. The authors concluded that the test material was safe and well tolerated.

B. *Bacillus subtilis* strain MB40 Safety Evaluation

1. Complete Genome Sequencing and Strain Lineage

Complete genome sequencing was conducted on isolated *Bacillus subtilis* MB40 colonies in order to perform DNA sequence-based testing for potential risk ranging from antibiotic resistance to toxin production (Kramer and Spears, 2015a). As discussed in Part 3, the complete genome sequence is available upon request and would be provided electronically (BIO-CAT Microbials, 2015).

2. Genome Analysis - Toxin Screening

Using the genome sequence, the potential of *Bacillus subtilis* MB40 to produce the major enterotoxins (Hbl, Nhe, CytK, entFM, and BceT) found in other disease/illness related *Bacillus* species was investigated using two *in silico* methods: virtual polymerase chain reaction (PCR) and nBLAST. Positive control genes were identified and used to demonstrate functionality of the nBLAST algorithm and virtual PCR tool. The *Bacillus cereus* genome was also analyzed as a positive control using both methods. No in-frame complete matches to the six major enterotoxins harbored by *Bacillus* species (Hbl, Nhe, CytK, entFM, and BceT) were generated for *Bacillus subtilis* MB40 using either nBLAST or virtual PCR (Kramer and Spears, 2015b).

3. Enterotoxin Testing

Following the *in-silico* evaluation described above, the absence of two of the major enterotoxins produced by *Bacillus* species (*Bacillus* Diarrheal Enterotoxin and *Bacillus cereus* enterotoxins was confirmed by commercially available assay kits manufactured by 3M (St. Paul, MN) and Oxoid (Hampshire, UK). Neither of the kits were able to detect the presence of either the toxin at their respective detection thresholds, while the positive and negative control samples yielded positive and negative results, respectively. Therefore, it was concluded that under production fermentation conditions *Bacillus subtilis* MB40 does not produce either of these toxins nor will they be present in the commercial product (Kramer and Spears, 2015b).

Bacillus subtilis has been shown to produce a protease, subtilisin, that is capable of causing sensitization in fermentation facility workers repeatedly exposed to high exposure levels (EPA, 1997). The level of residual subtilisin present in the *Bacillus subtilis* MB40 microbial preparation was demonstrated to be negligible (i.e. below analytical limits of detection) in a sensitive assay for alkaline protease activity (BIO-CAT; unpublished data on file). Subtilisin is considered to have “very low toxigenic properties” (EPA, 1997) and has GRAS status as a direct food additive at levels consistent with cGMP (21 CFR 184.1150).

4. Antibiotic Resistance

Three different methods were used to test *Bacillus subtilis* MB40 for antibiotic resistance. Results of all three tests were submitted to Health Canada for the OPTI-BIOME® Master File.

A preliminary evaluation was conducted using ResFinder, a web-based method that uses nBLAST for identification of acquired antimicrobial resistance genes in whole-genome data (Zankari et al., 2012).

As shown in Table 9, bioinformatic data generated by ResFinder suggested that *Bacillus subtilis* MB40 had a high likelihood of being resistant to Tetracycline and Aminoglycoside antibiotics.

Table 9. ResFinder Results for *Bacillus subtilis* MB40 Antibiotic Resistance

Antibiotic Group	% Identity	Query/HSP Length	Predicted Phenotype
Aminoglycoside Resistance gene aadK	100	855/855	Aminoglycoside Resistance
Beta-lactam	No resistance genes found		
Fluoroquinolone	No resistance genes found		
Fosfomycin	No resistance genes found		
Fusidic acid	No resistance genes found		
Glycopeptide	No resistance genes found		
MLS – macrolide, Lincosamide, and Streptogramin B	No resistance genes found		
Nitroimidazole	No resistance genes found		
Oxazolidinone	No resistance genes found		
Phenicol	No resistance genes found		
Rifampicin	No resistance genes found		
Sulphonamide	No resistance genes found		
Trimethoprim	No resistance genes found		
Tetracycline Resistance gene tet(L)	100	1377/1377	Tetracycline Resistance

Due to the potential for false positives or inclusive results from the ResFinder method, antibiotic sensitivity testing was performed in vivo based on published guidelines established by the Clinical Laboratory Standards Institute (CLSI) subcommittee on Antimicrobial Susceptibility Testing. *Bacillus subtilis* MB40 colonies were plated with various classes of antibiotics and incubated for 16-18 hours in ambient air at 35°C. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control test organisms according to CLSI protocols. Susceptibility or resistance to each antibiotic was determined based on the measured inhibition zones surrounding each antibiotic disc.

Both control organisms, *E. coli* and *S. aureus*, behaved within expected ranges as published by CLSI. *Bacillus subtilis* MB40 was susceptible to the majority of the antibiotics to which it was exposed (18 of 21), including those to which the *in silico* analysis predicted resistance (e.g. the aminoglycosides, gentamicin and kanamycin) (Table 10). *Bacillus subtilis* MB40 was determined to

have resistance to only one antibiotic, fosfomycin, and was neither susceptible nor resistant to rifampin. There is some difficulty in assessing the antibiotic susceptibility of *Bacillus subtilis* using the CLSI inhibition zone method because there is no given range for data interpretation for this genus and species. If *Bacillus subtilis* MB40 behaves similar to a *staphylococcus* it should be considered resistant to ampicillin; however, if *Bacillus subtilis* MB40 behaves more similar to an *enterococcus* then it should be considered susceptible to ampicillin.

Table 10. Results of Antibiotic Susceptibility Testing

TEST GROUP	DISC CODE	ZONE (MM)	ZONE INTERPRETATION
Aminoglycosides			
Kanamycin	K 30	35	S
Gentamicin	GM 10	33	S
Neomycin	N 30	29	S
Streptomycin	S 10	15	S
β-lactams: Penicillins			
Penicillin	P 10	29	S
Ampicillin	AM 10	28	S, R*
Amoxicillin/Clavulanic acid	AmC 30	31	S
β-lactams: Cephems			
Cephalothin	CF 30	52	S
Cefotaxime	CTX 30	25	S
Cefaclor	CEC 30	50	S
Ceftriaxone	CRO 30	26	S
Fluorquinolones			
Ciprofloxacin	CIP 5	30	S
Fosfomycins			
Fosfomycin + Glucose-6-Phosphate	FOS 200	6	R
Folate Pathway Inhibitors			
Sulfamethoxazole Trimethoprim	SXT	32	S
Glycopeptides			
Vancomycin	Va 5	17	S
Macrolides, Lincosamides, Streptogramins			
Clindamycin	CC 2	22	S
Erythromycin	E 15	33	S
Quinupristin/Dalfopristin	SYN 15	20	S
Phenicols			
Chloramphenicol	C 30	30	S

	DISC CODE	ZONE (MM)	ZONE INTERPRETATION
	RA 5	18	Int
	Te 30	19	S

Int – neither susceptible nor resistant; R – resistant; S – susceptible
* Susceptible if *Bacillus subtilis* MB40 behaves more similar to an *enterococcus* and resistant If *Bacillus subtilis* MB40 behaves similar to a *staphylococcus*

An additional study was performed to determine the minimum inhibitory concentration (MIC) of eight different antibiotics (vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol against *Bacillus subtilis* MB40. Testing was performed according to the Microdilution Broth Method outlined in CLSI Document M07-A10 (CLSI, 2015a). The concentration of *Bacillus subtilis* MB40 cells used per well was 7.50×10^5 CFU/mL. Staphylococcus aureus (all antibiotics except streptomycin) and *Enterococcus faecalis* (streptomycin only) were tested in tandem with Bacillus subtilis MB40 as a validity check for the assay at 1.3275×10^6 and 2.40×10^6 CFU/mL (respectively), and exhibited MICs within the CLSI quality control range. Ten different dilutions of each antibiotic were tested to determine each MIC. Results for Bacillus subtilis MB40 are shown in Table 11.

The results show that *Bacillus subtilis* MB40 showed acceptable susceptibility to all antibiotics tested in the assay except streptomycin.

Table 11. Minimum Inhibitory Concentrations of Antibiotics for *Bacillus subtilis* MB40

ANTIBIOTIC	MIC (μG/ML)		
	MB40	*S. AUREAS*	*E. FAECALIS*
Vancomycin	0.5	1	
Gentamicin	0.125	0.25	
Kanamycin	1	2	
Streptomycin	>32		32*
Erythromycin	0.125	0.25	
Clindamycin	2	0.125	
Tetracycline	4	0.5	
Chloramphenicol	4	8	

MB40 – *Bacillus subtilis* MB40; MIC – Minimal Inhibitory Concentration; mL – milliliter; μg – microgram*Inhibition at 32 μg/mL can be extrapolated to indicate susceptibility of streptomycin at 1000 μg/mL and a lack of high-level aminoglycoside resistance (per CLSI Document M100-S25) (CLSI, 2015b).

In conclusion, results of the antibiotic resistance tests that were performed with *Bacillus subtilis* MB40 showed resistance to fosfomycin, streptomycin and potentially ampicillin. The fact that

Bacillus subtilis MB40 was not resistant to streptomycin in the MIC test is not a unique finding for *Bacillus* species. A study performed by Adimpong et al. (2012) showed that out of 85 *Bacillus* species used for Sudanese bread production (*Bacillus subtilis subsp. subtilis* (n = 29), *Bacillus licheniformis* (n = 38) and *Bacillus sonorensis* (n = 18)), all were resistant to streptomycin.

5. Antibiotic Production

Bacillus subtilis is reported to produce 66 antibiotics, with 4-5% of its genome devoted to antibiotic synthesis (Sorokulova, 2013; Stein, 2005). Lantibiotics (peptide antibiotics) are among the many antimicrobial substances produced by members of the *Bacillus* genus (Lee and Kim, 2011; Mora et al., 2011). Lantibiotics are used in food preservation, but not orally administered as a treatment in human or veterinary medicine due to a lack of functional stability. These peptides are rapidly degraded through the digestive process rendering them of little use when orally administered (Edwards et al., 1999; Hansen, 1994). There is no indication that *Bacillus subtilis* MB40 produces antimicrobial substances that are used in medical or veterinary medicine and could potentially disrupt the normal intestinal microflora (Pariza et al., 2015). A cross-streak screening experiment in which *Bacillus subtilis* MB40 was plated with *Lactobacillus acidophilus and Lactobacillus casei,* confirmed that growth of these common gut bacteria was not inhibited by the presence of *Bacillus subtilis* MB40 (BIO-CAT Microbials; unpublished data on file).

6. Short-term Toxicity Study with *Bacillus subtilis* MB40

Short-term toxicity of Bacillus subtilis MB40 was evaluated in a 14-day oral gavage dose study in Sprague Dawley [Crl:CD(SD)] rats (Spears et al., 2020). Groups of 10 male and 10 female rats were administered *Bacillus subtilis* MB40 (supplied as a spray-dried powder at an activity level of 1.85×10^{11} CFU/g) by gavage at doses of 500, 1000, and 2000 mg/kg bw/day using concentrations of 50, 100, and 200 mg/mL prepared in deionized water. The doses were equivalent to 9.25×10^{10}, 1.85×10^{11} and 3.7×10^{11} CFU/kg bw/day. Based on average initial body weights, the doses in terms of CFU/day were 2.18×10^{10}, 4.33×10^{10}, and 8.51×10^{10}. A vehicle control group was concurrently administered deionized water on the same daily dosing regimen as the test article-treated groups. Test article formulations were prepared daily. The protocol was designed in general accordance with FDA Redbook 2000 Testing Guideline IV.C.3.a, *Short-Term Toxicity Studies with Rodents*.

Homogeneity of the test article formulations was confirmed prior to the initiation of dosing and target concentrations of the dosing formulations were verified by microbiological analysis of the first and last formulation preparations used. The activity (CFU/mL) of the analyzed formulation samples was within the laboratory's SOP-defined acceptance criteria (i.e. ±15% of target for all doses and intervals).

Animals were evaluated twice daily for mortality and moribundity. Clinical examinations were performed daily and detailed physical examinations were performed weekly. Individual body weights and food consumption were recorded weekly. Clinical pathology evaluations (hematology,

coagulation, serum chemistry, and urinalysis) were performed on all rats at the scheduled termination. Complete necropsies were conducted, and organ weights were measured for preselected organs. A standard listing of tissues and organs were collected from all animals for potential microscopic examination.

No mortality and no test article-related effects were reported for any of the aforementioned evaluated parameters at any dose of *Bacillus subtilis* MB40. Some statistically significant differences in hematology, coagulation, and serum chemistry parameters were reported when the control and test article-treated groups were compared but were considered non- test-article related because they were not dose-dependent, were generally within the laboratory's historical range and were likely due to individual animal variability. For example, higher mean prothrombin times were noted in all test article- treated male and female groups but increases did not occur in a dose-related manner and group means generally were within the laboratory's historical control range of study means, with the exception of the mid-dose group males. Higher mean alanine aminotransferase values were noted in all test article-treated female groups (statistically significant at 500 and 2000 mg/kg bw/day) but there was no dose-response and group means were within the laboratory's historical control range of study means.

Some statistically significant differences in organ weights absolute and/or relative were reported when the control and test article-treated groups were compared but were considered non-test article-related because of the lack of a dose-response and because group means were within the laboratory's historical control range and were likely due to individual animal variability. For example, higher mean testes and adrenal gland weights (absolute and relative to brain weight) were noted in the test article-treated male groups at 500 and/or 2000 mg/kg bw/day.

The NOAEL for *Bacillus subtilis* MB40 after oral administration to rats for 14 days was determined to be 2000 mg/kg bw/day (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day), the highest dose tested.

7. Human Clinical Safety and Tolerability Studies with *Bacillus subtilis* MB40

Study 1

In a single-blind, placebo lead-in study, the safety and tolerability of *Bacillus subtilis* MB40 was evaluated in normal, healthy adult volunteers (Spears et al., 2020). Thirty subjects were enrolled and 27 subjects (12 males and 15 females) completed the study. The completed subjects had an average age of 36.0 ± 10 years and an average weight of 75.6 ± 15.4 kg. Three subjects discontinued participation from the study after week 1 (two subjects) and week 2 (one subject) due to non-compliance with test product and completion of the study forms. The overall test product compliance of the subjects that completed the study was 99.2% ± 3.3%.

Subjects were initially given two placebo capsules per day for 7 days (placebo: 250 mg capsules containing only maltodextrin and other excipients). Subjects then received two test capsules per

day for 21 days (study product: 250 mg capsules containing 20 billion CFU/g of *Bacillus subtilis* MB40 [5 billion CFU/capsule] with maltodextrin and excipients). The total daily dose of *Bacillus subtilis* MB40 during the treatment period was 10×10^9 (10 billion) CFU/day. The dose level was selected in order to achieve a dose that would not exceed the levels considered to be the acceptable daily intake of other *Bacillus* species with GRAS status. Subjects received a total of 42 doses of the study product, *Bacillus subtilis* MB40, throughout the duration of the study.

A GI Symptom Assessment Questionnaire and Bristol Stool Chart Diary were completed daily throughout the placebo and study product administration periods. The GI Questionnaire was provided to record the frequency and severity of GI symptoms (nausea, abdominal pain, bloating, heartburn, vomiting, gas, diarrhea, constipation or indigestion) after taking the placebo or study product. The Stool Chart Diary was provided to record the day/time of every bowel movement and associated stool description according to the Bristol Stool Chart.

The first dose of each weekly supply of placebo or study product was administered with 240 mL (8 fluid ounces) of room temperature water and a light snack at the clinic on Days 1, 8, 15 and 22. Subjects were then discharged following completion of study procedures and given the remaining supply of placebo or study product, along with a GI Questionnaire and Stool Chart Diary for the rest of the week. Subjects were instructed to take remaining doses of placebo or study product in the morning and the evening within 30 minutes of a meal separated as close as possible to 12 hours.

Study Product compliance and Adverse Events (AE) were recorded and assessed on Days 8, 15, 22, and 29 along with a review of subject GI Questionnaires and Stool Chart Diaries. Physical examinations, vital signs assessments, and clinical laboratory tests (hematology and serum chemistry) were conducted at appropriate intervals throughout the study to permit evaluation of any medically significant changes from baseline as a result of the study product administration.

There were no clinically significant changes as a result of study product administration based on physical exam findings, clinical laboratory tests, and vital signs and no Serious Adverse Events were reported during the study. There were five reported AE during the study, all graded as level 1 (mild; AE were graded on a Scale of 1-4, with 4 being the most severe). Three cases of viral upper respiratory infection were reported by three different subjects and ascribed as not likely related to the administration of the study product. Two AE, a case of nausea and chills both reported by the same subject, were ascribed as likely related to the administration of the study product; however, these transient symptoms occurred during the middle of the 21-day treatment period (Study Days 19-21; Treatment Days 12-14) and resolved within 31 hours. Therefore, this mild case of nausea and chills in one subject does not indicate a safety concern with respect to administration of the study product, *Bacillus subtilis* MB40.

There were no significant changes in the total number of bowel movements per subject per week between the placebo week (average of 11.1 ± 4.6) and the three subsequent treatment weeks (week 2: 10.7 ± 3.6; week 3: 10.7 ± 3.8; week 4: 11.2 ± 4.3) with *Bacillus subtilis* MB40

administration. Each subject's Bristol Stool Chart description was scored Type 1 (hard) through Type 7 (watery). The Bristol Stool Chart score was consistent across all of the study weeks for each subject (average for the placebo week 1: 3.8 ± 0.1; averages for treatment weeks, week 2: 3.9 ± 0.1; week 3: 3.9 ± 0.1; week 4: 3.9 ± 0.2). The symptoms reported on the daily GI Questionnaires during the treatment period generally occurred with similar or lower incidence and severity compared to the placebo week.

The administration of *Bacillus subtilis* MB40 at 10 x 10^9 (10 billion) CFU/day for 21 days to 27 healthy volunteer subjects was concluded to be safe and well tolerated.

Study 2

In a multi-center, randomized, double-blind, placebo-controlled, parallel study, the efficacy and safety of *Bacillus subtilis* MB40 on abdominal discomfort, gas and bloating was evaluated in a healthy adult population (Penet et al., 2019). Following a two-week run in period, participants received either a single OPTI-BIOME® capsule containing 5 x 10^9 CFU of *Bacillus subtilis* MB40 plus excipients (maltodextrin, magnesium stearate, gelatin and silicon dioxide) or a single placebo capsule containing only the excipients, once daily for 28 days. Baseline demographics of participants in the two groups were well matched for age, gender, and the average bloating intensity and number of days with bloating during the run-in period. One hundred participants with an age range of 18-75 years were enrolled, and 75% of the participants were female.

Data from ninety-nine participants were analyzed in the Intent-to-Treat population (ITT ; n=50 received OPTI-BIOME® and n=49 received placebo) and from ninety-one participants were analyzed in the Protocol Compliant Population (PP; n=45 received OPTI-BIOME® and n=46 received placebo). Overall product compliance was 100%.

The change from baseline to week 4 in the weekly mean of the daily bloating, gas and abdominal discomfort scores was assessed by the modified Daily Abdominal Discomfort, Gas, and Bloating questionnaire. The change from baseline to week 4 in the modified GSRS was also assessed, as was the change from baseline to week 4 in the weekly mean consistency score as determined by the Bristol Stool Scale (BSS). The change from baseline to week 4 in the weekly mean number of bowel habits was determined from reports in the daily bowel habits diary. Quality of life was assessed by the modified RAND SF-36 questionnaire. Numerical efficacy endpoints were formally tested for significance between groups by an Analysis of Covariance. A within-group analysis on efficacy endpoints was done using a Student's paired samples t-test or Wilcoxon sign rank test.

The OPTI-BIOME® product was tolerated well among study participants. There were no adverse effects of treatment on any GI parameter evaluated. With respect to the safety analysis, all laboratory measures of complete blood count with differential, hematology, electrolyte count, liver and kidney function tests, and vitals remained within clinically normal levels during this study.

Thirty AE were reported by 22 participants in this study. Of these, 13 were reported by participants in the OPTI-BIOME® group and 17 were reported by participants in the placebo group. Of the 13 AE reported by those in the OPTI-BIOME® group, 8 were possibly related to the product: abdominal discomfort (1), constipation (3), diarrhea (1), dry mouth (1), flatulence (1), and increased appetite (1). All other AE were assessed as unlikely or not related to the product. Of the 17 AE reported by those in the placebo group, five were possibly related to the product: abdominal discomfort (1), constipation (2), infrequent bowel movements (1), and paresthesia (1). All other AE were assessed as either unlikely or not related to the product. All AE were resolved before the end-of-study.

Information from the two available studies are summarized in Table 12.

