3. Wash, put on the Extravidin conjugate, 1 hr incubate.
4. Wash, put on Gibco substrate, 12.5 min incubate
5. Put on Gibco amplifier, 2-10 min incubate.
6. Read plates on microplate reader

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1. Ferreira, J L., Maslanka, S, Johnson, E., and Goodnough, M. 2003. Detection of botulinal neurotoxins A, B, E, and F by amplified enzyme-linked Immunosorbent assay: collaborative study. *JAOAC International* **86**:314-331.
2. Solomon, H. and Lilly, T. 2001. FDA Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum*.
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A modification of the method described above is available in Laboratory Information Bulletin (LIB) No. 4292. The LIB describes a modification that uses digoxigenin labeled IgGs to detect type A, B, E, and F botulinal toxins. The digoxigenin label substitutes for the biotin label in the amplified ELISA and is detected using an anti-digoxigenin horse radish peroxidase conjugate and TMB substrate.

IV. **Detection of Type A, B, E, and F *Clostridium botulinum* Toxins Using Digoxigenin-labeled IgGs and the ELISA (DIG-ELISA).** Contact J. L. Ferreira (FDA) 404 253-2216, S. Sharma (FDA) 301 *436-1570*. S. Maslanka (CDC) 404 639-0895, or J. Andreadis (CDC) for questions regarding this method.

This method is a modification of the amplified-ELISA (amp-ELISA). Digoxigenin-labeled antitoxin IgG's are substituted for biotin-labeled IgG's and anti-digoxigenin horse radish peroxidase conjugate (HRP) is substituted for the streptavidin-alkaline phosphatase used in the amp-ELISA. An appropriate substrate (TMB) is used for the HRP enzyme. The A, B, E, and F botulinal toxins are detected at approximately 10 MLD/mL (0.12-0.25 ng/mL). Toxic cultures may be more antigenic than purified toxins and the level of detection using the DIG-ELISA may be more sensitive than the mouse bioassay. Both TPGY and CMM are tested since more toxin may be generated in one medium compared to the other and the confirmatory mouse bioassay also utilizes these media. Very toxic cultures (greater than approximately 10,000 MLD/mL) may give a positive absorbance for more than one toxin type in the amp-ELISA as well as the DIG-ELISA (crossing between types). Generally, a 10-fold dilution will show that the true toxin type will have a very high absorbance and the crossing type will have a negative absorbance. In either case the toxic sample must be confirmed using the mouse bioassay.

A. **Equipment and Materials**

1. Microplate, Dynex Immulon ll U-bottom, cat. No. 3655
2. Microtiter pipettors to deliver from 0.1- 2.0, 2-20, and 50-200 µl.
3. Multichannel pipettor, 8 or 12 place 50-200 µl
4. Pipets, disposable 1,5,10 ml
5. Glass test tubes 13X100 mm, 15X150 mm
6. Incubator, 35°C
7. Refrigerated centrifuge

8. Microplate washer
9. Microplate shaker
10. Microplate reader (read 450 nm)
11. Microtiter plate seals
12. Multichannel pipet reservoirs

B. Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)

1. Tryptone-peptone-glucose-yeast extract broth (TPGY).
2. Cooked meat medium (CMM).
3. 0.05M bicarbonate buffer: 0.8g Na_2CO_3 + 1.47g $NaHCO_3$ in 500 ml distilled
   H_2O, pH 9.6. Capsules to prepare 100 ml volume are available from Sigma.
4. 1% Casein buffer: Add 10.0g vitamin-free casein (Research Organics) + 7.65g NaCl, 0.724g Na_2HPO_4 (anhydrous), 0.21g KH_2PO_4 to 900 ml H_2O, and 3 ml of 1 M NaOH. Heat with stirring to ~ 80°C to dissolve casein. Check pH and adjust to 7.9 with 1 M NaOH, q.s. to 1 liter. Sterilize at 121°C for 20 min. Final pH is ~7.4-7.6. Casein blocker ready to use product is available from Pierce that gives slightly lower absorbance values than in-house prepared casein buffer. (SRL, Atlanta, GA).
5. Goat type A, B, E, or F digoxigenin-labeled antitoxin (SRL, Atlanta, GA).
6. Phosphate buffered saline with 0.005% Tween 20 wash buffer (PBST).
   1.2 g Na_2HPO_4 (anhydrous), 0.22g $NaH_2PO_4.H_20$, 8.5g NaCl per liter distilled H_2O. Adjust pH to 7.5 Add 50 µl