Table 12. Summary of Clinical Trials for Bacillus subtilis strain MB40

STUDY SETUP AND DETAILS	HUMAN STUDY RESULTS, SIGNIFICANCE, SAFETY	REFERENCE
Study Design: Single blind, placebo lead-in **Study Length**: 28 days (7 days placebo, then 21 days treatment) **Subjects**: n=30 healthy enrolled, 27 (12 M, 15 F) completed (36.0 ± 10 yrs.) **Dose, Delivery, and Frequency**: 2 capsules/day, each containing 5 billion cultures (total 10 billion/day)	**Outcome Measurements**: • Compliance • Daily GI Symptom Assessment Questionnaire for frequency and severity of GI symptoms (nausea, abdominal pain, bloating, heartburn, vomiting, gas, diarrhea, constipation or indigestion) • Day/time of every bowel movement and associated stool description according to the Bristol Stool Chart • Physical examination, vital signs and clinical laboratory tests (hematology and serum chemistry) • Adverse events **Results and Significance**: • Compliance of 99.2% ± 3.3%. • Symptoms reported on GI Questionnaires during the treatment period generally occurred with similar or lower incidence and severity compared to the placebo week. • Consistent Bristol stool form score across all of the study weeks for each subject • No clinically significant changes in physical examinations, vital signs assessments, and clinical laboratory tests • No serious AE • Two AE (mild nausea and chills both reported by the same subject) were ascribed as likely related to the administration of the study product. These AE resolved within 31 hours of reporting.	(Spears et al., 2020)
Study Design: Multicenter, randomized, double-blind, placebo-controlled, parallel **Study Length**: 28 days **Subjects**: n=99 healthy enrolled (n=50, 36 F and 14 M in treatment group and n=49, 38 F, 11M in in	**Outcome Measurements**: • Compliance • MDQ • Modified GSRS (diarrhea, constipation, abdominal discomfort, indigestion, and reflux symptom scores) • Bristol Stool Chart • Frequency of bowel movements • Quality of life (modified RAND SF-36 questionnaire)	(Penet et al., 2019)

STUDY SETUP AND DETAILS	HUMAN STUDY RESULTS, SIGNIFICANCE, SAFETY	REFERENCE
placebo group); Age: 18-75 yrs. **Dose, Delivery, and Frequency**: 1 capsule/day, containing 5 billion cultures	• Physical examination, vital signs assessments, and clinical laboratory tests (hematology and serum chemistry) • Adverse events **Results and Significance**[a] • Compliance 100% • No adverse effect of treatment on GSRS, MDQ, Bristol Stool Chart, or frequency of bowel movements • No adverse effect of treatment on quality of life, physical examination, vital signs, and clinical laboratory tests • No serious AE • Thirteen AE in treatment group and 17 in placebo group • Eight AE in treatment group possibly related to the product: abdominal discomfort (1), constipation (3), diarrhea (1), dry mouth (1), flatulence (1), and increased appetite (1). • All AE resolved before the end-of-study	

[a] Results are for total population
AE – adverse events; F – females; GI – gastrointestinal; GSRS – Gastrointestinal Symptoms Rating Scale; M – males; MDQ – Modified Daily Abdominal Discomfort, Gas, and Bloating Questionnaire

8. Safety Assessment of *Bacillus subtilis* MB40

The safety of *Bacillus subtilis* MB40 has been evaluated utilizing scientific procedures as outlined by Pariza et al. (2015) (Figure 4). Based on the outcome of the decision tree for determining the safety of microbial cultures for consumption by humans and animals including strain characterization and genome sequencing, screening for undesirable attributes and metabolites, and experimental evidence of safety by in appropriately designed safety evaluation studies, it was concluded that *Bacillus subtilis* MB40 is deemed to be safe for human consumption.

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Figure 4. Pariza et al. (2015) Decision Tree Analysis of *Bacillus subtilis* MB40

Question 1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?
YES; Strain MB40 is unambiguously characterized as *Bacillus subtilis* through 16S sequencing and complete *de novo* genome sequencing (Kramer and Spears, 2015a; additional details summarized below in "Safety Screening Tests with *Bacillus subtilis* MB40").
(The evaluation proceeded to Question 2.)

Question 2. Has the strain genome been sequenced?
YES; The genome of *Bacillus subtilis* MB40 was sequenced by Beckman Coulter Genomics and typed to be 99% similar to the parent strain *Bacillus subtilis* DSM 10 (aka Marburg strain 168) (Kramer and Spears, 2015a; additional details summarized below in "Safety Screening Tests with *Bacillus subtilis* MB40").
(The evaluation proceeded to Question 3.)

Question 3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?
YES; Using either nucleotide BLAST® or virtual PCR analysis of *Bacillus subtilis* MB40, no in-frame complete matches to the major enterotoxins found in other disease/illness related *Bacillus* species (i.e. Hbl, Nhe, CytK, entFM, or BceT) were generated. The absence of BDE and BceT in *Bacillus subtilis* MB40 was also confirmed via commercially available assay kits (Kramer and Spears, 2015b; additional details summarized below in "Safety Screening Tests with *Bacillus subtilis* MB40").
(The evaluation proceeded to Question 4.)

Question 4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?
YES; *Bacillus subtilis* MB40 contains no plasmid DNA which is typically associated with the transfer of antibiotic resistance genes. In an *in vivo* Antibiotic Sensitivity Test, *Bacillus subtilis* MB40 was susceptible to more than 85% of all antibiotics to which it was exposed, confirming the absence of most clinically relevant resistance genes in the MB40 genome (Spears et al., 2020; additional details summarized below in "Safety Screening Tests with *Bacillus subtilis* MB40").
(The evaluation proceeded to Question 5.)

Question 5. Does the strain produce antimicrobial substances?
NO; The antimicrobial active compounds produced by members of the *Bacillus* genus are primarily lantibiotics and lantibiotic-like peptides which are rapidly degraded through the digestive process and not suitable for oral administration as a treatment in human or veterinary medicine (see additional details and references provided below in "Safety Screening Tests with *Bacillus subtilis* MB40").
(The evaluation proceeded to Question 6.)

Question 6. Has the strain been genetically modified using rDNA techniques?
NO; *Bacillus subtilis* MB40 has not been genetically modified.
(The evaluation proceeded to Question 8a.)

Question 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 and characterizing component (not simply an 'incidental isolate')?
NO; *Bacillus subtilis* MB40 was isolated from the soil. However, it should be noted that *Bacillus subtilis* MB40 shares the same genus and species of *Bacillus subtilis var. nattō* used in the fermentation of soybeans into "nattō, a traditional Japanese food.
(The evaluation proceeded to Question to 13a.)

Question 13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
NO; The No-Observed-Adverse-Effect-Level (NOAEL) for *Bacillus subtilis* MB40 after oral administration to rats for 14 days was determined to be 2000 mg/kg bw/day (equivalent to 3.7×10^{11} CFU/kg bw/day), the highest dose tested (Spears et al., 2020; additional details summarized below in "Short-term Toxicity Study with *Bacillus subtilis* MB40"). The safety and tolerance of *Bacillus subtilis* MB40 was demonstrated in humans after repeated oral administration at 10×10^9 CFU/day (Spears et al., 2020; additional details summarized below in "Human Clinical Safety and Tolerability Study with *Bacillus subtilis* MB40")
(The evaluation proceeded to Step 14a.)

Step 14a: The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

C. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

"…reasonable certainty in the minds of competent scientists that the substance is not harmful under the conditions of its intended use."[1]

Amplification is provided in that the conclusion of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

"…General recognition of safety requires common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food that there is reasonable certainty that the substance is not harmful under the conditions of its intended use." "'Common knowledge' can be based on either 'scientific procedures' or on experience based on common use in food prior to January 1, 1958." [2]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[3]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive. General recognition of safety through scientific procedures shall be based upon the application of generally available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.

[1] See 21 CFR 170.3 (e)(i) and 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 11/1/19).
[2] See 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 11/1/19).
[3] See Footnote 3.

The apparent imprecision of the terms "appreciable," "at the time," and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Renwick, 1990; Rulis and Levitt, 2009; Lu, 1988).

As noted below, this safety assessment to ascertain GRAS status for BIO-CAT Microbials' *Bacillus subtilis* MB40 for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

D. FDA Safety Methodology

Safety assessment methodology has been defined by advances in the science of risk assessment. Risk assessment, simply defined, consists of an estimate of exposure to a chemical or food ingredient coupled with an assessment of assigning a safe dose or level of exposure. Exposure estimates are based on knowledge of how the chemical and ingredient will be used. Assigning a safe dose can be a highly scientific mathematical approach, or a judgment approach, or a blend of these two approaches. The approach is usually dictated by the quantity, quality and rigor of the safety data available. For example, assessment of carcinogenic risk is usually a highly mathematical approach relying on specialized safety data. GRAS assessments on history of use are more a function of judgment based on information about use, as opposed to analysis of safety data. For ingredients where there is no history of use, FDA has traditionally used an approach that relies on simple mathematics using safety data and some measure of scientific judgment (Kokoski et al., 1990). FDA primarily relies on the review of laboratory animal data. More recently, FDA is relying on human clinical information. FDA toxicologists first determine that the study does not demonstrate any indication of a carcinogenic effect. The next step is to carefully review the findings at each dose level and assign the dose level without adverse effects as the NOAEL or "no adverse effect level." The NOAEL, expressed as a weight of ingredient per kilogram of body weight of the experimental animal, is divided by an appropriate safety factor to obtain an ADI. The ADI is then compared to an EDI, expressed in the same units for sake of comparison. If the ADI comfortably exceeds the EDI, the ingredient is considered to be safe under intended conditions of use. If the ADI and EDI are close to being equivalent, or even if the EDI slightly exceeds the ADI, scientific judgment based on a variety of factors can be used to consider the ingredient to be safe under intended conditions of use (Frankos and Rodricks, 2001; Kokoski et al., 1990).

FDA sets data requirements based on concern levels that are largely based on levels of use in food in concert with chemical structures if the ingredient is structurally similar to a chemical with known toxicity of concern.[4] Detailed guidelines are given by FDA on design and conduct of the study, including number of animals per dose groups, and tissues and fluids to be examined. FDA also requires that the studies are conducted according to Good Laboratory Practice regulations.[5] These criteria are fairly conservative; except in the most trivial exposure situations, most new

[4] See a useful summary of FDA requirements by exposure level and chemical structure in FDA guidelines at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-summary-table-recommended-toxicological-testing-additives-used-food Accessed 11/1/2019.
[5] See https://www.fda.gov/ICECI/Inspections/NonclinicalLaboratoriesInspectedunderGoodLaboratoryPractices/default.htm. Accessed 11/1/2019

ingredients require a set of chronic and developmental toxicity studies, as well as a full battery of short-term studies for mutagenicity and genotoxicity. In these cases, FDA uses a 100-fold safety factor to calculate the ADI from the NOAEL. If only subchronic studies are available, FDA uses an additional uncertainty factor of ten, which translates to a safety factor of 1,000 (Frankos and Rodricks, 2001; Kokoski et al., 1990; Lu, 1988).

This methodology for setting an ADI has its limitations. The methodology cannot be used where estimated consumption exceeds 1.5 g /person/day because practical limitations preclude feeding rodents sufficiently high levels to achieve a margin of safety of 100-fold. In these cases, it has been suggested that there be an absence of adverse effects at doses approaching 2500 mg/kg bw/day, which is viewed as a practical limit in rodents (Borzelleca, 1992). In these instances, the safety evaluation needs to rely on scientific judgment from a variety of studies. In general, there needs to be a high NOAEL with lack of serious findings in the animal toxicology studies coupled with clean clinical studies in humans at the proposed use levels or good arguments based on ADME considerations or background occurrence in the diet.

FDA does not rigidly adhere to these guidelines for testing requirements in all cases. For purified natural extracts or natural products where the biological source is a common food, or the source is not of concern to FDA, the agency will usually agree with GRAS determinations for use at dietary levels of the extract or natural product that are equivalent to the average exposure to natural sources in the diet without requiring any new or additional toxicity data. However, if data in the literature indicate possible adverse effects, FDA will normally insist that more studies are undertaken to investigate the safety of the ingredient. Additional studies are normally required for allowance of higher use levels.

E. Common Knowledge Elements for GRAS Conclusions

The first common knowledge element for a GRAS conclusion requires that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge element for a GRAS conclusion requires that consensus exists within the broader scientific community.

1. Public Availability of Scientific Information

The key evidence for safety of *Bacillus subtilis* MB40 (nonclinical and clinical studies with *Bacillus subtilis* MB40 and other *Bacillus subtilis* strains) are publicly available. In addition, a GRN for *Bacillus subtilis* DE111 and twelve previous GRNs for enzymes produced from *Bacillus subtilis* (GRN 751, GRN 746, GRN 714, GRN 649, GRN 592, GRN 579, GRN 476, GRN 406, GRN 274, GRN 205, GRN 114, GRN 20) are available on the FDA's GRAS Notice Inventory website. EPA exemptions for *Bacillus subtilis* strains GB03, FMCH002, BU1814, MBI 600; CX-9060, QST 713, and QST 713 variant soil from tolerances in food crops are available in the Federal Register. This

GRAS evaluation satisfies the first common knowledge element, as the scientific information that is the basis of the GRAS determination for *Bacillus subtilis* MB40 is publicly available.

2. Scientific Consensus

The second common knowledge element for a GRAS conclusion requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use. BIO-CAT Microbials intends to add its *Bacillus subtilis* MB40 to a wide variety of foods. *Bacillus subtilis* MB40 will be added to foods at a maximum level of 2×10^9 CFU/serving, for a maximum estimated daily intake (EDI) of 36.4×10^9 (36.4 billion) CFU/day. This EDI does not present a safety concern to humans.

The traditional Japanese food “nattō” (fermented soybean) contains up to 1×10^9 viable spores of *Bacillus subtilis*/gram of nattō product. Based on a serving size of 175 g, a daily consumer of nattō would therefore consume up to 1.75×10^{11} CFU *Bacillus subtilis*/day (175 billion CFU/day) from this source only.

Ingestion of 4.8×10^{10} (48 billion) CFU/day *Bacillus subtilis* C-3102 for 28 days has been shown to be safe for healthy adults

A significant number of animal, clinical studies, and reviews consistently support safety of numerous *Bacillus subtilis* strains, and a GRN for *Bacillus subtilis* DE111 (GRN 831) and twelve previous GRNs for enzymes produced from *Bacillus subtilis* have been reviewed by FDA with “no question” responses in GRAS notifications (GRN 751, GRN 746, GRN 714, GRN 649, GRN 592, GRN 579, GRN 476, GRN 406, GRN 274, GRN 205, GRN 114, GRN 20). The estimated daily intake of *Bacillus subtilis* DE111 for GRN 831 is 1.3×10^{11} CFU/day.

While no NDINs have been accepted by FDA, these notifications were rejected for lack of information rather than safety concerns from presented information. *Bacillus subtilis* is present in some currently marketed dietary supplements, with recommended doses up to 10 billion CFU/day. Numerous *Bacillus subtilis* strains are permitted for use on crops by EPA and are exempted from tolerances. The classification as a Natural Health Product by Health Canada and the Qualified Presumption of Safety conclusion from EFSA also demonstrate the view of other regulatory authorities on the safe use of *Bacillus subtilis.*

In addition, the strain specific data available for *Bacillus subtilis* MB40, based on *in silico/in vitro*, animal and clinical data demonstrate a lack of safety concerns for this strain based on the following:

- *Bacillus subtilis* MB40 is adequately characterized phenotypically and lacks known genetic elements for virulence factors/toxins associated with pathogenicity
- The antibiotic resistance profile is acceptable compared to species of *Bacillus* that are used in food

- The No-Observed-Adverse-Effect-Level (NOAEL) for *Bacillus subtilis* MB40 after oral administration to rats for 14 days was determined to be 2000 mg/kg bw/day (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day in rats), the highest dose tested.

- Based on a conservative 100-fold safety factor for inter-and intra-species differences, the ADI of *Bacillus subtilis* MB40 in humans was calculated as 3.7×10^{9} CFU/kg bw/day (or 2.6×10^{11} (260 billion) CFU/day for a 70 kg person).

- Clinical studies with doses up to 1×10^{10} (10 billion) CFU *Bacillus subtilis* MB40 /day for up to 21 days demonstrate a lack of adverse effects.

- The estimated daily intake of *Bacillus subtilis* MB40 from proposed uses at potential maximum intakes is 3.64×10^{10} (36 billion) CFU/day, lower than the ADI.

Overall, the safety data in animals and humans for *Bacillus subtilis* MB40 support the conclusion that it is safe for human consumption.

BIO-CAT Microbials and the Expert Panel maintain that other well-qualified scientists would conclude that BIO-CAT Microbials' *Bacillus subtilis* MB40 is generally recognized as safe for use in food given the regulatory and safety data available and using well accepted toxicological principles.

F. Regulatory Framework

The regulatory framework for determining whether a substance can be considered generally recognized as safe (GRAS) in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) ("The Act"), is set forth at 21 CFR 170.30, which states:

> General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.
>
> General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

These criteria are applied below in an analysis of whether the use of *Bacillus subtilis* MB40 as an ingredient for selected foods is GRAS based upon scientific procedures.

G. Comparison of the Estimated Daily Intake to the Acceptable Daily Intake

The tolerated level of *Bacillus subtilis* MB40 in an adult human was determined to be 2.6×10^{11} CFU/day based on the 14-day study in rats.

The EDI of *Bacillus subtilis* MB40 from proposed uses was determined to be 3.64×10^{10} CFU/day, the maximum amount estimated from food use. Using this conservative upper estimate of intake, consumer intakes of *Bacillus subtilis* MB40 from the proposed uses would not exceed the acceptable intake level.

H. Discussion of Information Inconsistent with GRAS Conclusion

The authors of this GRAS determination are not aware of information that would be inconsistent with a finding that the proposed use of *Bacillus subtilis* MB40 as an ingredient in food is generally recognized as safe.

The regulatory framework for determining whether a substance is generally recognized as safe (GRAS) is in 21 CFR 170.30, which states that GRAS status through scientific procedures shall ordinarily be based upon published studies, which may be corroborated by unpublished studies and other data and information. These criteria have been applied to the existing data for *Bacillus subtilis* MB40.

I. GRAS Conclusion

The weight of the publicly available evidence from nonclinical and clinical studies with *Bacillus subtilis* MB40 and other *Bacillus subtilis* strains provides a basis upon which to conclude that the proposed uses of *Bacillus subtilis* MB40 described in this dossier satisfy the safety standard of Reasonable Certainty of No Harm and are safe. Based on the pivotal, published data and information that are generally available, one may conclude that the proposed uses of *Bacillus subtilis* MB40, produced consistent with current Good Manufactory Practice (cGMP) and meeting the food grade specifications presented above, are Generally Recognized As Safe (GRAS) based on scientific procedures. Support for these conclusions by a consensus of qualified experts in the general scientific community is provided in Appendix 3 (Expert Panel Report).

BIO-CAT's *Bacillus subtilis* MB40, when produced in accordance with FDA Good Manufacturing Practices requirements and when it meets those specifications presented by BIO-CAT in Table 2 is Generally Recognized As Safe when consumed at the levels and uses described herein. The quantity of a substance added to food should not exceed the amount reasonably required to accomplish its intended effect.

This declaration has been made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

PART 7. LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE

A. List of Acronyms and References

1. List of Acronyms

µm	Micromolar
AAFCO	Association of American Feed Control Officials
ADFI	Average gaily feed intake
ADG	Average daily gain
ADI	Acceptable Daily Intake
ADMI	Average daily dry matter intake
AE	Adverse Events
AOAC	Association for Official and Analytical Chemists
ATP	Adenosine triphosphate
BAM	Bacteriological Analytical Manual
BIO-CAT	BIO-CAT Microbials, LLC
BSS	Bristol Stool Scale
bw	body weight
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
cGMP	current Good Manufacturing Practice
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
DOM	Date of Manufacture
EDI	Estimated Dietary Intake
EFSA	European Food Safety Authority
EPA	US Environmental Protection Agency
FAME	Fatty Acid Methyl Ester
FC	Feed consumption
FCR	Feed conversion ratio
FDA	US Food and Drug Administration
GI	Gastrointestinal
GOS	galacto-oligosaccharides
GRAS	Generally Recognized as Safe
GSRS	Gastrointestinal Symptom Rating Scale
ICP	Inductively Coupled Plasma
ITT	Intent-to-Treat
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	kilogram
M	males
MDQ	Modified Daily Abdominal Discomfort, Gas, and Bloating Questionnaire
mg	milligram
min	Minute
mL	milliliter
n	number
nBLAST	Nucleotide Basic Local Alignment Search Tool
NDI	New Dietary Ingredient
NDINs	New Dietary Ingredient Notifications

NHP	Natural Health Product
NLT	not less than
NNHPD	Natural and Non-Prescription Health Products Directorate
NOAEL	No observed adverse effect level
NPN	Natural Product Number
PC	plate count
PCR	polymerase chain reaction
PDP	Principal Display Panel
PLA	Product Licence Application
PP	Per protocol
PP	Protocol Compliant Population
ppm	parts per million
RBC	Red blood cell count
SD	standard deviation
TOS	total organic solids
USDA	US Department of Agriculture
VFA	Volatile fatty acids

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B. Appendices

Appendix 1 Fatty acid profiling via MIDI Sherlock System Fatty Acid Methyl Ester (FAME)

Sherlock Samples Page 2

MIDI LABS/MICROBIAL ID, INC.
Volume: DATA0 File: E17A174.97C Samp Ctr: 9 ID Number: 1730
Type: Samp Bottle: 86 Method: RTSBA6
Created: 10/17/2017 1:04:11 PM Created By: kdohrman (Karen Dohrman)
Sample ID: C-BCM17-10 (02-BSMB40-ML6605

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.7066	182529	0.007	----	6.5635		----	< min rt	
0.7190	1.134E+9	0.023	----	6.6495	SOLVENT PEAK	----	< min rt	
1.7728	607	0.009	1.036	12.6231	13:0 iso	0.26	ECL deviates 0.000	Reference 0.001
2.0502	2771	0.008	0.999	13.6280	14:0 iso	1.14	ECL deviates 0.000	Reference 0.000
2.1566	1020	0.009	0.988	13.9999	14:0	0.41	ECL deviates 0.000	Reference -0.001
2.3482	77127	0.009	0.971	14.6316	15:0 iso	30.74	ECL deviates 0.000	Reference -0.002
2.3765	95201	0.008	0.968	14.7249	15:0 anteiso	37.85	ECL deviates 0.000	Reference -0.002
2.4598	533	0.009	----	14.9997	15:0	----	ECL deviates 0.000	
2.5899	1499	0.009	0.953	15.4158	16:1 w7c alcohol	0.59	ECL deviates 0.002	
2.6583	6690	0.009	0.949	15.6343	16:0 iso	2.61	ECL deviates 0.001	Reference -0.001
2.7050	5188	0.009	0.946	15.7838	16:1 w11c	2.02	ECL deviates 0.002	
2.7725	8324	0.009	0.942	15.9997	16:0	3.22	ECL deviates 0.000	Reference -0.003
2.8242	310	0.009	0.939	16.1642	15:0 iso 3OH	0.12	ECL deviates 0.002	
2.8539	359	0.008	0.938	16.2591	15:0 2OH	0.14	ECL deviates 0.004	
2.9033	4270	0.009	0.935	16.4164	17:1 iso w10c	1.64	ECL deviates 0.002	
2.9331	2343	0.009	0.934	16.5114	Sum In Feature 4	0.90	ECL deviates -0.001	17:1 anteiso B/iso I
2.9721	23610	0.009	0.932	16.6357	17:0 iso	9.04	ECL deviates -0.001	Reference -0.005
3.0029	23275	0.009	0.931	16.7336	17:0 anteiso	8.90	ECL deviates 0.001	Reference -0.003
3.3989	680	0.011	0.917	18.0001	18:0	0.26	ECL deviates 0.000	Reference -0.005
3.4898	500	0.013	0.914	18.2978	17:0 2OH	0.19	ECL deviates 0.010	
----	2343	---	----	----	Summed Feature 4	0.90	17:1 iso I/anteiso B	17:1 anteiso B/iso I

ECL Deviation: 0.003 Reference ECL Shift: 0.003 Number Reference Peaks: 10
Total Response: 253774 Total Named: 253774
Percent Named: 100.00% Total Amount: 243515

Matches:

Library	Sim Index	Entry Name
RTSBA6 6.21	**0.907**	**Bacillus-subtilis**
	0.682	Bacillus-amyloliquefaciens (Bacillus subtilis group)

Confidence Level/ Comment: SPECIES

Sherlock Version 6.3 [S/N 9030] Reviewed by: [redacted] 10/17/2017

Appendix 2 *Bacillus subtilis* MB40 Certificates of Analysis

Appendix 2.1 Lot No. OPTIMB40-MCO2

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

OPTI-BIOME® *Bacillus subtilis* MB40

Lot Number: OPTIMB40-MC02
Date of Manufacture: 1/2/2019

Test	Test Results	Product Acceptance Criteria	Method
Activity	138 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	5.61%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	US FDA BAM
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.03ppm	<0.5 ppm	ICP
Mercury	<0.01 ppm	<0.5 ppm	ICP
Cadmium	0.01 ppm	<0.5 ppm	ICP
Arsenic	0.05 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s):	Non-genetically modified *Bacillus subtilis*
Country of Origin:	USA
Additional Ingredients:	Maltodextrin from waxy maize
Shelf Life:	24 Months
Storage:	Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

BIO-CAT Microbials
689 Canterbury Rd
Shakopee, MN 55379
P 952.445.4251
F 952.445.7233

Chris Drahota
Quality Assurance Coordinator

Appendix 2.2 Lot No. OPTIMB40-PC24

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

OPTI-BIOME® *Bacillus subtilis* MB40

Lot Number: OPTIMB40-PC24
Date of Manufacture: 4/24/2019

Test	Test Results	Product Acceptance Criteria	Method
Activity	108 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	3.96%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	US FDA BAM
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.08 ppm	<0.5 ppm	ICP
Mercury	0.01 ppm	<0.5 ppm	ICP
Cadmium	0.04 ppm	<0.5 ppm	ICP
Arsenic	0.11 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s): Non-genetically modified *Bacillus subtilis*
Country of Origin: USA
Additional Ingredients: Maltodextrin from waxy maize
Shelf Life: 24 Months
Storage: Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

BIO-CAT Microbials
689 Canterbury Rd
Shakopee, MN 55379
P 952.445.4251
F 952.445.7233

2-17-2020
Chris Drahota
Quality Assurance Coordinator

Appendix 2.3 Lot No. OPTIMB40-CB13

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

OPTI-BIOME® *Bacillus subtilis* MB40

Lot Number:	OPTIMB40-CB13
Date of Manufacture:	3/13/2018

Test	Test Results	Product Acceptance Criteria	Method
Activity	119 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	4.95%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	US FDA BAM
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.03 ppm	<0.5 ppm	ICP
Mercury	0.01 ppm	<0.5 ppm	ICP
Cadmium	0.01 ppm	<0.5 ppm	ICP
Arsenic	0.07 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s):	Non-genetically modified *Bacillus subtilis*
Country of Origin:	USA
Additional Ingredients:	Maltodextrin from waxy maize
Shelf Life:	24 Months
Storage:	Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

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[redacted] 2-17-2020
Chris Drahota
Quality Assurance Coordinator

Appendix 2.4 Lot No. OPTIMB40-SA22

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

OPTI-BIOME® *Bacillus subtilis* MB40

Lot Number: OPTIMB40-SA22
Date of Manufacture: 5/22/2017

Test	Test Results	Product Acceptance Criteria	Method
Activity	115 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	5.69%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	<10 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	US FDA BAM
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.02 ppm	<0.5 ppm	ICP
Mercury	0.01 ppm	<0.5 ppm	ICP
Cadmium	0.01 ppm	<0.5 ppm	ICP
Arsenic	0.05 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s): Non-genetically modified *Bacillus subtilis*
Country of Origin: USA
Additional Ingredients: Maltodextrin from waxy maize
Shelf Life: 24 Months
Storage: Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

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[redacted] 2/7/2020
Chris Dranota
Quality Assurance Coordinator

Appendix 2.5 Lot No. OPTIMB40-SC11

BIO-CAT Microbials

CERTIFICATE OF ANALYSIS

OPTI-BIOME® *Bacillus subtilis* MB40

Lot Number: OPTIMB40-SC11
Date of Manufacture: 7/11/2019

Test	Test Results	Product Acceptance Criteria	Method
Activity	115 Billion CFU/g	Not less than 100 Billion CFU/g	US FDA BAM
Color	Light Tan	Light tan to dark tan	Organoleptic
Visual Inspection	Pass	Visually free from foreign material	Organoleptic
Texture	Pass	Crystalline, free flowing powder	Organoleptic
Odor	Pass	Strong fermentation	Organoleptic
ID*	Pass	> 98% homology	16S Sequencing
Moisture Content	4.25%	<10% moisture	Ohaus MB45
Microbial			
Yeast and Mold	30 CFU/g	≤300 CFU/g	US FDA BAM
Salmonella	Negative/25g	Negative/25g	US FDA BAM
Coliforms	<10 CFU/g	≤30 CFU/g	US FDA BAM
E. coli	Negative/25g	Negative/25g	AOAC 991.14
Listeria	Negative/25g	Negative/25g	US FDA BAM
S. aureus	<10 CFU/g	<10 CFU/g	US FDA BAM
Heavy Metals**			
Lead	0.03 ppm	<0.5 ppm	ICP
Mercury	0.01 ppm	<0.5 ppm	ICP
Cadmium	<0.01 ppm	<0.5 ppm	ICP
Arsenic	<0.03 ppm	<0.3 ppm	ICP

*Results determined from testing of *Bacillus subtilis* raw material
**Results determined from testing a minimum of every 5th lot

Product Information

Organism(s): Non-genetically modified *Bacillus subtilis*
Country of Origin: USA
Additional Ingredients: Maltodextrin from waxy maize
Shelf Life: 24 Months
Storage: Store in a cool, dry environment

The information on the Certificate of Analysis has been reviewed by BIO-CAT Microbials, LLC. Should we become aware of any discrepancies in the information provided we will notify our customer immediately. This Certificate shall not be reproduced except in full without written permission of BIO-CAT Microbials.

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2-17-2020
Chris Drahota
Quality Assurance Coordinator

Appendix 3 Expert Panel Report

Foreword

An independent panel of experts ("Expert Panel") was convened by GRAS Associates, LLC on behalf of their client, BIO-CAT Microbials, LLC (BIO-CAT) to evaluate the safety and Generally Recognized as Safe (GRAS) status of *Bacillus subtilis* MB40. The members of this Expert Panel† are qualified to serve in this capacity by qualification of scientific training and experience in the safety of food and food ingredients.

Discussion

A significant amount of safety information related to the consumption of Bacillus subtilis MB40 and other strains of Bacillus subtilis is generally available. and has been discussed in Part 6 of dossier.

The Expert Panel has reviewed the manufacturing process and specifications for producing *Bacillus subtilis* MB40, and all available published safety data in its evaluation of the GRAS status of *Bacillus subtilis* MB40. The Expert Panel notes that *Bacillus subtilis* MB40 is 99% homologous to *Bacillus subtilis* DSM 10 and is non-pathogenic and non-toxigenic. *Bacillus subtilis* MB40 is produced consistent with current Good Manufacturing Practices (cGMP) as a pure spore culture consisting of only *Bacillus subtilis* MB40 spores, fermentation media and maltodextrin (or other diluents). All stabilizers/additives (or diluents) used in the process are food grade. The Expert Panel concurs that specifications for *Bacillus subtilis* MB40 are adequate and that BIO-CAT has demonstrated that the product is produced according to the specifications and is stable for up to 30 months at room temperature.

There is a high presumption that *Bacillus subtilis* MB40 is safe for human consumption based on the following: (1) other strains of *Bacillus subtilis* are available in dietary supplements, have been determined to be GRAS, or are used to produce enzymes that have been determined to be GRAS; (2) the EPA permits the use of *Bacillus subtilis* on crops without tolerances; (3) EFSA has issued a Qualified Presumption of Safety Determination for the use of *Bacillus subtilis* as an animal feed additive based on the absence of toxigenic potential; and, (4) estimated consumption *Bacillus subtilis* from nattō is up to 1.75 x 10^{11} CFU (175 billion CFU) / day.

BIO-CAT Microbials, LLC also evaluated the safety of *Bacillus subtilis* MB40 using the procedure outlined by Pariza et al. (2015). Based on the outcome of the decision tree for determining the safety of microbial cultures for consumption by humans and animals, including strain characterization and genome sequencing, screening for undesirable attributes and metabolites,

† Dr. Falk is an independent consultant with over 20 years of experience in reviewing food safety issues, GRAS reviews, and new dietary ingredient notifications at the Life Science Research Office (LSRO) and LSRO Solutions. Dr. Douglas Archer is a microbiologist with extensive experience in food science and food safety. R. Martin holds a Ph.D. in Chemistry with over 38 years of experience evaluating safety of food ingredients within FDA.

and experimental evidence of safety by appropriately designed safety evaluation studies, it was concluded that *Bacillus subtilis* MB40 is deemed to be safe for human consumption.

The Expert Panel considered the following as evidence of safety for *Bacillus subtilis* MB40

- *Bacillus subtilis* MB40 is adequately characterized phenotypically and genetically and lacks known genetic elements for antibiotic resistance and virulence factors/toxins associated with pathogenicity;

- The No-Observed-Adverse-Effect-Level (NOAEL) for *Bacillus subtilis* MB40 after oral administration to rats for 14 days was determined to be 2000 mg/kg bw/day (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day in rats), the highest dose tested;

- The safety of *Bacillus subtilis* has been demonstrated in guinea pigs at a single oral dose of 1×10^{12} CFU/day;

- The results of feeding studies in pigs show no adverse effects of up to up to 1.2×10^{9} CFU *Bacillus subtilis*/day during gestation and 6.2×10^{9} CFU *Bacillus subtilis*/day during lactation on reproduction or development;

- Clinical studies with doses up to 1×10^{10} (10 billion) CFU *Bacillus subtilis* MB40 /day for up to 21 days demonstrate a lack of adverse effects. This result is corroborated by the results of published clinical safety studies with other strains of *B. subtilis* in which no adverse effects were reported after repeated administration of up to 4.8×10^{10} CFU/day to human volunteers;

- *Bacillus subtilis* DE111 has been notified as GRAS (GRN 831) at an estimated intake level of up to 1.3×10^{11} CFU/day;

- A daily consumer of nattō consumes up to 1.75×10^{11} CFU *Bacillus subtilis*/day (175 billion CFU/day) from this source only;

- Based on a conservative 100-fold safety factor for inter-and intra-species differences, the ADI of *Bacillus subtilis* MB40 in humans was calculated as 3.7×10^{9} CFU/kg bw/day (or 2.6×10^{11} (260 billion) CFU/day for a 70 kg person) from the NOAEL of the 14-day study in rats;

- Clinical studies with *Bacillus subtilis* MB40, clinical and nonclinical studies with other *Bacillus subtilis* strains and the GRAS Notice for a different *Bacillus subtilis* strain (GRN 831) support the safety and appropriateness of the ADI for *Bacillus subtilis* MB40;

- The estimated daily intake of *Bacillus subtilis* MB40 from proposed uses at potential maximum intakes is 3.64×10^{10} (36 billion) CFU/day. Using this conservative upper estimate of intake, consumer intakes of *Bacillus subtilis* MB40 from the proposed uses would not exceed the ADI.

In summary, a compelling case can be made that scientific consensus exists regarding the safety of BIO-CAT Microbials' *Bacillus subtilis* MB40 in support of a GRAS conclusion under the conditions of its intended use.

Conclusion

The Expert Panel critically reviewed the data provided by BIO-CAT Microbials for their *Bacillus subtilis* MB40, as well as publicly available published information obtained from peer-reviewed journals and other safety assessments prepared by other Expert Panels and well-respected international regulatory bodies.

BIO-CAT Microbials' *Bacillus subtilis* MB40, manufactured as described in Part 2.B of the Supplement, and declared within the subject notification meets FDA's definition of safety in that there is "reasonable certainty of no harm under the intended conditions of use" as described herein is generally recognized as safe (GRAS).

Robert L. Martin, Ph.D.

Michael Falk, Ph.D.

Douglas L. Archer, Ph.D.

END

From:	Amy Mozingo
To:	Hice, Stephanie
Cc:	William J. Rowe
Subject:	RE: GRN 000955 - Questions for Notifier
Date:	Tuesday, January 19, 2021 4:08:00 PM
Attachments:	image001.png GA_BioCat_GRN 000955 Addendum FINAL_01.19.21.pdf

Dear Dr. Hice,
Please find attached the responses to FDA's questions on GRAS Notice No. 000955.
Should you need any further clarification please let us know.
Best Regards,
Amy Mozingo

Amy Mozingo, MS
Director of Operations, GRAS Associates
a Nutrasource Pharmaceutical and Nutraceutical Services company
O: 301-461-8929| C: 772-532-3454

LinkedIn | **Twitter** | **Blog**

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From: William J. Rowe <wrowe@nutrasource.ca>
Sent: Thursday, January 7, 2021 3:37 PM
To: Amy Mozingo <amozingo@gras-associates.com>
Subject: Fwd: GRN 000955 - Questions for Notifier

Sent from my iPhone

Begin forwarded message:

From: "Hice, Stephanie" <Stephanie.Hice@fda.hhs.gov>
Date: January 7, 2021 at 3:35:56 PM EST
To: "William J. Rowe" <wrowe@nutrasource.ca>
Subject: GRN 000955 - Questions for Notifier

Dear Mr. Rowe,

During our review of GRAS Notice No. 000955, we noted further 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,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
stephanie.hice@fda.hhs.gov

GRAS ASSOCIATES
Food Safety Regulatory Services,
A Nutrasource Company

11810 Grand Park Avenue
Suite 500
North Bethesda, MD 20852
Phone: 1-301-461-8929
Fax: 1-888-531-3466

Cover Letter

January 19, 2021

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

Attention: Dr. Stephanie Hice
Staff Fellow (Biologist)
Division of Food Ingredients
stephanie.hice@fda.hhs.gov

Dear Dr. Hice,

GRAS Associates, LLC, acting as the Agent for BIO-CAT Microbials, LLC, submits this Addendum to answer questions/comments received for the GRAS Notification number 000955 for OPTI-BIOME® *Bacillus subtilis* MB40.

William J. Rowe
President
Agent for BIO-CAT Microbials, LLC
GRAS Associates, LLC
11810 Grand Park Ave, Suite 500
North Bethesda, MD 20852
wrowe@nutrasource.ca

Table of Contents

List of Tables

Foreward

The purpose of this Addendum is to address questions posed by FDA during the GRAS Dossier review regarding OPTI-BIOME® Bacillus subtilis MB40. This addendum will address each question point by point as laid out in the request.

Questions/Comments Regarding GRN 000955:

January 7, 2021
Questions:

1. Please provide an approximate ratio of spores to vegetative cells. Additionally, please describe how the production of spores is ensured.

Response: The final powder product of *B. subtilis* MB40 is as close to 100% spores as can be measured. After fermentation, acid is added to the culture to drop the pH to 4.5. At this pH, spores will survive but any vegetative cells will not. Additionally, the stabilized culture is concentrated via centrifugation and then spray dried. Any possible remaining vegetative cells will not survive during the spray drying process. To ensure the final preparation is entirely spores, a total aerobic enumeration is compared to an aerobic enumeration that has been heat treated (80^{o}C for 5 min). Only spores will survive the heat treatment. If the total aerobic count and the heat-treated spore count are the same, the preparation is 100% spores. If the total aerobic count is higher than the spore count, then there are vegetative cells present in the preparation.

2. For the administrative record, please explain what "… periodically monitored for genetic drift" means (page 6).

Response: Every year, samples of all B. subtilis MB40 stock vials are submitted to a 3rd party lab for 16S rRNA sequencing to confirm identity. Additionally, the complete genome sequencing has been completed twice (once in 2015 and again in 2019). These genome sequences have been aligned and compared to ensure no gene additions or deletions after generations of growth.

3. Please describe whether *Bacillus subtilis* strain BS-MB40 PTA-122264 produces any secondary metabolites, and whether this poses a safety concern.

Response: The *B. subtilis* MB40 genome has been analyzed using antiSMASH for the following secondary metabolites.

Table 1. Secondary Metabolite Analysis Using antiSMASH

Cluster type	Most similar known cluster	Similarity
NRPS (Non-ribosomal peptide synthases)	Surfactin	82%
NRPS (Non-ribosomal peptide synthases)	Plipastatin	30%
NRPS (Non-ribosomal peptide synthases)	Fengycin	93%

Cluster type	Most similar known cluster	Similarity
NRPS (Non-ribosomal peptide synthases)	Bacillibactin	100%
NRPS (Non-ribosomal peptide synthases)	Tridecaptin	40%
Other	Bacilysin	100%
TranAT-PKS	Bacillaene	100%
Sactipeptide, ranthipeptide	Sporulation killing factor	100%
Sactipeptide	Subtilosin A	100%
Terpene	N/A	N/A
Glycocin	Sublancin 168	100%

Based on the genomic data (antiSMASH), LC/MS was performed on extracts of *B. subtilis* MB40 culture to determine the presence of predicted secondary metabolites. Results are below.