of Tween 20/L PBS. Sterilize at 121°C for 20 min. 10 × PBST: 12.0g Na_2HPO_4 (anhydrous), 2.2g $NaH_2PO_4.H_20$, 85.0g NaCl per liter distilled H_2O. Adjust pH to 7.5 Add 500 µl of Tween 20/L PBS. 1 × PBST is then prepared by adding 100 ml of 10X PBST to 900 ml of distilled H_2O and mixing before use. 10X PBS is available commercially from GibCo.

7. Anti-digoxigenin HRP poly conjugate (Roche Applied Science).
8. Tetra methyl benzidine (Ultra-TMB) (Pierce).
9. 0.5 M H_2SO_4.
10. Botulinal complex toxin standards A, B, E, and F. (Metabiologics Inc., Madison, WI)

C. **DIG-ELISA Procedure**

1. **Preparation of samples.**
    a. **Cultural sample preparation.** Food samples or anaerobic isolates picked from agar plates are inoculated into TPGY (**without trypsin**) and CMM as recommended in Chapter 17 of the Bacteriological Analytical Manual (2001). TPGY broth and cooked meat media are incubated for 5 days at 26°C and 35°C respectively. Cultures are centrifuged at 7,000 × g and 4°C for 30 min, supernatant pH is adjusted to 7.4-7.6 using 1 N NaOH or 1N HCl. Samples and controls are analyzed **in duplicate** for TPGY and for CMM. Analyze undiluted and 1:5 dilutions of each culture supernatant. 1:5= 0.2 ml culture + 0.8 ml casein buffer.

b. **Food sample preparation.** If a food has a liquid packing medium, the liquid may be removed, centrifuged as above to remove solids and/or fats and the supernatant/ aqueous layer directly analyzed by ELISA after pH adjustment to 7.4-7.6. If the food is a solid or semi-solid, the toxin must be extracted. An equal amount of food (20 g) and casein buffer (20 mL) are mixed by grinding with a mortar and pestle or by other means to mix the food and buffer. The food-buffer slurry (1:2 dilution) is centrifuged at 7,000 × g for 30 min at 4°C. The aqueous supernatant is removed and adjusted to pH 7.4-7.6 if necessary using 1 N NaOH or 1 N HCl. Some foods such as Honey may also require dilution to remove ELISA inhibitors. Honey has previously been tested at a 1:5 dilution with satisfactory results. Normal food that does not contain botulinal toxin can be spiked with known standard toxin(s) at 2ng toxin/mL (~100 MLD/mL) of the food extract in casein buffer to monitor the possible inhibition of the ELISA by the food. Botulinal neurotoxin standards were diluted in casein buffer and used as controls or for spiking foods prior to analysis.

2. **Preparation of microtiter plates.** Coat each well of the microtiter plate with 100 µl of appropriate dilution of goat type A, E, or F or rabbit type B antitoxin diluted in bicarbonate buffer. Prepare the number of needed microtiter plate wells to test the sample. Dilute the stock antitoxins according to the accompanying directions. Store plate with coating buffer overnight at 4°C with

plastic seal cover on top of plate to prevent evaporation.

3. **ELISA analysis of samples.**

    a. Remove plate from 4°C storage and wash plate 5 times in PBST with 45 second hold between each aspiration. Use a commercial plate washer or other mechanical device; avoid using a squeeze bottle to wash.

    b. Block plate in casein buffer with by filling all wells to the top of the plate (~300 µl/well) and incubate for 60-90 min at 35°C. Prepare the sample and control dilutions while the plate is being blocked.

    Negative controls: Duplicate wells are tested with all reagents except toxin (pH adjusted undiluted sterile CMM and TPGY broth if used and casein control). Casein buffer control is used as a system control.

    Positive controls: Duplicate wells are tested using standard toxins type A, B, E, and F diluted in pH adjusted sterile TPGY and CMM (if used) at a concentration of 2 ng/mL. The LD_{50}/ng will vary depending on toxin type.

    ELISA Food Inhibition controls: Type A, B, E, and F neurotoxins can be used to spike a food at 2 ng/mL of the supernatant obtained from the food-casein buffer slurry. Duplicate wells are tested for each toxin type. Results are compared to the positive control that consists of toxin spiked into casein to demonstrate if the product inhibits the

ELISA. The product may be diluted further to remove inhibitory substances but will lower the sensitivity of the test.

c. Wash the blocked plate as above and then add the toxic samples and controls (100 µl/well). Work from the left side of the plate to the right side when adding the reagents.

d. Incubate toxin-containing samples and controls for 2 hr. at 35°C. Prepare the type A, B, E, and F digoxigenin-labeled antibody reagents according to directions while incubating the samples. Do not make more than you need!

e. Wash plate 5 times in PBST as above.

f. Add the diluted digoxigenin-labeled goat antibody (100 µl/well) and incubate for 60 min at 35°C.

g. Wash plate 5 times in PBST as above.

h. Add the anti-digoxigenin poly HRP conjugate diluted 1:5,000 in casein buffer (100 µl/well), and incubate for 60 min at 35°C.

i. Wash 5 times in PBST then tamp the plate several times on a paper towel to remove any residual wash buffer.

j. Add 100 µl of the TMB (substrate at room temperature) solution, incubate 20-30 min at 35°C. Positive sample wells will begin to turn a blue-green color. High toxin samples will develop color within a few minutes. The analysis can be stopped with 100 µl of stop reagent at any time (within 20-30 min) after addition of the substrate when

positive controls give appropriate sensitivity (absorbance ≥ 1.0) and negative controls are acceptable (absorbance not greater than ~ 0.39). The plate should be taken to the plate reader immediately after addition of the stop solution. Measure absorbance at 450 nm on microplate reader.

Results: A positive test is an absorbance value that is >0.20 above the absorbance observed in the negative controls (sterile uninoculated TPGY broth or CMM or negative food sample). As in any ELISA, higher background absorbance will result if plates are insufficiently washed.

D. **Confirmation of positive ELISA samples.** The DIG-ELISA was designed for screening TPGY and CMM culture media that may contain type A, B, E, and/or F botulinal toxins. Some food matrices may be inhibitory to the test or may generate false positive results. Samples that are positive or are inhibitory to the DIG-ELISA test must be confirmed using the mouse bioassay.

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2. Solomon, H. and Lilly, T. 2001. FDA Bacteriological Analytical Manual. Chapter 17, *Clostridium botulinum*.
3. Ferreira, J.L., Maslanka, S., Andreadis J. 2002. Detection of type A, B, E, and F *Clostridium botulinum* toxins using digoxigenin-labeled

IgGs and the ELISA. FDA/ORA Laboratory Information Bulletin Vol. 18, Number 10, 4292:1-10.

Flow Diagram for DIG-ELISA

Day 1

Coat microtiter plates with capture IgG and store overnight at 4°C.

Day 2

1. Wash plates, block, put on toxic samples and controls, 2 hr incubate.
2. Wash, put on digoxigenin-labeled IgG's, 1 hr incubate.
3. Wash, put on the anti-digoxigenin HRP conjugate, 1 hr incubate.
4. Wash, put on TMB substrate, 20-30 min incubate.
5. Stop the reaction with stop reagent.
6. Measure absorbance on plates with microplate reader at 450 nm.

V. **Specific Detection of Clostridium botulinum Types A, B, E, and F Using the Polymerase Chain Reaction (PCR)** For additional information on this PCR method, contact Kathy E. Craven or Joseph L. Ferreira at FDA, ORA, Southeast Regional Laboratory, 60-8th Street, N.E., Atlanta, GA 30309. Telephone: (404) 253-1200; FAX: (404)253-1210.