Table 2. Secondary Metabolite Analysis Using LC/MS

	Compound	Presence/absence via LC/MS
Non-ribosomal peptides	Surfactin	Present
	Plipastatin	Present
	Fengycin	Present
	Bacillibactin	Absent (Predicted by genome)
	Bacilysin	Present
	Lichenysin	Absent
Polyketides	Bacillaene	Absent (Predicted by genome)
	Macrolactin	Absent

Harwood et al. (2018) mentions that surfactin is known to be produced by *B. subtilis*. Surfactin has surfactant properties and lyses mammalian cells (including red blood cells) *in vitro* at concentrations of 40 μM– 60 μM; at concentrations up to 25 μM its cytolytic activity is not considered to be significant. Oral administration of 500 mg/kg bw/day surfactin C to pregnant ICR mice resulted in no maternal toxicity, fetotoxicity or teratogenicity, indicating that fairly large amounts of the material would have to be administered to rats to cause toxicity. In the 14-day rat study for *B. subtilis* MB40 (Spears et al., 2020), there was no evidence of toxicity to tissues or cells (including red blood cells) at up to 2000 mg/kg bw/day *B. subtilis* MB40 (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day), the highest dose tested.

According to Harwood et al. (2018), plipastatin and fengycin are biosurfactant antifungal cyclic lipodecapeptides known to be produced by *B. subtilis*. Harwood states that plipastatin is widely advocated as a replacement for chemical fungicides because of its biodegradability and lack of reported toxicity to plants and animals. As mentioned above, there is no evidence of toxicity to tissues or cells in rats receiving up to 2000 mg/kg bw/day *B. subtilis* MB40 (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day) for 14 days (Spears et al., 2020). Further, in clinical studies that were conducted with *B. subtilis* MB40, no adverse effects were noted (including GI effects) (Penet et al., 2019; Spears et al., 2020).

Bacilysin is also known to be produced by *B. subtilis* (Harwood et al., 2018). Bacilysin consists of L-alanine and the non-proteinogenic amino acid L-anticapsin and has activity against gram negative and gram positive pathogenic bacteria (Nannan et al., 2020). As mentioned above, there is no evidence of toxicity to tissues or cells in rats receiving up to 2000 mg/kg bw/day *B. subtilis* MB40 (equivalent to 3.7×10^{11} CFU/kg bw/day or 8.51×10^{10} CFU/day) for 14 days. Further, in clinical studies that were conducted with *B. subtilis* MB40, no adverse effects were noted (including GI effects).

As stated in the GRAS dossier, *B. subtilis* has Qualified Presumption of Safety (QPS) status. Further, a different strain of *B. subtilis* recently has been determined GRAS for use in human food (FDA GRN 905, 2020). This strain was not analyzed for its ability to produce secondary metabolites. As mentioned in Harwood et al. (2018), surfactin, plipastatin/fengycin and bacilysin are produced by 99%, 97% and 93% of *B. subtilis* strains tested. It is therefore likely that the strain of *B. subtilis* that was recently determined GRAS produces surfactin and there is a good possibility that it also produces plipastatin/fengycin and/or bacilysin. As mentioned by Harwood et al. (2018), "although widely used commercial strains of *B. subtilis* and *B. licheniformis* produce well-characterised secondary metabolites (PKs and NRPs) and AMPs, there are no well-authenticated reports of human or animal toxicity associated with these compounds. Indeed, each year the Japanese consume ~7 billion helpings of natto, a soybean-based food fermented using a surfactin producing natto variant of *B. subtilis*."

In conclusion, there is no evidence to suggest that levels of surfactin, plipastatin, fengycin or bacilysin that could be produced from *B. subtilis* MB40 at the proposed level of intake of the ingredient in the dossier would not be safe for humans.

4. For the administrative record, please briefly specify how the purity of the initial inoculum is ensured, and state whether the fermentation process is conducted in a contained, sterile environment.

Response: The initial inoculation of the growth media is conducted in a positive pressure class 100,000 cleanroom where handling of all strains, flasks, and necessary tools is completed in a biosafety cabinet by a trained lab personnel wearing a "coverall" garment and footwear designated only for use in the cleanroom space. Additionally, the process of inoculation is witnessed by a second trained lab personnel and their initials are included with the documentation of the inoculum preparation. A sample of every

inoculum is tested for purity via streaking to trypticase soy agar (TSA) plates and pathogen contamination via streaking to selective media.

The stock vials are also tested at least once per year via 16S sequencing to ensure strain identity.

The production fermentation is conducted in a contained and sterile environment.

5. Please clarify if "Activity (CFU/g)" listed in Tables 2 and 3, as well as in Appendix 2, refers to Total Viable Spore Count (pages 11-12, 52-56).

Response: References to "counts" or "activity" in terms of CFU/g of *B. subtilis* MB40 powder means total viable spores.

6. The notifier states that the method used to detect *Staphylococcus aureus* is AOAC 2003.07 (page 11), which corresponds to enumeration of *S. aureus* in frozen lasagna, custard, frozen mixed vegetables, frozen hashbrowns, and frozen batter-coated mushrooms. Please clarify if this method is appropriate and fit for purpose.

Response: This is actually outdated information. In the initial testing for *S. aureus,* we utilized 3M petrifilm and this was the AOAC number listed in association with that 3M product. However, we have not utilized Petrifilm for the purposes of testing for *S. aureus* for quite some time. As is listed on the sample CoA, FDA BAM (chapter 12) methods are used.

7. The notifier states that the method used to detect "activity", yeast and mold, *Salmonella* serovars, coliforms and *Listeria* spp. is "FDA BAM" (page 11). For the administrative record, please provide the chapter number from the FDA Bacteriological Analytical Manual used for the referenced methods.

Response:

Activity (total aerobic enumeration): Chapter 3

Coliforms: Chapter 4

Salmonella: Chapter 5

Listeria: Chapter 10

Yeast and Mold: Chapter 18

8. Please specify whether *Listeria* refers to *Listeria monocytogenes* (page 11).

Response: The *Listeria* assay used will identify the presence of the genus *Listeria* which includes *Listeria monocytogenes* but is not limited to only *Listeria monocytogenes*.

9. The notifier indicates that inductively coupled plasma (ICP) is used to analyze for heavy metals but does not provide the exact method(s) (page 11). Please indicate the method(s) used to analyze for heavy metals. If standards method(s) are used, please provide the complete and appropriate citation(s).

Response: A contract lab (SORA Labs) is used for this work. The following response is from their lab:

"We have an internally-validated method derived from the following sources:

1) Ruth E. Wolf and Monique Adams, 2015, Multi-Elemental Analysis of Aqueuos Geochemical Samples by Quadrupole Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), USGS, U.S. Department of the Interior and U.S. Geological Survey Open – File Report 2015-1010, p 1-34, http://dx.doi.org/10.3133/ofr20151010.

2). United States Pharmacopeia 39/ National Formulatory 34, Vol.1, General Chapters <232>, <2232> and <233>, (2016).

3). Method 6020A, ICP-MS, EPA 6020A, Revision 1, EPA January, 1998.

4). AOAC Official Method 993.14 Trace Elements in Water and Wastewaters. ICP-MS, 1993, AOAC 993.14.

5). Food and Nutrition Board, Food Chemicals Codex, Institute of Medicine, (National Academy Press, Washington DC, tenth ed., 2016), pp. 1422-1424. (General Test and Assays: ICP)."

10. Table 2 (page 11) indicates that heavy metal testing is performed on every lot, but Table 3 (page 12) indicates that heavy metal testing is done on every 5th lot. Please clarify the frequency in which heavy metal testing is performed on *B. subtilis* strain BS-MB40 PTA-122264 spore preparation.

Response: Up to this point, because of the relative infrequent rate at which *B. subtilis* MB40 is produced, every lot of material is tested to establish a pattern of consistency. When production increases to a point where more than 5 lots of material is produced per year, a transition to skip lot testing will occur.

11. Please include a statement indicating that all analytical methods used to analyze the batches for conformance with the stated specifications have been validated for that particular purpose.

Response: The methods were selected specifically because they represent industry standards for the analysis (most of them coming from the FDA BAM methods). Internal validations of all SOPs, media, and other testing materials are performed to ensure they are functioning as expected and laboratory personnel undergo skill assessments to ensure they can properly perform the analyses. Additionally, the results of internal analyses match the results of 3rd party testing.

12. On page 5, the notifier indicates that *B. subtilis* strain BS-MB40 PTA-122264 spore preparation will be used as an ingredient in a wide variety of conventional foods, including baked goods, non-alcoholic beverages, juice, cereal, chewing gum, coffee, tea, condiments, confections, dairy analogs, fats and oils, herbs, frozen dairy products, pasta, candy, milk, processed fruits, processed vegetables and vegetable juices, jams and jellies, and sugar and sweet sauces at a maximum level of 2×10^9 colony forming units (CFU)/serving. However, Table 5 (page 15) includes several additional food

categories. Please clarify the food categories in which *B. subtilis* strain BS-MB40 PTA-122264 spore preparation is intended to be used.

Response: The language on page 5 was not meant to be totally inclusive for the purpose of brevity. Note that the word "including" is not meant to imply total inclusivity. The foods in Table 5 are the foods to which the strain will be added.

13. The notifier states that the intended use of *B. subtilis* strain BS-MB40 PTA-122264 spore preparation is GRAS based on scientific procedures (21 CFR 170.30(a) and (b)), however includes a discussion in Part 5, Experience Based on Common Use in Foods (pages 16-20). Please note that the information provided in Part 5 does not meet the regulatory definition of "Common Use in Foods" as defined by 21 CFR Part 170.245. We note that the provided discussion should be incorporated into Part 6, Narrative, as defined by 21 CFR Part 170.250. For the administrative record, please make a statement that corrects this reference.

Response: The GRAS is based on scientific procedures and not based on common use in food prior to 1958. As stated by FDA, additional information provided in Part 5 does not meet the regulatory definition of "Common Use in Foods" as defined by 21 CFR Part 170.245 and therefore is incorporated into the Part 6 narrative as instructed by FDA.

14. On page 17, the notifier lists the date of closure for GRN 000831 as October 7, 2019. We note that October 7, 2019 corresponds to the date of our correction letter, however, the date of closure is August 13, 2019. For the administrative record, please make a statement that corrects this reference.

Response: The date of closure listed for GRN 000831 in the GRAS dossier is incorrect and should be August 13, 2019.

15. On page 17, the notifier lists the intended use level for GRNs 000746 and 000751 as "… minimum levels necessary to achieve the intended technical effect". We note that the intended use levels listed in our response letters for GRNs 000746 and 000751 are "… at a maximum level of 20 mg Total Organic Solids (TOS)/kg flour" and "… at levels up to 49.5 mg Total Organic Solids per kg (mg TOS/kg) starch raw material", respectively. For the administrative record, please make a statement that corrects this reference.

Response: The reference links provided are those for the GRAS notifications which state that the intended levels are the at the minimum levels necessary to achieve the intended technical effect. The maximum levels stated above by the FDA are mentioned in the GRAS dossiers.

16. On page 19, the notifier lists the date of closure for NDI 277 as June 15, 2005. We note that the stamped date of closure reflected on the letter is May 27, 2005. For the administrative record, please make a statement that corrects this reference.

Response: The stamped date of closure on the FDA response letter is May 27, 2005.

17. On pages 30-31, the notifier states, "… results of the antibiotic resistance tests that were performed with *Bacillus subtilis* MB40 showed resistance to fosfomycin, streptomycin and potentially ampicillin".

a. Please describe whether this poses a potential safety concern.

Response: The results of all studies that were done for fosfomycin resistance are conflicting. No resistance genes were found for fosfomycin in strain *B. subtilis* MB40, yet the CLSI inhibition zone method showed that the strain was resistant to fosfomycin, when compared to the results for *E. coli* and *E. faecalis* (the only evaluated species for fosfomycin in this assay). The key for the test is appended to this response. As stated in the dossier, "there is some difficulty in assessing the antibiotic susceptibility of *Bacillus subtilis* using the CLSI inhibition zone method because there is no given range for data interpretation for this genus and species." Because no resistance genes for this antibiotic were found in the *B. subtilis* MB40, the result in the CLSI test should be considered a false positive due to lack of an appropriate strain for evaluation, and not a potential safety concern for humans.

Further, the results for ampicillin in the CLSI test as reported are somewhat misleading as reported in the dossier. In the CLSI test key, results for ampicillin resistance to five different species are reported (*Enterobacteriaciae*, *Enterococcus*, *Listeria monocytogenes, Haemophilis* and *Staphylococcus*). *B. subtilis* MB40 is susceptible to ampicillin if it is considered an *Enterobacteriaciae*, *Enterococcus*, *Listeria monocytogenes or Haemophilis*, and resistant only if considered a *Staphylococcus*. Further, the zone of inhibition reported (28 mm) is the upper limit for a conclusion of resistance to *Staphylococcus*, and 29 mm is the lower limit for a conclusion of susceptibility. Therefore, the susceptible result for ampicillin in the CLSI test using *Staphylococcus* as a comparator should be considered a false positive, and therefore not a safety concern. In support of this conclusion, *B. subtilis* strain DE111, which was recently notified as GRAS to FDA (in GRN 831), also used the CLSI inhibition test and found a slightly lower zone of inhibition (26 mm) for ampicillin. This result also was reported in FDA GRN 831 (2019) as both susceptible and resistant, and the FDA did not specifically point out that this could be a potential safety concern in GRN 831.

The weight of overall evidence (two of three tests) indicates that the strain is resistant to streptomycin. This is not a potential safety concern for humans since streptomycin is given parenterally (i.m. or i.v.) in humans for its indications.[1] Although this drug is used to control growth of bacteria in the GI tract of production animals[2], it is not used for this purpose in humans. Therefore, resistance of the organism to streptomycin is not a safety concern for humans.

[1] See Streptomycin Dosage Guide with Precautions – Drugs.com: https://www.drugs.com/dosage/streptomycin.html. Accessed Jan. 15, 2021.
[2] See Streptomycin Oral Solution for veterinary use – Drugs.com: https://www.drugs.com/vet/streptomycin-oral-solution.html#:~:text=STREPTOMYCIN%20ORAL%20SOLUTION%20is%20indicated%20as%20an%20aid,designed%20to%20be%20added%20to%20the%20drinking%20water. Accessed Jan. 15, 2021.

b. Further, the notifier states, "The fact that *Bacillus subtilis* MB40 was not resistant to streptomycin in the MIC test is not a unique finding for *Bacillus* species" (page 31). For the administrative record, please clarify this discrepancy.

Response: The word "not" in front of the word "resistant" is a typographical error. MB40 is resistant to streptomycin in the MIC test, and this is not a unique finding for *Bacillus* species.

18. Please provide an updated literature search that discusses the safety of *B. subtilis*, including the date (month and year) the literature search was performed and discuss whether there are any study results that may be contradictory to a GRAS conclusion. 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. 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

Response: The following discussion is appended to Section 6.1.

The original literature search for safety information on *B. subtilis* was conducted through January 2020 and has been updated to January 2021. The search located five relevant studies, plus a notified GRAS on *B. subtilis* that became publicly available during this period.

The GRAS dossier is for *Bacillus subtilis* SG188 (a spore formulation) and is incorporated by reference into Table 6 of the GRAS dossier for *Bacillus subtilis* MB40 as follows:

Table 3. Amendment to Original Table 6. Summary of *Bacillus subtilis* in FDA GRAS Inventory

SUBSTANCE	GRN # / CLOSURE DATE	INTENDED USE	USE RATE	COMPANY/ REFERENCE	FDA RESPONSE
Bacillus subtilis SG188	GRN 905 June 8, 2020	For use as an ingredient in beverages, such as milk drinks, protein high energy sports drinks, hot beverages and juices; and dry and shelf-stable products such as cereals, cookies, gums and confectionary	Up to 1 x 10^9 spores per serving	SporeGen Ltd, FDA GRN 905 (2020)	FDA had no questions

GRN 905 states "In an extreme case, an individual might consume as many as 5 servings of foods containing the bacterium in a day, thus ingesting up to $5x10^9$ viable spores.

Three new articles were located about the use of *Bacillus subtilis* in animal feed and are incorporated by reference into Table 8 of the GRAS dossier for *Bacillus subtilis* MB40 as follows:

Table 4. Amendment to Original Table 8. Results of *Bacillus subtilis* studies in livestock

SPECIES	CONCENTRATION/ DOSE/DURATION	ENDPOINTS MEASURED	RESULTS	REFERENCE
Weaned piglets experimentally infected with an enterotoxigenic *E. coli*	Bacillus subtilis DSM 32540 (1 × 10^9 CFU/kg feed, approx. 7.2 × 10^8 CFU/day based on overall ADFI of 742 g/day) for 28 days in infected animals	BW, ADG, ADFI, GTF, diarrhea score, total and differential WBC, TNF-α and haptoglobin, intestinal morphology, bacterial translocation to mesenteric lymph nodes and spleen, microbial count in intestine hemolytic coliforms	No adverse effect on any parameter measured compared to infected controls	He et al. (2020a)
Weaned piglets experimentally infected with an enterotoxigenic *E. coli*	Bacillus subtilis DSM 32540 (2.56 × 10^9 CFU/kg feed, approx. 1.5 × 10^9 CFU/day based on overall ADFI of 598 g/day) for 28 days in infected animals	BW, ADG, ADFI, GTF, diarrhea score, alertness score, hematology, TNF-α and haptoglobin, intestinal morphology; bacterial translocation to mesenteric lymph nodes and spleen, microbial count in intestine hemolytic coliforms	No adverse effect on any parameter measured compared to infected controls	He et al. (2020b)
Weaned piglets experimentally infected with an enterotoxigenic *E. coli*	Bacillus subtilis DSM 32540 (1.3 × 10^6 CFU/g feed, approx. 5.2 × 10^8 CFU/day based on overall ADFI of 598 g/day) for 21 days in infected animals	BW, ADG, ADFI, GTF, fecal score, frequency of diarrhea, intestinal morphology, liver, stomach, small intestine, cecum, colon and spleen weight, pH and VFA content of cecal digesta	No adverse effect on any parameter measured compared to infected controls	Park et al. (2020)

ADFI – average gaily feed intake; ADG – average daily gain, ADFI – average daily feed intake; BW – body weight, CFU – colony forming units; FC – feed consumption; GTF – gain to feed ratio; TNF-α – tumor necrosis factor alpha; VFA – volatile fatty acid; WBC – white blood cell count

The results show that up to 1.5 × 10^9 CFU/day of *B. subtilis* has no adverse effect on the performance of piglets infected with an enterotoxigenic *E. coli*.

The following study is added to Section 6.2 – Clinical Safety Data on *Bacillus subtilis* (other strains), by reference.

Rui and Ma (2020) conducted a retrospective study in 72 children (age 5-11) with antibiotic-associated diarrhea (AAD). The study was not randomized or placebo-

controlled. Groups of 36 subjects received routine treatment with or without *Bacillus subtilis* and *Enterococcus faecium* granules (two 1-gram packs daily for 7 days). The number of CFU ingested per day cannot be determined from the information given in the paper. The primary outcomes were duration of diarrhea (days), and number of dressings needed every day. The secondary outcomes were abdominal pain intensity (as measured by a 10-point visual analog scale, stool consistency (as assessed by Bristol Stool Scale) and any adverse events. There were no adverse effects of the intervention on primary or secondary outcomes and no adverse events were reported.

As mentioned above, FDA would like the La Jeon et al. (2012) and Harwood et al. (2018) studies to be discussed in the paper.

The Harwood et al. (2018) study is discussed above under Question 3 and the information is not reiterated here.

The La Jeon et al. (2012) paper is an “unusual” case report of a 71-year old male that visited the emergency department with chest pain that was first noticed after swallowing tablets containing *Bacillus subtilis* and *Bacillus Licheniformis* 3 hr before admission. The pain progressively worsened and was associated with dyspnea. His medical history included a mild drinking habit and past pulmonary tuberculosis. He was also taking medicine for chronic obstructive pulmonary disease (COPD). A computed tomography (CT) scan of the chest showed a pleural effusion in the left lower lobe and an esophageal perforation was suspected. The authors stated that they believed that the tablets were the cause of the perforation. Colonies identified as members of the genus *Bacillus* were isolated from blood and pleural fluid and 16S rRNA sequence analysis confirmed the presence of *Bacillus subtilis* and *Bacillus Licheniformis* in these fluids.

As mentioned in the GRAS dossier on Page 27, *Bacillus subtilis* is not considered pathogenic or toxigenic to humans, animals, or plants (EPA, 1997). Based on a review of literature citing human infections with *Bacillus subtilis* (de Boer and Diderichsen, 1991), almost all cases of *Bacillus subtilis* infection were related to drug abusers or debilitated patients. Also, as mentioned in Section 6B, no in-frame complete matches to the six major enterotoxins harbored by *Bacillus* species (Hbl, Nhe, CytK, entFM, and BceT) were generated for *Bacillus subtilis* MB40 using either nBLAST or virtual PCR (Kramer and Spears, 2015b). Further, the absence of two of the major enterotoxins produced by *Bacillus* species (*Bacillus* Diarrheal Enterotoxin and *Bacillus cereus* enterotoxins was confirmed by commercially available assay kits manufactured by 3M (St. Paul, MN) and Oxoid (Hampshire, UK).