Clostridium botulinum organisms generally produce one of four neurotoxin types (A, B, E, and F) associated with human illness. Neurotoxin type determination is important in determining the identification of the bacterium. A PCR method was developed to identify 24 hour botulinal cultures as potential type A, B, E and F neurotoxin producers as well as culture of other clostridial species which also produce botulinal neurotoxins. Components of the PCR

and amplification conditions were adjusted for optimal amplification of toxin gene target regions enabling the simultaneous testing for types A, B, E, and F in a single thermal cycler. Each primer set was specific for its corresponding toxin type. Additionally, a DNA extraction procedure was included to remove inhibitory substances that may affect amplification. This procedure is rapid, sensitive, and specific for the identification of toxigenic *C. botulinum*.

Because of the severity of neuroparalytic illness caused by botulinal neurotoxin, a rapid diagnosis for the specific toxin type is necessary during illness outbreaks suspected of being foodborne. The PCR technique has also been used to detect multiple botulinal toxin-producing types within a single PCR assay (4,6). The PCR assay for the toxin gene type is determined after a 24-hour anaerobic culture to obtain vegetative cells. ELISA procedures may require up to five days of culture growth before toxin is detected (5,9). The PCR method may also be used in conjunction with the mouse bioassay to determine toxin type. For example, a culture that is PCR positive for the type A toxin gene would require mouse protection/testing confirmation only for toxin type A.

A. **Equipment and Materials**

1. Programmable automatic thermocycler
2. Horizontal gel electrophoresis apparatus
3. Electrophoresis constant-voltage power supply
4. Heating plate
5. Incubators, 35°C
6. Water bath, 37°C and 60°C
7. Freezer, -20 and -70°C

8. Speed Vacuum, optional
9. Microwave
10. Sterile disposable inoculating loops
11. Microcentrifuge tubes, 1.5 and Thin Walled PCR reaction tubes, 0.2 ml or 0.5 ml
12. Variable digital micropipettors (e.g., 0.5-20 µl, 20-200 µl, 100-1,000µl)
13. Aerosol-resistant pipet tips
14. Microcentrifuge
15. UV transilluminator
16. Polaroid camera and Polaroid film 3000 ISO or comparable Gel Documentation System

B. Media (/food/laboratory-methods/media-index-bam) and Reagents (/food/laboratory-methods/reagents-index-bam)

Molecular biology grade reagents are recommended and are available from various manufacturers.

1. Tryptone-peptone glucose yeast extract broth (TPGY).
2. Phosphate-buffered saline, pH 7.4 (PBS)
3. Tris EDTA, pH 8.0 (1X TE). 10mM Tris-HCL, 1mM EDTA, pH 8.0 in distilled water
4. Proteinase K- 10 mg Proteinase K/ml 1× TE
5. Lysozyme-10 mg Lysozyme/ml 1 × TE
6. 3 M Sodium Acetate, pH 5.2
7. 95% ethanol
8. 2'-Deoxynucleoside-5'-triphosphates (dATP, dCTP,

dGTP, dTTP); stock solution 2.5 mM of each dNTP

9. *Taq* DNA polymerase (available from various vendors) or Amplitaq® (Perkin-Elmer)
10. 10 × Reaction Buffer B-500mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), 1.0 % Triton X-100
11. 15 mM $MgCl_2$
12. *Clostridium botulinum* neurotoxin oligonucleotide primers types A, B, E, and F, 10 µM stock solutions (2).
13. Light mineral oil, optional
14. Sterile deionized water, RNase and DNase free
15. 10× TBE (0.9 M Tris-borate, 0.02 M EDTA, pH 8.3)
16. Agarose (nucleic acid electrophoresis grade)
17. Ethidium bromide solution, 10 mg/ml
18. 6× sample loading buffer
19. DNA molecular weight markers (e.g., 123 bp ladder or 100 bp ladder)

C. **Procedure for amplification of *C. botulinum* neurotoxin A, B, E, and F gene fragments from presumptive *C. botulinum* isolates using TPBY enrichment broth**

Food sample preparation and enrichment (Chapter 17, Part l Mouse Bioassay, Section D).

1. **DNA isolation Procedures.** Boil sterile 10 ml portions of Tryptone-Peptone-Glucose-Yeast Extract Broth (TPGY) in a water bath for 10 min and quickly cool to room temperature just prior to use. Inoculate TPGY with presumptive *C. botulinum* isolates using a

disposable sterile inoculating loop and incubate overnight at 35° C. Remove a 1.4 ml aliquot from each of the cultures and dispense into separate sterile micro-centrifuge tubes. Centrifuge at 14,000 × g for 2 min and discard supernatant. Wash the bacterial pellets in 1.0 ml PBS, pH 7.4 and centrifuge at 14,000 × g for 2 min. Discard supernatant and resuspend pellets in 400 µl PBS and 100 ml of 10 mg lysozyme/ml 10mM Tris, 1 mM EDTA, pH 7.4 (TE). Incubate for 15 min at 37° C in a water bath, inverting tubes every 5-7 min during incubation. Add 10 µl of 10 mg Proteinase K/ml TE to suspensions and incubated for 1 h in a 60° C water bath. Invert tubes every 10-15 min during the incubation period. Boil suspensions for 10 min in a water bath and centrifuge for 2 min at 14,000 rpm. Transfer supernatants to sterile 1.5 ml micro-centrifuge tubes. Add 50 µl aliquot of 3 M Sodium acetate and 1.0 ml of 95% ethanol to supernatants, mix by inversion, and cool at -70 °C (or -20°C) for 30 min. Centrifuge the ethanol-salt preparations at 14,000 rpm. Discard supernatants and dry pellets using a DNA Speed-Vacuum (Savant Instruments, Inc., Holbrook, NY). Re hydrate pellets in 200-µl sterile TE buffer and store immediately at -20° C until PCR analysis is performed.

2. **Alternative DNA isolation/preparation procedures.** Cell lysis by boiling can also be performed to simplify the procedure. *C. botulinal* cultures are grown 24 hours as previously described. Remove a 1.4 ml aliquot and centrifuge at 14,000 × g for 2 min. Boil the suspension in a water bath for 10 min and centrifuge at 14,000 × g for 2 min to remove cell debris. Remove the

supernatants and place into a sterile microcentrifuge tube. Store at -20°C until PCR analysis is performed. Commercial DNA extraction kits such as Gene Clean II (BIO 101,Inc., La Jolla, CA) and S&S Elu-Quick (Schleicher & Schuell, Keene, NH) may be used if the cells are sufficiently lysed. Manufacturers' protocol supplied with kits are followed. The method used for lysis of gram positive organisms prior to extraction of the DNA for PCR is important. Unless DNA concentrations are determined before PCR analysis, it may be necessary to test dilutions of the DNA sample to avoid false negative results caused by too little or too much DNA when using commercially available kits. We recommend the use of no more than 344 ng of total DNA be used for the PCR analysis.

Note: DNA purification before amplification is recommended to reduce the possibility of inhibitory substances in cultures from affecting the PCR and to increase the concentration of target DNA. Purification of DNA removes inhibitory substances that may affect PCR amplification. Simple boiling of the cell culture may not remove all inhibitors from the PCR DNA preparation for all cultures. No PCR inhibition was observed due to the TPGY medium itself. The use of the described extraction procedure that incorporates Proteinase K and lysozyme consistently lysed *C. botulinum* cells (2). The amount of isolated DNA yielding positive results using this amplification method ranged from approximately 0.34 ng- 5,160 ng DNA per 100-µl total volume PCR reaction. Using DNA concentrations outside this range may result in false negative results.