In the FDA comments section for FDA GRN 905 (2020) the authors stated “occasionally there are documented reports of what, prima facie, appears as a genuine [*Bacillus subtilis*] infection. For example, Jeon et al. (2012) describe a case of bacteremia following an esophageal perforation caused by *B. subtilis* and *B. licheniformis*. Similarly, a recent report (Gu et al., 2019) identified a strain of *B. subtilis* isolated from a deep-sea hydrothermal vent that has virulence potential in animals. In this case the precise mechanism whereby *B. subtilis* can invade vertebrate cells was not identified. As

discussed by Harwood et al. (2018), *Bacillus species* can secrete molecules that have cytotoxic potential."

The authors of FDA GRN 905 (2020) also stated "while it is possible that the strains involved may have carried unique features enabling pathogenicity, it does illustrate that even non-pathogenic microorganisms can under some occasions participate in potentially lethal infection requiring clinical treatment. Most importantly, these studies demonstrate the need to conduct safety analysis on a strain-by-strain basis." *Bacillus subtilis* MB40 has been tested for toxicity in rats for 14 days and there was no evidence of toxicity at doses up to 3.7×10^{11} CFU/kg bw/day (8.51×10^{10} CFU/day).

None of the studies mentioned above provide contradictory information to a GRAS conclusion.

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Appendix 1 – BD BBL Sensi-Disc Antimicrobial Susceptibility Instructions

BD Key begins on the following page.

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BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs

English:	pages	1 – 4	Deutsch:	Seiten	7 – 9	Español:	páginas	11 – 13	8840621
Français :	pages	4 – 7	Italiano:	pagine	9 – 11	Português	páginas	13 – 16	2011/07

Pokyny vám poskytne místní zástupce společnosti BD. / Kontakt den lokale BD repræsentant for at få instruktioner. / Kasutusjuhiste suhtes kontakteeruge oma kohaliku BD esindajaga. / Επικοινωνήστε με τον τοπικό αντιπρόσωπο της BD για οδηγίες. / A használati utasítást kérje a BD helyi képviseletétől. / Naudojimo instrukcijų teiraukitės vietos BD įgaliotojo atstovo. / Kontakt din lokale BD-representant for mer informasjon. / Aby uzyskać instrukcje użytkowania, skontaktuj się z lokalnym przedstawicielstwem BD. / Inštrukcie získate u miestneho zástupcu spoločnosti BD. / Kontakta lokal Becton Dickinson-representant för anvisningar. / Свържете се с местния представител на BD за инструкции. / Contactați reprezentantul dumneavoastră local BD pentru instrucțiuni. / Talimatlar için yerel BD temsilcilerinize danışın. / Obratite se svom lokalnom predstavniku kompanije BD za uputstva. / Для получения инструкций свяжетесь с местным представителем компании BD. / Өзіңіздің жергілікті БД өкіліне жүгініп нұсқау алыңыз. / Kontaktiraj lokalnog predstavnika BD za upute.

Refer to product label for CE mark availability. / Informace o dostupnosti značky CE najdete na etiketě na výrobku. / Produktet er tilgængeligt, hvis CE-mærkningen findes på etiketten. / Toote saadavuse teadasaamiseks kontrollige CE–märgi olemasolu etiketil. / Pour vérifier le marquage CE du produit, se référer à l'étiquette. / Siehe Produktetikett für die Verfügbarkeit CE markierter Produkte. / Το προϊόν είναι διαθέσιμο εάν η ετικέτα φέρει τη σήμανση CE. / Lásd a termék címkéjén levő CE jel meglétét. / Prodotto disponibile se l'etichetta reca il marchio CE. / CE žymės ieškokite ant produkto etiketės. / Se på produktmerkingen om det har CE-merket. / O dostępności produktu informuje znak CE na etykiecie. / Verificar a disponibilidade do produto pela marca CE no rótulo. / Pre kontrolu prítomnosti CE značky nahliadnite do etikety výrobku. / "Ver disponibilidad del marcado CE en la etiqueta del producto". / Produkten finns tillgänglig om dess etikett är CE-märkt. / Виж етикета на продукта за наличност на знак CE. / Verificați prezența marcajului CE pe eticheta produsului. / CE işaretiyle ilgili olarak ürün etiketine başvurun. / Potražite oznaku CE na nalepnici proizvoda. / Проверьте наличие знака CE на этикетке продукта. / Potražite CE oznaku na nalepnici proizvoda. / Samo proizvodi sa CE oznakom dostupni su u Europi.

INTENDED USE – These discs are used for semi-quantitative *in vitro* susceptibility testing by the agar disc diffusion test procedure of common, rapidly growing and certain fastidious bacterial pathogens. These include the *Enterobacteriaceae*, *Staphylococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Enterococcus* spp., *Vibrio cholerae* and, by modified procedures, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae* and other streptococci. NOTE: Special procedures are required for testing pneumococci, enterococci and methicillin/oxacillin-resistant staphylococci, for performing β-lactamase tests and for screening and confirmatory tests for ESBLs; see the "RESULTS" section.

For zone diameter interpretive criteria adopted in France, refer to the instructions in the French language section of this insert.

SUMMARY AND EXPLANATION – Agar diffusion methods employing dried filter paper discs impregnated with specific concentrations of antimicrobial agents were developed in the 1940s. In order to eliminate or minimize variability in this testing, Bauer et al. developed a standardized procedure in which Mueller Hinton Agar was selected as the test medium.[1,2]

Various regulatory agencies and standards-writing organizations subsequently published standardized reference procedures based on the Bauer-Kirby method. Among the earliest and most widely accepted of these standardized procedures were those published by the U.S. Food and Drug Administration (FDA)[3] and the World Health Organization (WHO).[4,5] The procedure was adopted as a consensus standard by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) and is periodically updated.[6,7] **The latest CLSI documents should be consulted for current recommendations.**

PRINCIPLES OF THE PROCEDURE – Discs containing a wide variety of antimicrobial agents are applied to the surface of Mueller Hinton Agar plates (or Haemophilus Test Medium Agar for *H. influenzae*, GC II Agar with **IsoVitaleX™** Enrichment for *N. gonorrhoeae* or Mueller Hinton Agar with 5% Sheep Blood for *S. pneumoniae*, β-hemolytic and viridans group streptococci) that have been inoculated with pure cultures of clinical isolates. Following incubation, the plates are examined and the zones of inhibition surrounding the discs are measured and compared with established zone size ranges for individual antimicrobial agents in order to determine the agent(s) most suitable for use in antimicrobial therapy.

REAGENTS – **Sensi-Disc™** brand discs are 6-mm discs prepared by impregnating high quality absorbent paper with accurately determined amounts of antibiotic or other chemotherapeutic agents. Discs are clearly marked on both sides with letters and numbers designating the agent and the drug content. (See chart giving concentrations of reactive ingredients.) The drug content of discs is assayed by the methods established by the FDA or by methods similar or comparable to those published in the United States *Federal Register*.

Sensi-Disc agents are furnished in cartridges containing 50 discs each. The last disc in each cartridge is marked "X" and contains the drug as coded. Cartridges are for use in **BBL™ Sensi-Disc™** Dispensers; these include a Single Disc Dispenser, an 8-Place Dispenser for 100 mm-style Petri dishes, 6- and 8-Place Self-Tamping Dispensers for 100 mm-style dishes and a Self-Tamping 12-Place Dispenser for 150 mm-style plates.

Warnings and Precautions: For *in vitro* Diagnostic Use.

Follow directions for use; disc performance depends not only on disc potency, but on use of proper inoculum and control cultures, functional pretested plates, proper storage temperature and other factors.

Observe aseptic techniques and established precautions against microbiological hazards throughout all procedures. Sterilize cultures, containers and other contaminated materials after use.

Storage Instructions:

1. On receipt, store discs at -20 – +8°C. If the laboratory refrigerator is frequently opened and closed, and a suitable temperature is **not** maintained, place there a supply sufficient only for use within a week. Some discs (e.g., β-lactams) should preferably be kept frozen at -20°C.
2. Allow containers to come to room temperature before opening. Return unused discs to the refrigerator when application of discs has been completed. Once opened, discs should be placed in a tightly sealed, desiccated container for storage.
3. Use the oldest discs first.
4. Discard expired discs. Also, cartridges from which discs have been frequently removed during a week and discs left out overnight in the laboratory should be discarded, or else the discs should be tested for acceptable performance prior to continued use.
5. If the discs form incorrect zones with the recommended control organisms, the entire procedure should be checked; faulty zone size may be due to the disc, the inoculation, the preparation or depth (about 4 mm) of medium, or other factors.

The expiration date applies only to discs in intact containers, stored as directed.

SPECIMENS – Specimens should not ordinarily be employed in this test. See Directions, which include preparation of inoculum. If possible, cultures should be derived from specimens obtained from patients prior to the initiation of antimicrobial therapy.

PROCEDURE

Material Provided: Sensi-Disc™ susceptibility test discs as labeled.

Materials Required But Not Provided: Ancillary culture media, reagents, quality control organisms and laboratory equipment required to perform disc diffusion susceptibility testing by the standardized procedure. Prepare a 0.5 McFarland turbidity standard by adding 0.5 mL of 0.048 M $BaCl_2$ [1.175% (wt/vol) $BaCl_2 \cdot 2H_2O$] to 99.5 mL of 0.18 M [0.36N] H_2SO_4 [1% (vol/vol)]. Verify by using a spectrophotometer with a 1-cm light path and matched cuvette; absorbance at 625 nm should be 0.08 – 0.13.

Directions, Including User Controls:[6]

1. Preparation of inoculum with test and control cultures.
   a. Perform a Gram stain. Use only pure cultures.
   b. Select three to five similar colonies and transfer with inoculation needle or loop into 4 – 5 mL of a suitable broth such as **Trypticase™** Soy Broth (or Mueller Hinton Broth for fastidious organisms).
   c. Incubate the broth cultures at 35°C for 2 – 6 h, if necessary, to develop a turbidity equivalent to the 0.5 McFarland turbidity standard (approximately 1 to 2 x 10^8 CFU/mL). Alternatively, make a direct broth or saline suspension of colonies selected from an agar plate incubated overnight (a nonselective medium such as blood agar, or chocolate agar for *H. influenzae* and *N. gonorrhoeae*, should be used). **The direct colony suspension method is preferred for *Staphylococcus* spp., *S. pneumoniae* and other streptococci, *Haemophilus* spp. and *N. gonorrhoeae*.**[6]
   d. Dilute, if required, to obtain turbidity equivalent to the 0.5 McFarland turbidity standard. For diluent, use sterile broth or saline. Alternatively, standardize the inoculum photometrically; to facilitate inoculum adjustment of rapidly growing organisms, the **Prompt™** Inoculation System (volumetric inoculum preparation device) may be used.[8]

   Overnight broth cultures should not be used as inoculum.
2. Inoculation.
   a. Within 15 min, dip a sterile cotton swab into the properly adjusted inoculum and rotate it firmly several times against the upper inside wall of the tube to express excess fluid.
   b. Streak the entire agar surface of a Mueller Hinton Agar (or other appropriate agar) plate three times, turning the plate 60° between streakings to obtain even inoculation.
   c. The lid may be left ajar for 3 – 5 min, but no more than 15 min, to allow for any surface moisture to be absorbed before applying the drug-impregnated discs.
3. Select appropriate discs (such as recommended in reference 7, Tables 1A and 1B of M100 [M2]).
4. Apply the discs by means of a **BBL™** dispenser, using aseptic precautions. Deposit discs so that the centers are at least 24 mm apart. It is preferable to deposit penicillin and cephalosporin discs so that they are no less than 10 mm from the edge of the Petri dish, and their centers are at least 30 mm apart. Avoid placing such discs adjacent to one another. With *H. influenzae, N. gonorrhoeae* and *S. pneumoniae*, use no more than nine discs per 150 mm plate or four discs per 100 mm plate. If discs have been placed on the agar with other than the Self-Tamping Dispensers, press them down with a sterile needle or forceps to make contact with the surface.
5. Within 15 min, place the plates agar side up in a 35 ± 2°C incubator (for *Staphylococcus* spp., testing at temperatures above 35°C may not detect methicillin-resistant staphylococci (MRS); for *N. gonorrhoeae,* incubate at 36 ± 1°C [do not exceed 37°C]). *Haemophilus* spp., *N. gonorrhoeae, S. pneumoniae* and other streptococci should be incubated in an atmosphere enriched with 5% CO_2.
6. Examine the plates after 16 – 18 h of incubation (20 – 24 h for *N. gonorrhoeae, S. pneumoniae* and other streptococci). A full 24 h of incubation is recommended for *Staphylococcus* spp. to detect methicillin/nafcillin/oxacillin/vancomycin-resistant staphylococci and *Enterococcus* spp. for vancomycin resistance. The diameters of the zones of complete inhibition are measured, as determined by gross visual inspection. Zones are measured to the nearest whole millimeter. For further details in measuring zones of inhibition, consult the reference.[6] If only isolated colonies grow, the inoculum is too light and the test should be repeated. Zones around discs containing different drugs are not comparable for the purpose of comparing activity of drugs. See the Zone Diameter Interpretive Chart, which gives expected values from testing common aerobes. Zone measurement may be simplified by using a **BBL™ Sensi-Disc™** Zone Interpretation Set.
7. Control tests using prescribed cultures should be included each day susceptibility testing is performed or weekly if satisfactory performance can be documented according to the CLSI standard.[6] Typical zone sizes of *E. coli* ATCC™ 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766, *N. gonorrhoeae* ATCC 49226, *S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218 (β-lactamase-producing strain), *E. faecalis* ATCC 29212 (for quality control testing of gentamicin 120 µg and streptomycin 300 µg discs) and *Klebsiella pneumoniae* ATCC 700603 (for screening and confirmatory tests for ESBLs) are given in the chart (or footnotes) and indicate the correct performance of the entire procedure. *E. faecalis* ATCC 29212 (or 33186) is also recommended for evaluating new lots of Mueller Hinton Agar for low thymine and thymidine content (see footnote tt). *H. influenzae* ATCC 10211 is recommended as a useful additional quality control strain to verify the growth promotion properties of Haemophilus Test Medium Agar.[7]

RESULTS [6,7] – NOTE: Recommended interpretive criteria are based on usual dosage regimens and routes of administration in the U.S.

Beginning in 2006, CLSI established zone diameter interpretive ranges for *Neisseria meningitidis*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. For these ranges, consult CLSI M100-S21[7] or the latest M100 supplement available. In addition, CLSI guideline M45 – *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria* – can be consulted to obtain information for testing a variety of organisms including *Campylobacter*, *Corynebacterium* spp., *Bacillus* spp., etc.[9] For organisms not found in the accompanying table, or the references mentioned, studies are not yet adequate to develop reproducible definitive standards for interpretation of results. If necessary, a dilution method usually will be the most appropriate testing method, which may require submitting the organism to a reference laboratory.

In some instances, CLSI has implemented new zone diameter ranges for interpretive or quality control criteria. When this has occurred, footnote "aa" has been added indicating that the FDA-approved zone diameters provided differ from the current CLSI recommendations.

Compare recorded zone diameters with those in the chart; results with a specific organism may be reported as Resistant, Intermediate or Susceptible. For some organism/antimicrobial agent combinations, the absence or rare occurrence of resistant strains precludes defining any results categories other than "Susceptible." For strains yielding results suggestive of a "nonsusceptible" category, organism identification and antimicrobial susceptibility test results should be confirmed. Subsequently, the isolates should be saved and submitted to a reference laboratory that will confirm results using a CLSI reference dilution method.[6]

A rapid β-lactamase test (e.g., using **Cefinase™** discs) may yield clinically relevant information earlier than results of a disc diffusion test with *Haemophilus* spp., *N. gonorrhoeae* and *Moraxella catarrhalis*; it is the only reliable test for detecting β-lactamase-producing *Enterococcus* spp. A positive β-lactamase test predicts resistance to penicillin, ampicillin and amoxicillin among *Haemophilus* spp., *N. gonorrhoeae* and *M. catarrhalis* and resistance to penicillin, including amino-, carboxy- and ureido-penicillins among staphylococci and enterococci. A negative β-lactamase test does not rule out resistance due to other mechanisms. Do not test members of the *Enterobacteriaceae*, *Pseudomonas* spp. and other aerobic gram-negative bacilli because the results may not be predictive of susceptibility to the β-lactams most often used for therapy. Accurate detection of β-lactamase in staphylococci may require induction of the enzyme and incubation of a nitrocefin-based test for up to 1 h. Induction can be easily accomplished by testing the growth from the zone margin surrounding an oxacillin disc test. Care must be exercised to ensure accurate results, including testing of known positive and negative control strains at the time clinical isolates are examined.[6]

Enterobacteriaceae: When fecal isolates of *Salmonella* and *Shigella* spp. are tested, only ampicillin, a quinolone and trimethoprim/sulfamethoxazole should be reported routinely. In addition, chloramphenicol and a third generation cephalosporin should be tested and reported for extraintestinal isolates of *Salmonella* spp. For *Salmonella* and *Shigella* spp., aminoglycosides and first and second generation cephalosporins and cephamycins may appear active *in vitro* but are not effective clinically and should not be reported as susceptible.[7]

Enterobacter, *Citrobacter*, and *Serratia* may develop resistance during prolonged therapy with third generation cephalosporins. Therefore, isolates that are initially susceptible may become resistant within 3 to 4 days after initiation of therapy. Testing of repeat isolates may be warranted.[7]

Extended-spectrum β-lactamases (ESBLs) are enzymes produced by gram-negative bacilli that arise by mutation in genes for common plasmid-mediated β-lactamases. Strains of *Klebsiella* spp. and *E. coli* that produce ESBLs may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparent *in vitro* susceptibility to some of these agents. Some of these strains will show zones of inhibition below the normal susceptible population but above the standard breakpoints for certain extended-spectrum cephalosporins or aztreonam; such strains should be screened for potential ESBL production by using the ESBL screening breakpoints before reporting results for penicillins, extended-spectrum cephalosporins or aztreonam. Other strains may test intermediate or resistant by standard breakpoints to one or more of these agents. In all strains with ESBLs the zone diameters for one or more of the extended-spectrum cephalosporins or aztreonam should increase in the presence of clavulanic acid as determined in phenotypic confirmatory testing. For all confirmed ESBL-producing strains, the test interpretation should be reported as resistant for all penicillins, cephalosporins, and aztreonam. See footnote t for ESBL screening and confirmatory tests. The decision to perform ESBL screening tests on all urine isolates should be made on an institutional basis, considering prevalence, therapy and infection control issues.[7] To screen *Proteus mirabilis* for ESBL production, see M100.[7]

Non-*Enterobacteriaceae*: Non-*Enterobacteriaceae* other than *P. aeruginosa*, *Acinetobacter* spp., *B. cepacia* and *S. maltophilia* should be tested by the dilution method (see M7[10]). For *B. cepacia* and *S. maltophilia*, consult CLSI M100-S21 for zone diameter interpretative standards and quality control.

P. aeruginosa may develop resistance during prolonged therapy with all antibiotics. Isolates that are initially susceptible may become resistant within 3 to 4 days after initiation of therapy and testing of repeat isolates may be warranted.[7]

The susceptibility of *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis can be reliably determined by the disc method, but may require extended incubation up to 24 h before reporting as susceptible.[7]

***Staphylococcus* spp.:** *Staphylococcus* spp. may develop resistance during prolonged therapy with quinolones. Therefore, isolates that are initially susceptible may become resistant within 3 to 4 days after initiation of therapy. Testing of repeat isolates may be warranted.[7]

Methods for the detection of methicillin-resistant staphylococci include the oxacillin disc test, the cefoxitin disc test and tests for *mec*A or the protein encoded by *mec*A, the penicillin-binding protein 2a (PBP 2a, also called PBP 2′). In the past, the presence of resistance to other classes of agents was an indication of methicillin (oxacillin) resistance. However, some methicillin-resistant *S. aureus* (MRSA), such as those found in community-associated infections, are not multiply-resistant.[6]

MRSA and methicillin-resistant, coagulase-negative staphylococci should be reported as resistant (or not reported) to all other penicillins, carbapenems, cephems, and β-lactam/β-lactamase inhibitor combinations, regardless of *in vitro* test results with those agents. This is because most cases of documented methicillin-resistant infections have responded poorly to β-lactam therapy, and convincing clinical data have yet to be presented that document clinical efficacy for β-lactams versus MRS. For oxacillin-susceptible *S. aureus* and coagulase-negative staphylococci, results for parenteral and oral cephems, β-lactam/β-lactamase inhibitor combinations, and carbapenems, if tested, should be reported according to the results generated using routine interpretive criteria. For oxacillin-resistant *S. aureus* and coagulase-negative staphylococci (MRS), other β-lactam agents; i.e., penicillins, β-lactam/β-lactamase inhibitor combinations, cephems, and carbapenems, may appear active *in vitro*, but are not effective clinically. Results for these drugs should be reported as resistant or should not be reported. This is because most cases of documented MRS infections have responded poorly to β-lactam therapy, or because convincing clinical data have yet to be presented that document clinical efficacy for those agents. Routine testing of urine isolates of *S. saprophyticus* is not advised, because infections respond to concentrations achieved in urine of antimicrobial agents commonly used to treat acute, uncomplicated urinary tract infections (e.g., nitrofurantoin, trimethoprim/sulfamethoxazole or a fluoroquinolone).[6,7]

To obtain information on predicting *mec*A-mediated resistance in *Staphylococcus* spp. using cefoxitin (30 µg), consult CLSI M100-S21.

Similarly, to obtain information on testing *Staphylococcus* spp. for inducible clindamycin resistance consult M100-S21.

***Enterococcus* spp.:** Enterococci may be resistant to penicillin and ampicillin because of the production of low-affinity, penicillin-binding proteins (PBPs), or the production of β–lactamase. The disc diffusion test can accurately detect isolates with altered PBPs, but it will not reliably detect β-lactamase producing strains. The latter strains are best detected by using a direct β-lactamase test;[6] e.g., with **Cefinase** nitrocefin discs or chromogenic cephalosporin discs.

For *Enterococcus* spp., cephalosporins, aminoglycosides (except for high level resistance screening), clindamycin and trimethoprim/sulfamethoxazole may appear active *in vitro* but are not effective clinically and isolates should not be reported as susceptible.

***Haemophilus* spp.:** Only results of testing with ampicillin, one of the third-generation cephalosporins, chloramphenicol and meropenem should be reported routinely with cerebrospinal fluid isolates of *H. influenzae*.

Amoxicillin/clavulanic acid, azithromycin, clarithromycin, cefaclor, cefprozil, loracarbef, cefdinir, cefixime, cefpodoxime, cefuroxime axetil and telithromycin are oral agents that may be used as empiric therapy for respiratory tract infections due to *Haemophilus* spp. The results of susceptibility tests with these antimicrobial agents are often not useful for management of individual patients. However, susceptibility testing of *Haemophilus* spp. with these compounds may be appropriate for surveillance or epidemiologic studies.

***Streptococcus* spp. other than *S. pneumoniae*:** Susceptibility testing of penicillins and other β-lactams approved by the U.S. Food and Drug Administration for treatment of *S. pyogenes* or *S. agalactiae* is not necessary for clinical purposes and need not be done routinely, since as with vancomycin, resistant strains have not been recognized. Interpretive criteria are provided for pharmaceutical development, epidemiology or monitoring for emerging resistance. Any strain found to be intermediate or resistant should be referred to a reference laboratory for confirmation.

To obtain information on testing β-hemolytic streptococci for inducible clindamycin resistance consult M100-S21.[7]

LIMITATIONS OF THE PROCEDURE

1. The test as herein described applies primarily to rapidly growing aerobic pathogens. For fastidious bacteria other than *H. influenzae*, *N. gonorrhoeae*, *S. pneumoniae* and other streptococci, consult M100 (*N. meningitidis*) or M45.[7,13] Otherwise, test by the dilution method. Testing of anaerobes requires special procedures.[11]
2. The classifications of Resistant, Intermediate and Susceptible vary only by one millimeter, which is within normal laboratory error. Some cultures may give a borderline zone that varies from day to day or from laboratory to laboratory; such cultures are relatively uncommon.
3. For detecting pneumococcal and enterococcal resistance, strictly adhere to CLSI recommended methods.[6]
4. Antimicrobial agents other than those listed in the Chart may be in current use. Susceptibility tests employing these agents should be interpreted on the basis of presence or absence of a definite zone of inhibition and should be considered as only qualitative until such time as interpretive zones have been established. All zone diameters should be recorded.
5. ESBL confirmatory testing is only valid when the four discs (cefotaxime, cefotaxime/clavulanic acid, ceftazidime, ceftazidime/clavulanic acid) are used simultaneously. Individual usage of these discs is not recommended by CLSI.[6,7]
6. Accurate results are a function of the correct storage and maintenance of quality control organisms. This is especially true for *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603, because spontaneous loss of the plasmid encoding the β-lactamase has been documented. Refer to CLSI standard M2 for recommendations on the correct storage and maintenance of quality control organisms.[6]
7. The ability to detect vancomycin-resistant *Staphylococcus aureus* (VRSA) with this product is unknown. Additional testing methods as recommended by the Centers for Disease Control and Prevention (CDC) should be used when performing susceptibility testing on *S. aureus* isolates, particularly methicillin-resistant *S. aureus* (MRSA). These tests include nonautomated MIC methods (e.g., broth microdilution or agar dilution) and a vancomycin agar screen test (Brain Heart Infusion Agar with 6 µg/mL of vancomycin). These methods require a full 24 h of incubation to detect VRSA. For additional information, refer to the CDC web site.[12]

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Zone Diameter Interpretive Chart †

Antimicrobial Agent	Code	Disc Potency	Zone Diameter Interpretive Standards (mm) Resistant	Intermediate[a]	Susceptible[b]	Control Zone Diameter Limits (mm) *E. coli* ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853	*H. influenzae* ATCC 49247[c]	*H. influenzae* ATCC 49766[c]	*N. gonorrhoeae* ATCC 49226[d]	*S. pneumoniae* ATCC 49619[e]
Amdinocillin [f]	AMD-10	10 µg				23 – 29	—	—				
Enterobacteriaceae			≤15	—	≥16							
Amikacin	AN-30	30 µg				19 – 26	20 – 26	18 – 26				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci			≤14	15 – 16	≥17							
Amoxicillin/ Clavulanic Acid [g,h,i]	AmC-30	20/10 µg				18 – 24[g,ii]	28 – 36	—				
Enterobacteriaceae			≤13	14 – 17	≥18							
Staphylococcus spp. [j]			≤19	—	≥20							
Haemophilus spp. [c,k]			≤19	—	≥20				15 –23[c]	—		
Ampicillin [h,l]	AM-10	10 µg				16 – 22	27 – 35	—				
Enterobacteriaceae[ii] and *V. cholerae* [m]			≤13	14 – 16	≥17							
Staphylococci [j,ii]			≤28	—	≥29							
Enterococcus spp. [n,o,ii]			≤16	—	≥17							
Listeria monocytogenes [f]			≤19	—	≥20							
Haemophilus spp. [c,k,p]			≤18	19 – 21	≥22				13 – 21[c]	—		
Streptococci (non-*S. pneumoniae*, β-hemolytic only) [e,i,aaa,ccc]			—	—	≥24[ii]							30 – 36[e]
Ampicillin/ Sulbactam [g,h,i]	SAM-20	10/10 µg				19 – 24[g,ii]	29 – 37	—				
Enterobacteriaceae, P. aeruginosa, Acinetobacter [q] and staphylococci [j]			≤11	12 – 14[ii]	≥15[ii]							
Haemophilus spp. [c,k]			≤19	—	≥20				14 –22[c]	—		
Azithromycin	AZM-15	15 µg				—	21 – 26	—				
Staphylococcus spp. [r]			≤13	14 – 17	≥18							
Haemophilus spp. [c]			—	—	≥12				13 – 21[c]	—		
S. pneumoniae and other streptococci [e,r,s]			≤13	14 – 17	≥18							19 – 25[e]
Azlocillin	AZ-75	75 µg				—	—	24 – 30				
P. aeruginosa			≤17	—	≥18							
Aztreonam	ATM-30	30 µg				28 – 36	—	23 – 29				
Enterobacteriaceae, [t] *P. aeruginosa & Acinetobacter*			≤15	16 – 21	≥22							
Haemophilus spp. [c]			—	—	≥26				30 – 38[c]	—		
Bacitracin [f]	B-10	10 U	≤ 8	9 – 12	≥13	—	12 – 22	—				
Carbenicillin	CB-100	100 µg				23 – 29	—	18 – 24				
Enterobacteriaceae and *Acinetobacter*			≤19[ii]	20 – 22[ii]	≥23							
P. aeruginosa			≤13	14 – 16	≥17							
Cefaclor [h,i]	CEC-30	30 µg				23 – 27	27 – 31	—				
Enterobacteriaceae [u] and staphylococci [j]			≤14	15 – 17	≥18							
Haemophilus spp. [c,k]			≤16	17 – 19	≥20				—	25 – 31[c]		
Cefamandole	MA-30	30 µg				26 – 32	26 – 34	—				
Enterobacteriaceae and staphylococci [j]			≤14	15 – 17	≥18							
Cefazolin	CZ-30	30 µg				21 – 27[ii]	29 – 35	—				
Enterobacteriaceae [u] and staphylococci [j]			≤14	15 – 17	≥18							
Cefdinir [h]	CDR-5	5 µg				24 – 28	25 – 32	—				
Enterobacteriaceae[kk] and methicillin-susceptible staphylococci [j]			≤16	17 – 19	≥20							
Haemophilus spp. [c]			—	—	≥20				—	24 – 31[c]		
Cefepime [h,i]	FEP-30	30 µg				31 –37[ii]	23 – 29	24 – 30				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci [j]			≤14	15 – 17	≥18							
Haemophilus spp. [c]			—	—	≥26				25 –31[c]	—		
N. gonorrhoeae [d]			—	—	≥31						37 – 46[d,ii]	
Viridans Streptococci (non-*S. pneumoniae*) [e,ccc]			≤ 21[ii]	22 – 23[ii]	≥24[ii]							28 – 35[e]
Streptococci (non-*S. pneumoniae*, β-hemolytic only)[aaa,ccc]			—	—	≥24[ii]							
Cefixime [h]	CFM-5	5 µg				23 – 27	—	—				
Enterobacteriaceae [v]			≤15	16 –18	≥19							
Haemophilus spp. [c]			—	—	≥21				25 – 33[c]	—		
N. gonorrhoeae [d]			—	—	≥31						37 – 45[d]	
Cefmetazole	CMZ-30	30 µg				26 – 32	25 – 34	—				
Enterobacteriaceae and staphylococci [j]			≤12	13 – 15	≥16							
N. gonorrhoeae [d]			≤27	28 – 32[w]	≥33						31 – 36[d]	
Cefonicid	CID-30	30 µg				25 – 29	22 – 28	—				
Enterobacteriaceae and staphylococci [j]			≤14	15 – 17	≥18							
Haemophilus spp. [c,k]			≤16	17 – 19	≥20				—	30 – 38[c]		
Cefoperazone	CFP-75	75 µg				28 – 34	24 – 33	23 – 29				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci [j]			≤15	16 – 20	≥21							
Cefotaxime [h]	CTX-30	30 µg				29 – 35	25 – 31	18 – 22				
Enterobacteriaceae,[t,x] *P. aeruginosa, Acinetobacter* and staphylococci [j]			≤14	15 – 22[ii]	≥23[ii]							
Haemophilus spp. [c]			—	—	≥26				31 – 39[c]	—		
N. gonorrhoeae [d]			—	—	≥31						38 – 48[d]	
Viridans Streptococci (non-*S. pneumoniae*) [e,i,ccc]			≤25	26 – 27	≥28							31 – 39[e]
Streptococci (non-*S. pneumoniae*, β-hemolytic only)[aaa,ccc]			—	—	≥24[ii]							
Cefotaxime/ Clavulanic Acid [t]	CTX/CLA	30/10 µg										
Cefotetan	CTT-30	30 µg				28 – 34	17 – 23	—				
Enterobacteriaceae and staphylococci [j]			≤12	13 – 15	≥16							
N. gonorrhoeae [d]			≤19	20 – 25[w]	≥26						30 – 36[d]	
Cefoxitin	FOX-30	30 µg				23 – 29	23 – 29	—				
Enterobacteriaceae and staphylococci [j,aa]			≤14	15 – 17	≥18							
N. gonorrhoeae [d]			≤23	24 – 27[w]	≥28						33 – 41[d]	
Cefpodoxime [h,i]	CPD-10	10 µg				23 – 28	19 – 25	—				
Enterobacteriaceae [t,u,v] and staphylococci [j]			≤17	18 – 20	≥21							
Haemophilus spp. [c]			—	—	≥21				25 – 31[c]	—		
N. gonorrhoeae [d]			—	—	≥29						35 – 43[d]	
Cefprozil [h,i]	CPR-30	30 µg				21 – 27	27 – 33	—				
Enterobacteriaceae [u,y] and staphylococci [j]			≤14	15 – 17	≥18							
Haemophilus spp. [c,k]			≤14	15 – 17	≥18				—	20 – 27[c]		
Ceftazidime	CAZ-30	30 µg				25 – 32	16 – 20	22 – 29				
Enterobacteriaceae,[t] *P. aeruginosa, Acinetobacter* and staphylococci [j]			≤14	15 – 17	≥18							
Haemophilus spp. [c]			—	—	≥26				27 – 35[c]	—		
N. gonorrhoeae [d]			—	—	≥31						35 – 43[d]	
Ceftazidime/ Clavulanic Acid [t]	CAZ/CLA	30/10 µg										
Ceftibuten [h,i]	CTB–30	30 µg				27 – 35	—	—				
Enterobacteriaceae [z,ii]			≤17	18 – 20	≥21							
Haemophilus spp. [c]			—	—	≥28				29 – 36[c,ii]	—		
Ceftizoxime [h,i]	ZOX-30	30 µg				30 – 36	27 – 35	12 – 17				
Enterobacteriaceae,[t,x] *P. aeruginosa,*[ii] *Acinetobacter* and staphylococci [j]			≤14	15 – 19	≥20							
Haemophilus spp. [c]			—	—	≥26				29 – 39[c]	—		
N. gonorrhoeae [d]			—	—	≥38						42 – 51[d]	
Ceftriaxone [h]	CRO-30	30 µg				29 – 35	22 – 28	17 – 23				
Enterobacteriaceae,[t,x] *P. aeruginosa, Acinetobacter* and staphylococci [j]			≤13	14 – 20[ii]	≥21[ii]							
Haemophilus spp. [c]			—	—	≥26				31 – 39[c]	—		
N. gonorrhoeae [d]			—	—	≥35						39 – 51[d]	
Viridans Streptococci (non-*S. pneumoniae*) [e,i,ccc]			≤24	25 – 26	≥27							30 – 35[e]
Streptococci (non-*S. pneumoniae*, β-hemolytic only)[aaa,ccc]			—	—	≥24[ii]							
Cefuroxime (sodium) [h,i]	CXM-30	30 µg				20 – 26	27 – 35	—				
Enterobacteriaceae [u] and staphylococci [j] (parenteral)			≤14	15 – 17	≥18							
Haemophilus spp. [c,k]			≤16	17 – 19	≥20				—	28 – 36[c]		
N. gonorrhoeae [d]			≤25	26 – 30[w]	≥31						33 – 41[d]	
Cephalothin	CF-30	30 µg				15 – 21	29 – 37	—				
Enterobacteriaceae [u] and staphylococci [j]			≤14	15 – 17	≥18							
Chloramphenicol	C-30	30 µg				21 – 27	19 – 26	—				
Enterobacteriaceae,[r] *P. aeruginosa,*[r] *Acinetobacter* [r], staphylococci,[r] enterococci [r,yy] and *V. cholerae* [bb,r]			≤12	13 – 17	≥18							
Haemophilus spp. [c,r]			≤25	26 – 28	≥29				31 – 40[c]	—		
S. pneumoniae [e,r]			≤20	—	≥21							23 – 27[e]
Streptococci (non-*S. pneumoniae*) [e,r]			≤17	18 – 20	≥21							
Cinoxacin	CIN-100	100 µg				26 – 32	—	—				
Enterobacteriaceae			≤14	15 – 18	≥19							
Ciprofloxacin	CIP-5	5 µg				30 – 40	22 – 30	25 – 33				
Enterobacteriaceae,[ddd] *P. aeruginosa, Acinetobacter*, staphylococci and enterococci			≤15	16 – 20	≥21							
Haemophilus spp. [c]			—	—	≥21				34 – 42[c]	—		
N. gonorrhoeae [d]			≤27	28 – 40[w]	≥41						48 – 58[d]	
Clarithromycin	CLR-15	15 µg				—	26 – 32	—				
Staphylococcus spp. [r]			≤13	14 – 17	≥18							
Haemophilus spp. [c]			≤10	11 – 12	≥13				11 – 17[c]	—		
S. pneumoniae and other streptococci [e,r,s]			≤16	17 – 20	≥21							25 – 31[e]
Clindamycin [r]	CC-2	2 µg				—	24 – 30	—				
Staphylococcus spp.[ii]			≤14	15 – 20	≥21							
S. pneumoniae and other streptococci [e]			≤15	16 – 18	≥19							19 – 25[e]
Colistin [aa,dd]	CL-10	10 µg	≤8	9 – 10	≥11	11 – 15	—	—				
Doripenem	DOR-10	10 µg				28 – 35[aa]	33 – 42[eee]	29 – 35[aa]	21 – 31[jj]	—	—	30 – 38[cc]
Enterobacteriaceae[aa]			—	—	≥23							
Pseudomonas aeruginosa[f]			—	—	≥24							
Acinetobacter baumannii[f]			—	—	≥17							
Streptococcus anginosus group (*S. constellatus* and *S. intermedius*)[e,f]			—	—	≥24							
Doxycycline [ee]	D-30	30 µg				18 – 24	23 – 29	—				
Enterobacteriaceae[aa], *P. aeruginosa, Acinetobacter*[aa], staphylococci and enterococci [yy]			≤12	13 – 15	≥16							
Enoxacin	ENX-10	10 µg				28 – 36	22 – 28	22 – 28				
Enterobacteriaceae[ddd] and staphylococci[ff]			≤14	15 – 17	≥18							
N. gonorrhoeae[d]			≤ 31	32 – 35	≥36						43 – 51[d]	

Zone Diameter Interpretive Chart †

Antimicrobial Agent	Code	Disc Potency	Zone Diameter Interpretive Standards (mm) Resistant	Intermediate[a]	Susceptible[b]	Control Zone Diameter Limits (mm) E. coli ATCC 25922	S. aureus ATCC 25923	P. aeruginosa ATCC 27853	H. influenzae ATCC 49247[c]	H. influenzae ATCC 49766[c]	N. gonorrhoeae ATCC 49226[d]	S. pneumoniae ATCC 49619[e]
Ertapenem[h,i]	ETP-10	10 µg				29 – 36	24 – 31	13 – 21				28 – 35
Enterobacteriaceae and *Staphylococcus* spp.[j]			≤15	16 – 18	≥19							
Haemophilus spp.[c]			—	—	≥19				20 – 28	27 – 33		
Erythromycin	E-15	15 µg				—	22 – 30	—				
Staphylococcus spp. [r] and enterococci [r,yy]			≤13	14 – 22[ii]	≥23[ii]							
S. pneumoniae and other streptococci [e,r,s]			≤15	16 – 20	≥21							25 – 30[e]
Fosfomycin [z]	FOS-200	200 µg				22 – 30[ii]	25 – 33	—				
E. coli and *E. faecalis* only			≤12	13 – 15	≥16							
Gatifloxacin	GAT-5	5 µg				30 – 37	27 – 33	20 – 28[mm]				
Enterobacteriaceae[ddd] and *Staphylococcus* spp.[aa]			≤14	15 – 17	≥18							
P. aeruginosa, *Acinetobacter* spp. and enterococci[z]			≤14[ii]	15 – 17[ii]	≥18[ii]							
H. influenzae[c] and *H. parainfluenzae*[c]			—	—	≥18				33 – 41[c]	—		
N. gonorrhoeae[d]			≤33	34 – 37	≥38						45 – 56[d]	
S. pneumoniae and other streptococci (non-*S. pneumoniae*, β-hemolytic only)[e]			≤17[ii]	18 – 20[ii]	≥21[ii]							24 – 31[e]
Gemifloxacin	GEM-5	5 µg				29 – 36	27 – 33[ii]	19 – 25[mm,ii]				
Enterobacteriaceae[ll,ddd]			≤15	16 – 19	≥20							
H. influenzae[c] and *H. parainfluenzae*[c]			—	—	≥18				30 – 37	—		
S. pneumoniae[e]			≤19	20 – 22	≥23							28 – 34
Gentamicin												
Testing enterococci for high level resistance [n,o,gg]	GM-120	120 µg	6	7 – 9[hh]	≥10	—	—	—				
Enterobacteriaceae, *P. aeruginosa, Acinetobacter* and staphylococci	GM-10	10 µg	≤12	13 – 14	≥15	19 – 26	19 – 27	16 – 21				
Imipenem [h,i]	IPM-10	10 µg				26 – 32	—	20 – 28				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci [j]			≤13	14 – 15	≥16							
Haemophilus spp. [c]			—	—	≥16				21 – 29[c]	—		
Kanamycin	K-30	30 µg				17 – 25	19 – 26	—				
Enterobacteriaceae and staphylococci			≤13	14 – 17	≥18							
Levofloxacin	LVX–5	5 µg				29 – 37	25 – 30	19 – 26				
Enterobacteriaceae[ddd], *P. aeruginosa, Acinetobacter*, staphylococci [aa] and enterococci			≤13	14 – 16	≥17							
Haemophilus spp. [c]			—	—	≥17				32 – 40[c]	—		
S. pneumoniae and other streptococci (non-*S. pneumoniae*, β-hemolytic only) [e]			≤13	14 – 16	≥17							20 – 25[e]
Linezolid	LZD-30	30 µg				—	25 – 32[ii]	—				
Staphylococcus spp.			—	—	≥21							
Enterococcus spp.			≤20	21 – 22	≥23							
S. pneumoniae and other streptococci [e]			—	—	≥21							25 – 34[e,ii]
Lomefloxacin	LOM-10	10 µg				27 – 33	23 – 29	22 – 28				
Enterobacteriaceae[ddd], *P. aeruginosa, Acinetobacter* and staphylococci			≤18	19 – 21	≥22							
Haemophilus spp. [c]			—	—	≥22				33 – 41[c]	—		
N. gonorrhoeae [d]			≤26	27 – 37	≥38						45 – 54[d]	
Loracarbef [h,i]	LOR-30	30 µg				23 – 29	23 – 31	—				
Enterobacteriaceae [u,kk] and staphylococci [j]			≤14	15 – 17	≥18							
Haemophilus spp. [c,k]			≤15	16 – 18	≥19				—	26 – 32[c]		
Meropenem [h,i]	MEM-10	10 µg				28 – 34	29 – 37[ii]	27 – 33				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci [j]			≤13	14 – 15	≥16							
Haemophilus spp. [c]			—	—	≥20				20 – 28[c]	—		
Mezlocillin [ii]	MZ-75	75 µg				23 – 29	—	19 – 25				
Enterobacteriaceae and *Acinetobacter*			≤17	18 – 20	≥21							
P. aeruginosa			≤15	—	≥16							
Minocycline [ee]	MI-30	30 µg				19 – 25	25 – 30	—				
Enterobacteriaceae[aa], *P. aeruginosa, Acinetobacter*[aa], staphylococci and enterococci [yy]			≤14	15 – 18	≥19							
Moxalactam	MOX-30	30 µg				28 – 35	18 – 24	17 – 25				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci [j]			≤14	15 – 22	≥23							
Moxifloxacin	MXF-5	5 µg				28 – 35	28 – 35	—[aa]				
Enterobacteriaceae[f,ddd] and *Staphylococcus* spp.[aa]			≤15	16 – 18	≥19							
H. influenzae[c] and *H. parainfluenzae*[c]			—	—	≥18				31 – 39[c]	—		
S. pneumoniae[e]			≤14	15 – 17	≥18							25 – 31[e]
Nafcillin	NF-1	1 µg				—	16 – 22	—				
Staphylococcus aureus [j,nn]			≤10	11 – 12	≥13							
Nalidixic Acid	NA-30	30 µg				22 – 28	—	—				
Enterobacteriaceae [z]			≤13	14 – 18	≥19							
Neomycin [f]	N-30	30 µg	≤12	13 – 16	≥17	17 – 23	18 – 26	—				
Netilmicin	NET-30	30 µg				22 – 30	22 – 31	17 – 23				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci			≤12	13 – 14	≥15							
Nitrofurantoin	F/M-300	300 µg				20 – 25	18 – 22	—				
Enterobacteriaceae, staphylococci and enterococci			≤14	15 – 16	≥17							
Norfloxacin [ii]	NOR-10	10 µg				28 – 35	17 – 28	22 – 29				
Enterobacteriaceae[ddd], *P. aeruginosa, Acinetobacter*, staphylococci and enterococci			≤12	13 – 16	≥17							
Novobiocin [f]	NB-30	30 µg	≤17	18 – 21	≥ 22	—	22 – 31	—				
(Mueller Hinton agar with sheep blood for veterinary use)			≤14	15 – 16	≥17							
Ofloxacin	OFX-5	5 µg				29 – 33	24 – 28	17 – 21				
Enterobacteriaceae[ddd], *P. aeruginosa, Acinetobacter* and staphylococci [aa]			≤12	13 – 15	≥16							
Haemophilus spp. [c]			—	—	≥16				31 – 40[c]	—		
N. gonorrhoeae [d]			≤24	25 – 30[w]	≥31						43 – 51[d]	
S. pneumoniae [e] and other streptococci (non-*S. pneumoniae*, β-hemolytic only) [e]			≤ 12	13 – 15	≥16							16 – 21[e]
Oxacillin	OX-1	1 µg				—	18 – 24	—				
Staphylococcus aureus [j,nn,oo]			≤10	11 – 12	≥13							
Staphylococci, coagulase-negative [j,nn]			≤17	—	≥18							
S. pneumoniae (for penicillin G susceptibility) [e,h]			—	—	≥20							≤12[e,bbb]
Oxolinic Acid [f]	OA-2	2 µg	≤10	—	≥11	20 – 24	10 – 13	—				
Penicillin [h]	P-10	10 U				—	26 – 37	—				
Staphylococcus spp. [j,pp]			≤28	—	≥29							
Enterococcus spp. [n,o]			≤14	—	≥15							
L. monocytogenes [f]			≤19	20 – 27	≥28							
N. gonorrhoeae [d,qq,ii]			≤26	27 – 46[w]	≥47						26 – 34[d]	
Streptococci (non-*S. pneumoniae*, β-hemolytic only) [e,i,rr,aaa,ccc]			—	—	≥24[ii]							24 – 30[e]
Piperacillin	PIP-100	100 µg				24 – 30[g]	—	25 – 33				
Enterobacteriaceae and *Acinetobacter*			≤17	18 – 20	≥21							
P. aeruginosa			≤17	—	≥18							
Piperacillin/ Tazobactam [g]	TZP-110	100/10 µg				24 – 30[g]	27 – 36	25 – 33[ii]				
Enterobacteriaceae and *Acinetobacter* [ii]			≤17	18 – 20	≥21							
Staphylococcus spp. [j,ii] and *P. aeruginosa* [ii]			≤17	—	≥18							
Polymyxin B [aa,dd]	PB-300	300 U	≤8	9 – 11	≥12	12 – 16	—	—				
Quinupristin/Dalfopristin	SYN-15	4.5/10.5 µg				—	21 – 28[ii]	—				
Staphylococcus spp., *Enterococcus faecium* and *S. pyogenes*[e] only			≤15	16 – 18	≥19							19 – 24[e,ii,cc]
Rifampin	RA-5	5 µg				8 – 10	26 – 34	—				
Staphylococcus spp. and *Enterococcus* spp. [yy]			≤16	17 – 19	≥20							
Haemophilus spp. [c]			≤16	17 – 19	≥20				22 – 30[c]	—		
S. pneumoniae [e]			≤16	17 – 18	≥19							25 – 30[e]
Sparfloxacin	SPX–5	5 µg				30 – 38	27 – 33	21 – 29[ii]				
Staphylococcus spp.			≤15	16 – 18	≥19							
S. pneumoniae [e]			≤15[ii]	16 – 18 [ii]	≥19							21 – 27[e]
Spectinomycin	SPT-100	100 µg				—	—	—				
N. gonorrhoeae [d]			≤14	15 – 17[w]	≥18						23 – 29[d]	
Streptomycin												
Testing enterococci for high level resistance [n,o,gg]	S-300	300 µg	6	7 – 9[hh]	≥10	—	—	—				
Enterobacteriaceae	S-10	10 µg	≤11	12 – 14	≥15	12 – 20	14 – 22	—				
Sulfisoxazole [tt]	G-.25	250 µg				15 – 23	24 – 34	—				
Enterobacteriaceae, P. aeruginosa, Acinetobacter, staphylococci and *V. cholerae* [m]			≤12	13 – 16	≥17							
Telavancin	TLV-30	30 µg				—	16 – 20	—				17 – 24
Staphylococcus aureus (including methicillin-resistant isolates) [f]			—	—	≥15							
Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus anginosus group (*S. anginosus, S. constellatus* and *S. intermedius*)[e,f]			—	—	≥15							
Enterococcus faecalis (vancomycin-susceptible isolates only) [f]			—	—	≥15							
Telithromycin	TEL-15	15 µg				—	24 – 30	—				
S. aureus			—[aa]	—[aa]	≥22							
Haemophilus spp.[c]			≤11	12 – 14	≥15				17 – 23	—		
S. pneumoniae[e]			≤15	16 – 18	≥19							27 – 33
Tetracycline [ee]	Te-30	30 µg				18 – 25	24 – 30	—				
Enterobacteriaceae[aa], *P. aeruginosa, Acinetobacter*[aa], staphylococci, enterococci [yy] and *V. cholerae* [m]			≤14	15 – 18	≥19							
Haemophilus spp. [c]			≤25	26 – 28	≥29				14 – 22[c]	—		
N. gonorrhoeae [d,uu]			≤30	31 – 37[w]	≥38						30 – 42[d]	
S. pneumoniae and other streptococci [e]			≤18	19 – 22	≥23							27 – 31[e]
Ticarcillin	TIC-75	75 µg				24 – 30	—	21 – 27[ii]				
Enterobacteriaceae and *Acinetobacter*			≤14	15 – 19	≥20							
P. aeruginosa			≤14	—	≥15							
Ticarcillin/ Clavulanic Acid [g]	TIM-85	75/10 µg				24 –30[g,ii]	29 – 37	20 – 28				
Enterobacteriaceae and *Acinetobacter*			≤14	15 - 19	≥20							
P. aeruginosa			≤14	—	≥15							
Staphylococcus spp. [j]			≤22	—	≥23							
Tigecycline	TGC-15	15 µg				20 – 27	20 – 25	9 – 13[mm]	23 – 31[jj]	—	30 – 40[zz]	
Enterobacteriaceae[f,ss]			≤14	15 - 18	≥19							
S. aureus (including MRSA)[f]			—	—	≥19							
E. faecalis (vancomycin-susceptible isolates only)[f]			—	—	≥19							
Streptococcus spp. (other than *S. pneumoniae*)[e,f]			—	—	≥19							23 – 29[cc]
Tobramycin	NN-10	10 µg				18 – 26	19 – 29	19 – 25				
Enterobacteriaceae, P. aeruginosa, Acinetobacter and staphylococci			≤12	13 – 14	≥15							
Trimethoprim	TMP-5	5 µg				21 – 28	19 – 26	—				
Enterobacteriaceae and staphylococci			≤10	11 – 15	≥16							
Trimethoprim/ Sulfamethoxazole [tt]	SXT	1.25 µg 23.75 µg				23 – 29[ii]	24 – 32	—				
Enterobacteriaceae, P. aeruginosa, Acinetobacter, staphylococci and *V. cholerae* [m]			≤10	11 – 15	≥16							
Haemophilus spp. [c]			≤10	11 – 15	≥16				24 – 32[c]	—		
S. pneumoniae [e]			≤15	16 – 18	≥19							20 – 28[e]
Vancomycin	Va-30	30 µg				—	17 – 21	—				
Staphylococcus spp. [vv,ii]			—	—	≥15							
Enterococcus spp. [n,o,ww]			≤14	15 – 16	≥17							
S. pneumoniae [xx] and other streptococci [e]			—	—	≥17							20 – 27[e]

† Adapted in part from CLSI Document M100-S21 (M2): Disk Diffusion Supplemental Tables, *Performance Standards for Antimicrobial Susceptibility Testing*, with permission. The complete standard may be obtained from the Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA. Values not in M100-S21 are explained in other footnotes. For appropriate MIC correlates, refer to M100-S21.[6,7,9]

a The "Intermediate" category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. The "Intermediate" category implies clinical applicability in body sites where the drugs are physiologically concentrated (e.g., quinolones and β-lactams in urine), or when a higher than normal dosage of a drug can be used (e.g., β-lactams). The "Intermediate" category also includes "buffer zone" which should prevent small uncontrolled technical factors from causing major discrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins.

b Policies regarding generation of cumulative antibiograms should be developed in concert with the infectious disease service, infection control personnel, and the pharmacy and therapeutics committee. Under most circumstances, the percentage of susceptible and intermediate results should not be combined into the same statistics.

c These zone diameter standards and quality control limits apply only to tests with *Haemophilus* spp. using Haemophilus Test Medium (HTM) incubated in 5% CO_2 (16-18 h). *H. influenzae* ATCC 10211 is recommended as a useful additional quality control strain to verify the growth promotion properties of HTM. The zone margin should be considered as the area showing no obvious growth visible with the unaided eye. Faint growth of tiny colonies that may appear to fade from the more obvious zone should be ignored in the measurement. When testing *Haemophilus* with amoxicillin/clavulanic acid on HTM, include *E. coli* ATCC 35218 as the control strain. The acceptable limits for *E. coli* ATCC 35218 are 17-22 mm for amoxicillin/clavulanic acid when incubated in ambient air.

d These zone diameter standards and quality control limits are applicable only to tests performed using GC agar base and 1% defined growth supplement (e.g., **BBL™** GC II Agar with **IsoVitaleX™** Enrichment) incubated in 5% CO_2 (20 – 24 h).

e These zone diameter standards and quality control limits are applicable only to tests performed using Mueller Hinton agar supplemented with 5% defibrinated sheep blood incubated in 5% CO_2 (20 – 24 h). Interpretive standards apply to *S. pneumoniae* and other streptococci as indicated. Results may be inaccurate if specified criteria are applied to organisms other than those listed. Interpretive criteria for streptococci other than *S. pneumoniae* are proposed based on population distributions of various species, pharmacokinetics of the antimicrobial agents, previously published literature and the clinical experience of certain members of the CLSI subcommittee. Systematically collected clinical data were not available for review with many of the compounds in the group.[7] Despite the lack of reliable disc diffusion interpretive criteria for *S. pneumoniae* with certain β-lactams, *S. pneumoniae* ATCC 49619 is the strain designated for quality control of all disc diffusion tests with all *Streptococcus* spp.

f FDA-approved zone size recommendations from drug manufacturers not included in CLSI M100-S21 (M2-A10).[7]

g Another *E. coli* (ATCC 35218) has been designated for quality control of discs containing combinations of β-lactams and β-lactamase inhibitors. This strain produces a β-lactamase which should be inactivated by the inhibitor. When used in conjunction with ATCC 25922, both components of the combination discs can be monitored. Control limits with this strain for amoxicillin/clavulanic acid are 17-22 mm, for ampicillin is 6 mm (i.e., no zone), for ampicillin/sulbactam are 13-19 mm, for piperacillin are 12-18 mm, for piperacillin/tazobactam are 24-30 mm, for ticarcillin is 6 mm (i.e., no zone) and for ticarcillin/clavulanic acid are 21-25 mm. The *E. coli* ATCC 35218 control strain contains a plasmid-encoded β-lactamase (non-ESBL); therefore, the organism is resistant to many penicillinase-labile drugs, but susceptible to β-lactam/ β-lactamase inhibitor combinations. The plasmid must be present in the control strain for the quality control test to be valid; however, the plasmid may be lost during storage at refrigerator or freezer temperatures. See "Limitations of the Procedure" and M2 for additional details.

h Isolates of pneumococci with oxacillin zone sizes of ≥ 20 mm are susceptible (MIC ≤ 0.06 µg/mL) to penicillin and can be considered susceptible to ampicillin, amoxicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, cefaclor, cefdinir, cefepime, cefetamet, cefixime, cefotaxime, cefprozil, ceftibuten, ceftriaxone, cefuroxime, cefpodoxime, ceftizoxime, ertapenem, imipenem, loracarbef, and meropenem for approved indications, and these agents need not be tested. Penicillin and cefotaxime or ceftriaxone or meropenem MICs should be determined for those isolates with oxacillin zone sizes ≤ 19 mm because zones of ≤ 19 mm occur with penicillin-resistant, intermediate, or certain susceptible strains. Isolates should not be reported as penicillin resistant or intermediate based solely on an oxacillin zone ≤ 19 mm. Amoxicillin, ampicillin, cefepime, cefotaxime, ceftriaxone, cefuroxime, ertapenem, imipenem, and meropenem may be used to treat pneumococcal infections; however, reliable disc diffusion susceptibility tests with these agents do not yet exist. Their *in vitro* activity is best determined using an MIC method. Penicillin and cefotaxime or ceftriaxone or meropenem should be tested by a reliable MIC method (such as that described in CLSI document M7[9]) and reported routinely with CSF isolates of *S. pneumoniae*. Such isolates should also be tested against vancomycin using the MIC or disc method. With isolates from other sites, the oxacillin disc screening test may be used. If the oxacillin zone size is ≤ 19 mm, penicillin and cefotaxime or ceftriaxone MICs should be determined. To determine susceptibility of streptococci other than *S. pneumoniae* to cefdinir, use the 10-unit penicillin disc; isolates with penicillin zone sizes ≥ 28 mm are susceptible to penicillin and can be considered susceptible to cefdinir.

i A streptococcal isolate that is susceptible to penicillin can be considered susceptible to ampicillin, amoxicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, cefaclor, cefazolin, cefdinir, cefepime, cefprozil, cefotaxime, ceftibuten (group A streptococci only), ceftriaxone, cefuroxime, cefpodoxime, ceftizoxime, cephalothin, cephapirin, cephradine, imipenem, loracarbef, and meropenem for approved indications, and need not be tested against those agents. Viridans streptococci isolated from blood and normally sterile body sites (e.g., cerebrospinal fluid, blood, bone, etc.), should be tested for penicillin or ampicillin susceptibility using an MIC method.

j Penicillin-susceptible staphylococci are also susceptible to other penicillins, β-lactam/β-lactamase inhibitor combinations, cephems, and carbapenems approved for use by the FDA for staphylococcal infections. Penicillin-resistant, oxacillin-susceptible strains are resistant to penicillinase-labile penicillins but susceptible to other penicillinase-stable penicillins, β-lactam/β-lactamase inhibitor combinations, relevant cephems, and carbapenems. Oxacillin-resistant staphylococci are resistant to all currently available β-lactam antibiotics. Thus, susceptibility or resistance to a wide array of β-lactam antibiotics may be deduced from testing only penicillin and oxacillin. Routine testing of other penicillins, β-lactam/β-lactamase inhibitor combinations, cephems, and carbapenems is not advised.[7] For oxacillin-resistant staphylococci, report as resistant or do not report.

k Rare, β-lactamase-negative, ampicillin-resistant (BLNAR) strains of *Haemophilus influenzae* should be considered resistant to amoxicillin/clavulanic acid, ampicillin/sulbactam, cefaclor, cefetamet, cefonicid, cefprozil, cefuroxime, and loracarbef despite apparent *in vitro* susceptibility of some BLNAR strains to these agents.

l Class representative for ampicillin and amoxicillin.

m For *V. cholerae*, the results of disc diffusion tests for ampicillin, tetracycline, trimethoprim/sulfamethoxazole and sulfonamides (i.e., percentage of susceptible, intermediate, and resistant) correlate well with results determined by broth microdilution. Tetracycline results can be used to predict the likely susceptibility of isolates to doxycycline; do not use disc test for doxycycline or erythromycin because there is poor correlation with MIC results.

n Ampicillin is the class representative for ampicillin and amoxicillin. Ampicillin results may be used to predict susceptibility to amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin and piperacillin/tazobactam among non-β-lactamase-producing enterococci. Enterococci susceptible to penicillin are predictably susceptible to ampicillin, amoxicillin, ampicillin/sulbactam, amoxicillin/clavulanic acid, piperacillin, and piperacillin/ tazobactam for non-β-lactamase-producing enterococci. However, enterococci susceptible to ampicillin cannot be assumed to be susceptible to penicillin. If penicillin results are needed, testing of penicillin is required. Because ampicillin or penicillin resistance among enterococci due to β-lactamase production is not reliably detected using routine disc or dilution methods, a direct, nitrocefin-based β-lactamase test is recommended for blood and cerebrospinal fluid isolates. A positive β-lactamase test predicts resistance to penicillin, as well as amino-, carboxy- and ureido-penicillins. Certain penicillin- or ampicillin-resistant enterococci may possess high-level resistance (i.e., penicillin MICs ≥ 128 µg/mL or ampicillin MICs ≥ 64 µg/mL). The disc test will not differentiate those with normal resistance from this high-level resistance. For enterococci recovered from blood and CSF, the laboratory should consider determining the actual MIC for penicillin or ampicillin since enterococcal strains with normal lower level resistance (penicillin MICs ≤ 64 µg/mL and ampicillin MICs ≤ 32 µg/mL) should be considered potentially susceptible to synergy with an aminoglycoside (in the absence of high-level aminoglycoside resistance) whereas strains with higher level resistance may be resistant to such synergy.[6]

o Synergy between ampicillin, penicillin or vancomycin and an aminoglycoside can be predicted for enterococci by using a high-level aminoglycoside (gentamicin and streptomycin) screening test. Other aminoglycosides need not be tested because their activities against enterococci are not superior to gentamicin and streptomycin.

p The results of ampicillin susceptibility tests should be used to predict the activity of amoxicillin. The majority of isolates of *H. influenzae* that are resistant to ampicillin and amoxicillin produce a TEM-type-β-lactamase. In most cases, a direct β-lactamase test can provide a rapid means of detecting ampicillin and amoxicillin resistance.

q May be reported for *Acinetobacter* spp. resistant to other agents.

r Not routinely reported on isolates from the urinary tract.

s Susceptibility and resistance to azithromycin, clarithromycin and dirithromycin can be predicted by using erythromycin.

t See discussion of ESBLs under "RESULTS." For screening and confirmatory tests for ESBLs in *Klebsiella pneumoniae*, *K. oxytoca* and *E. coli*, see "RESULTS" section and reference 7. Screening breakpoints (Mueller Hinton agar, standard disc diffusion procedure, 35 ± 2°C, ambient air, 16 – 18 h) are: aztreonam (≤ 27 mm), ceftazidime (≤ 22 mm), cefotaxime (≤ 27 mm), cefpodoxime (≤ 17 mm) and ceftriaxone (≤ 25 mm). Quality control recommendations are *E. coli* ATCC 25922 (as listed in the chart); *K. pneumoniae* ATCC 700603 (aztreonam 9 – 17 mm), ceftazidime (10 – 18 mm), cefotaxime (17 – 25 mm), cefpodoxime (9 – 16 mm) and ceftriaxone (16 – 24 mm).[7] The use of more than one antimicrobial agent for screening improves the sensitivity of detection. Phenotypic confirmatory testing requires the use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid. A ≥ 5 mm zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone = ESBL. Quality Control recommendations are: negative strain *E. coli* ATCC 25922 which produces a ≤ 2 mm increase in zone diameter for antimicrobial agent tested alone versus its zone diameter when tested in combination with clavulanic acid; positive strain *K. pneumoniae* ATCC 700603 which produces a ≥ 3 mm increase in cefotaxime zone diameter and a ≥ 5 mm increase in ceftazidime zone diameter. See "Limitations of the Procedure." See reference 7 for details of the procedure.

u Cephalothin can be used to predict activity of cephalothin, cephapirin, cephradine, cephalexin, cefaclor and cefadroxil. Cefazolin, cefuroxime, cefpodoxime, cefprozil, and loracarbef (urinary isolates only) may be tested individually because some isolates may be susceptible to these agents when resistant to cephalothin.

v Not applicable for testing *Morganella* spp.

w For *N. gonorrhoeae*, an intermediate result for an antimicrobial agent indicates either a technical problem that should be resolved by repeat testing or a lack of clinical experience in treating organisms with these zones. The latter seems to be the case for cefmetazole, cefotetan, cefoxitin, and spectinomycin. Strains with intermediate zones with the other agents have a documented lower clinical cure rate (85 - 95%) compared to >95% for susceptible strains.

x Cefotaxime, ceftizoxime or ceftriaxone should be tested and reported on isolates from CSF in place of cephalothin and cefazolin.

y Because certain strains of *Providencia* spp. have been reported to give false-susceptible results with cefprozil discs, strains of this genus should not be tested and reported with this disc.

z Indicated for urine isolates only. In addition to testing urine isolates, nalidixic acid may be used to test for reduced fluoroquinolone susceptibility in isolates from patients with extraintestinal *Salmonella* infections. See footnote ddd.

aa FDA-approved zone diameters for interpretive and/or quality control criteria that differ from CLSI recommendations.

bb For *V. cholerae*, use with caution as the disc diffusion test may misclassify many organisms (higher minor error rate).

cc No criteria have been established to support testing of this drug with *Streptococcus pneumoniae*. The control range is listed for quality control purposes only.

dd Colistin and polymyxin B diffuse poorly in agar and the accuracy of the diffusion method is thus less than with other antibiotics. Resistance is always significant, but when treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

ee Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline or minocycline or both.

ff FDA-approved for *S. saprophyticus* and *S. epidermidis* (not *S. aureus*).

gg For control limits of gentamicin 120 µg and streptomycin 300 µg discs, use *E. faecalis* ATCC 29212 (gentamicin: 16 - 23[ii] mm; streptomycin: 14 - 20[ii] mm).

hh If the zone is 7 - 9 mm, the test is inconclusive and an agar dilution or broth microdilution screen test should be performed to confirm resistance.

ii CLSI-recommended zone sizes that differ from FDA-approved zone size recommendations.

jj No criteria have been established to support testing of this drug with *H. influenzae*. The control range is listed for quality control purposes only.

kk Because certain strains of *Citrobacter*, *Providencia*, and *Enterobacter* spp. have been reported to give false susceptible results with cefdinir and loracarbef discs, strains of these genera should not be tested and reported with these discs.

ll FDA-approved for *K. pneumoniae*.

mm No criteria have been established to support testing of this drug with *Pseudomonas aeruginosa*. The control range is listed for quality control purposes only.

nn If a penicillinase-stable penicillin is tested, oxacillin is the preferred agent and results can be applied to the other penicillinase-stable penicillins, cloxacillin, dicloxacillin, flucloxacillin, methicillin and nafcillin. Oxacillin is preferred because it is more resistant to degradation in storage, and because it is more likely to detect heteroresistant staphylococcal strains. Cloxacillin discs should not be used because they may not detect oxacillin-resistant *S. aureus*. Cefoxitin may be tested instead of oxacillin (see M100-S21). After incubation for a full 24 h, examine for light growth within the zone of inhibition of the oxacillin disc using transmitted light (plate held up to light). Any discernable growth within the zone of inhibition is indicative of oxacillin resistance.

oo If oxacillin intermediate results are obtained for *S. aureus*, perform testing for *mec*A or PBP 2a, the cefoxitin disc test, an oxacillin MIC test or the oxacillin-salt agar screening test. Report the result of the alternative test rather than the intermediate result.

pp Penicillin-resistant, oxacillin-susceptible strains of *Staphylococcus aureus* produce β-lactamase and the testing of the 10-unit penicillin disc instead of the ampicillin disc is preferred. Penicillin should be used to test the susceptibility of all β-lactamase-labile penicillins, such as ampicillin, amoxicillin, azlocillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin. Likewise, a positive β-lactamase test predicts resistance to these agents.[6] For oxacillin-resistant staphylococci, report as resistant or do not report.

qq A positive β-lactamase test predicts resistance to penicillin, ampicillin, and amoxacillin. A β-lactamase test will detect one form of penicillin resistance in *N. gonorrhoeae* and also may be used to provide epidemiologic information. Strains with chromosomally-mediated resistance can be detected only by additional susceptibility testing, such as the disc diffusion method or the agar dilution MIC method. Gonococci with 10-unit penicillin disc zone diameters of ≤19 mm are likely to be β-lactamase-producing strains. However, the β-lactamase test remains preferable to other susceptibility methods for rapid, accurate recognition of this plasmid-mediated penicillin resistance.

rr Susceptibility tests on *S. pyogenes* to penicillin are seldom necessary since this microorganism has continued to be universally susceptible to penicillin. However, some strains of *S. agalactiae* may give penicillin-intermediate results.[7]

ss Tigecycline has decreased *in vitro* activity against *Morganella* spp., *Proteus* spp. and *Providencia* spp.

tt The sulfisoxazole disc can be used to represent any of the currently available sulfonamides. Blood-containing media (except for lysed horse blood) are generally not suitable for testing sulfonamides or trimethoprim. Mueller Hinton agar should be as thymidine-free as possible for sulfonamide and/or trimethoprim testing. To determine whether the Mueller Hinton agar has sufficiently low levels of thymine and thymidine, *Enterococcus faecalis* ATCC 29212 or ATCC 33186 may be tested with the trimethoprim-sulfamethoxazole disc (see ref. 13). An inhibition zone of ≥ 20 mm that is essentially free of fine colonies indicates a sufficiently low level of thymine and thymidine.[6]

uu Gonococci with 30-µg tetracycline disc zone diameters of ≤ 19 mm usually indicate a plasmid-mediated tetracycline-resistant *N. gonorrhoeae* (TRNG) isolate. These strains should be confirmed by a dilution test (MIC ≥ 16 µg/mL) and/or referred to a public health laboratory for epidemiologic investigation.

vv All staphylococcal isolates with vancomycin zone diameters of 14 mm or less should be tested by a reference MIC method. The disc diffusion procedure will not differentiate strains with reduced susceptibility to vancomycin (MICs 4 to 8 µg/mL) from susceptible strains (MICs 0.5 to 2 µg/mL) even when incubated 24 h. Additionally, vancomycin-resistant *S. aureus* (VRSA) strains (MICs ≥ 16 µg/mL) may produce only subtle growth around a vancomycin disc. The vancomycin agar screen test described for enterococci (Brain Heart Infusion Agar with 6 µg/mL Vancomycin) may be used to enhance the sensitivity of detecting vancomycin-intermediate and vancomycin-resistant strains of *S. aureus* incubating the plates for a full 24 h at 35°C.[6] Use of a susceptible quality control strain, such as *E. faecalis* ATCC 29212 is critical to ensure specificity. *E. faecalis* ATCC 51299 may be used as a positive (i.e., resistant) control. Until further data on the prevalence or clinical significance of these isolates is known, laboratories may choose to examine MRSA strains more carefully for elevated MICs to vancomycin.[6] Currently, there are insufficient data to recommend using this agar screen test for coagulase-negative staphylococci. Send any staphylococci determined to have an elevated MIC to vancomycin (≥ 4 µg/mL) to a reference laboratory.

ww When testing vancomycin against enterococci, plates should be held a full 24 h and examined using transmitted light; the presence of a haze or any growth within the zone of inhibition indicates resistance. Organisms with intermediate zones should be tested by an MIC method as described in CLSI document M7. See also the vancomycin agar screen test described in the MIC Table 2D (M100-S21).[9]

xx No *S. pneumoniae* strain with a vancomycin zone diameter of inhibition <17 mm has been observed; submit such strains to a reference laboratory.[7]

yy Because of limited alternatives, chloramphenicol, erythromycin, tetracycline (or doxycyline or minocycline) and rifampin may be used for vancomycin-resistant enterococci (VRE) and consultation with an infectious disease practitioner is recommended.[7]

zz No criteria have been established to support testing of this drug with *N. gonorrhoeae*. The control range is listed for quality control purposes only.

aaa Strains of β-hemolytic streptococci with ampicillin, cefepime, cefotaxime, ceftriaxone or penicillin zone diameters of less than 24 mm have not been observed; submit such strains to a reference laboratory.

bbb Deterioration in oxacillin disc content is best assessed with *S. aureus* ATCC 25923, with an acceptable zone diameter of 18 - 24 mm.

ccc For ampicillin, cefepime, cefotaxime, ceftriaxone and penicillin, **Streptococci, β-hemolytic only** includes the large-colony-forming pyogenic strains of streptococci with group A (*S. pyogenes*), C or G antigens and strains with group B (*S. agalactiae*) antigen. For cefepime, cefotaxime and ceftriaxone, **Viridans Streptococci** includes small-colony-forming β-hemolytic strains with group A, C, F or G antigens (*S. anginosus*, previously termed *S. milleri*) as well as *S. mitis, S. oralis, S. sanguis, S. salivarius, S. intermedius, S. constellatus, S. mutans* and *S. bovis***.**

ddd Fluoroquinolone-susceptible strains of *Salmonella* that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal salmonellosis. Extraintestinal isolates of *Salmonella* should also be tested for resistance to nalidixic acid. For isolates that test susceptible to fluoroquinolones and resistant to nalidixic acid, the physician should be informed that the isolate may not be eradicated by fluoroquinolone treatment. A consultation with an infectious disease practitioner is recommended.

eee No criteria have been established to support testing of this drug with *S. aureus*. The control range is listed for quality control purposes only.

BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs
(Disques Sensi-Disc BBL pour antibiogramme)

Français

APPLICATION – Ces disques sont utilisés pour une évaluation semi-quantitative *in vitro* de la sensibilité aux antibiotiques des agents pathogènes bactériens courants à croissance rapide ainsi que de certaines espèces exigeantes, par un antibiogramme par diffusion sur disque en gélose.
Les microorganismes concernés incluent : les *Enterobacteriaceae*, les genres *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Enterococcus*, *Vibrio cholerae* et, avec des procédures modifiées, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae* et d'autres streptocoques.
REMARQUE : Des procédures particulières sont nécessaires pour tester les pneumocoques, les entérocoques et les staphylocoques résistants à la méticilline/oxacilline et pour réaliser des tests des β-lactamases et des tests de dépistage et de confirmation pour les ESBL ; voir la section « Résultats ».

Pour la France: Les résultats seront interprétés en fonction du dernier communiqué du Comité de l'antibiogramme de la Société Française de Microbiologie. Un guide d'interprétation conforme au communiqué du Comité de l'antibiogramme de la Société Française de Microbiologie pourra être fourni sur simple demande à la société Becton Dickinson France S.A.S., Tel: 04 76 68 36 36.

RESUME ET EXPLICATION – Les méthodes de diffusion en gélose utilisant des disques en papier filtre séchés contenant des concentrations déterminées en agents antimicrobiens ont été mises au point au cours des années 40. Afin d'éliminer ou de minimiser la variabilité inhérente à ce type de test, Bauer et al. ont mis au point une procédure standardisée dans laquelle la gélose Mueller Hinton était le milieu choisi pour le test.[1,2]
Divers organismes de réglementation et de rédaction des normes ont ensuite publié des procédures standardisées de référence en se basant sur la méthode Bauer-Kirby. Les normes de la Food and Drug Administration (FDA)[3] américaine et de l'Organisation mondiale de la santé (OMS)[4,5] figurent parmi les procédures standardisées les plus anciennes et les plus suivies. La procédure a été adoptée comme norme consensuelle par le Clinical and Laboratory Standards Institute (CLSI, anciennement NCCLS) et fait l'objet de mises à jour périodiques.[6,7] **Les documents du CLSI les plus récents doivent être consultés pour prendre connaissance des recommandations actuelles.**

PRINCIPES DE LA METHODE – Des disques contenant toute une gamme d'agents antimicrobiens sont déposés sur la surface de la gélose Mueller Hinton (ou de la gélose du test d'identification d'*Haemophilus* pour *H. influenzae*, de la gélose GC II enrichie d'**IsoVitaleX** pour *N. gonorrhoeae* ou de la gélose Mueller Hinton avec 5 % de sang de mouton pour *S. pneumoniae*, les streptocoques β hémolytiques et du groupe viridans) dans des boîtes de Pétri ensemencées avec des cultures pures d'isolats cliniques. Après incubation, les boîtes de Pétri sont examinées et les zones d'inhibition entourant les disques sont mesurées et comparées aux gammes de taille de zone établies pour les différents agents antimicrobiens afin de déterminer l'agent ou les agents les plus adéquats pour le traitement antimicrobien.

REACTIFS – Les disques **Sensi-Disc** sont des disques de 6 mm fabriqués à partir de papier absorbant de haute qualité imprégné d'antibiotiques ou d'autres agents chimiothérapeutiques en quantités déterminées de manière précise. Les disques sont clairement identifiés des deux côtés par des lettres et des chiffres désignant l'agent et sa concentration. (Voir le tableau des concentrations des composants actifs.) La teneur en agent des disques est mesurée par les méthodes définies par la FDA ou par des méthodes similaires ou comparables à celles publiées dans le *Federal Register* américain.

From:	Amy Mozingo
To:	Hice, Stephanie
Cc:	William J. Rowe
Subject:	RE: GRN 000955 - Questions for Notifier
Date:	Monday, February 8, 2021 2:17:35 PM
Attachments:	image001.png 2021-01-27 GRN 955 Questions for Notifier_Response 2021-08-08.pdf Penet et al. Study evaluating the efficacy of MB40 to Reduce Abdominal Discomfort, Gas, and Bloating.pdf

Dear Dr. Hice,
Please find attached the responses to FDA's questions along with the full text PDF of the Penet et al 2019 publication.
Best Regards,
Amy Mozingo

Amy Mozingo, MS
Director of Operations, GRAS Associates
a Nutrasource Pharmaceutical and Nutraceutical Services company
O: 301-461-8929| C: 772-532-3454

LinkedIn | **Twitter** | **Blog**

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From: William J. Rowe <wrowe@nutrasource.ca>
Sent: Wednesday, January 27, 2021 10:52 AM
To: Amy Mozingo <amozingo@gras-associates.com>
Subject: Fwd: GRN 000955 - Questions for Notifier

FYI for follow up thanks

Sent from my iPhone

Begin forwarded message:

From: "Hice, Stephanie" <Stephanie.Hice@fda.hhs.gov>
Date: January 27, 2021 at 10:49:47 AM EST
To: "William J. Rowe" <wrowe@nutrasource.ca>
Subject: RE: GRN 000955 - Questions for Notifier

Dear Mr. Rowe,

During our review of GRAS Notice No. 000955, we noted further 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,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
stephanie.hice@fda.hhs.gov

January 27, 2021

Questions/Comments Regarding GRN 000955:

Questions:

1. On page 34 of the notice, the notifier references a study by Penet et al., 2019. On page 49 of the notice, the study was listed as "in press" in the list of references. In the January 19, 2021 amendment, the notifier references the same study by Penet et al., 2019 (page 5), which is listed as "in press" in the list of references (pages 13-14). We note that, while the study is discussed in the notice, the full text of this paper is not accessible.

    a. Please provide instructions regarding how to access the full text of the paper and clarify if it is peer-reviewed. If it is unpublished, we note that unpublished studies are generally only used to corroborate published studies and information that serves as the basis for a notifier's GRAS conclusion. Therefore, please clarify if the basis of the notifier's GRAS conclusion is impacted.

        Response: The Penet article has been published in a peer-reviewed journal. The reference is Penet C, Kramer R, Little R, et al. A Randomized, Double-blind, Placebo-controlled, Parallel Study Evaluating the Efficacy of Bacillus subtilis MB40 to Reduce Abdominal Discomfort, Gas, and Bloating. Alternative Therapies in Health and Medicine. 2019 Nov. A link can be found here: A Randomized, Double-blind, Placebo-controlled, Parallel Study Evaluating the Efficacy of Bacillus subtilis MB40 to Reduce Abdominal Discomfort, Gas, and Bloating. - Abstract - Europe PMC

    b. Please explain why, given the adverse events (AEs) reported in the treatment group from this study (listed in Table 12, page 35 of the notice), the notifier concludes that "no adverse effects were noted (including GI effects)" in their January 19, 2021 response to our question #3.

        Response: Adverse events did occur in the Penet et al. 2019 study but the incidence did not differ between treatment and placebo groups. The response to FDA's question #3 is therefore changed to "no treatment-related adverse effects were noted (including GI effects)". Penet et al. (2019) stated that the effects in the treatment group were "possibly related" to treatment but also stated that the effects in the placebo group were also "possibly related" to treatment. Therefore, their use of "possibly related to treatment" is a misnomer. As stated in Penet et al., 2019, of the 13 AE reported by those in the OPTI-BIOME® group, 8 were "possibly related" to the product: abdominal discomfort (1), constipation (3), diarrhea (1), dry mouth (1), flatulence (1), and increased appetite (1). Of the 17 AE reported by those in the placebo group, five were "possibly related" to the product: abdominal discomfort (1), constipation (2), infrequent bowel movements (1), and paresthesia (1). Also, as stated in the Penet et al. 2019 study, "adverse event reporting between the groups was similar with 6 of 8 and 4 of 5 possibly related events attributable to gastrointestinal disorders in the MB40 and placebo groups respectively. All other adverse events were assessed as 'unlikely' or 'not related' to the study products."

    c. Please clarify if these AEs indicate a safety concern from consuming *Bacillus subtilis* strain BS-MB40 PTA-122264 spore preparation.

        Response: The AEs reported in Penet et al. (2019) do not indicate a safety concern and therefore do not impact the conclusion of GRAS.

Penet, “A Randomized, Double-blind, Placebo-controlled, Parallel Study Evaluating the Efcacy of Bacillus subtilis MB40 to Reduce Abdominal Discomfort, Gas, and Bloating”, ALTERNATIVE THERAPIES (2019). (12 pages)