This method is rapid and reliable for the identification of type A, B, E and F toxin-producing clostridial strains. PCR results for typing clostridial toxin genes were obtained in approximately 4 hours following a 24-hour incubation of the culture. This method is not limited by culture production of the neurotoxin which requires up to five days incubation prior to analysis by ELISA or the mouse bioassay (3,5). The PCR products also can be toxin gene typed or confirmed by using type-specific oligonucleotide or polynucleotide DNA probes.

Oligonucleotide Primers. Desalted oligonucleotide primers are obtained from commerical suppliers. Primers were derived from published DNA sequences for *C. botulinum* structural genes encoding types A, B, E, and F neurotoxins (1, 3, 7, 8). The forward (F) and reverse (R) PCR primer sequences are:

Type A
F 5' -GTG ATA CAA CCA GAT GGT AGT TAT AG -3'
R 5' -AAA AAA CAA GTC CCA ATT ATT AAC TTT -3'

Type B
F 5' -GAG ATG TTT GTG AAT ATT ATG ATC CAG -3'
R 5'- GTT CAT GCA TTA ATA TCA AGG CTG G -3'

Type E
F 5'- CCA GGC GGT TGT CAA GAA TTT TAT -3'
R 5'- TCA AAT AAA TCA GGC TCT GCT CCC -3'

Type F
F 5'-GCT TCA TTA AAG AAC GGA AGC AGT GCT-3'
R 5'- GTG GCG CCT TTG TAC CTT TTC TAG G -3'

PCR reaction preparation. Primer sets for each of the types are used in separate PCR reactions. PCR reactions are performed in a 100 µl volume mixture containing , 1 × PCR buffer [10 mM Tris-HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100], 2.5 mM $MgCl_2$, 0.5 µ'M concentration of each primer set (A, B, E, or F), 200 µM concentration of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 2.5 U *Taq* DNA polymerase, and 2 µl of sample DNA. If necessary add approx. 50-70 µl of sterile mineral oil. Thermal cyclers equipped with heated covers will not require the addition of a mineral oil overlay. If PCR reaction volumes are decreased to 50 µl, the amount of template should be decreased to 1.0 µl.
Note: It is recommended to add sample DNA to the PCR reaction mixture last in order to decrease potential contamination of PCR reagents. Positive and negative controls should be included in each analysis. Negative controls containing all of the reagents but lacking template DNA processed as described above are used to monitor for contamination with *C. botulinum* amplicons.

Temperature cycling. PCR conditions for simultaneous amplification of toxin gene fragments A, B, E, and F are:

One cycle at 95°C for 5 min
Thirty cycles of 94 °C for 1 min (denaturation)
60°C for 1 min (annealing)
72°C for 1 min (extension)
Final incubation of 72 °C for 10 min
Holding temperature of 4°C

Multiplex PCR for the amplification of A and E or B and F toxin gene fragments has been performed successfully using these primers but with lower PCR product yields (4). These four primer pairs can not be used together in one multiplex reaction because the primers are incompatible.

Agarose gel analysis of PCR products. Prepare a 1.2-1.5 % agarose gel in 0.5 × TBE containing 0.5 µg ethidium bromide/ml agarose. Agarose may be melted in 0.5 × TBE using a microwave. Cast gel and allow to solidify. Mix 10 µl portions of PCR products with approximately 2.0 µl 6× gel loading dye and load onto gel submerged in 1 × TBE. An appropriate molecular weight marker must be included on each gel in order to determine the approximate molecular weight of PCR products. Molecular weight markers should contain fragments which bracket the target sequence size. Apply a constant voltage of 10 V/cm and allow amplified fragments to migrate until appropriate band separation is achieved.

A short-wave UV light is used to visualize bands relative to the molecular weight marker. Predicted fragment lengths for each toxin gene fragment are: Type A, 983-bp; Type B, 492-bp; Type E, 410-bp, and Type F, 1137-bp. Photographs of the gels are used to document the results using either a polaroid camera or a comparable gel documentation system.

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BAM: Aerobic Plate Count

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Bacteriological Analytical Manual
Chapter 3
Aerobic Plate Count

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Chapter Contents

The aerobic plate count (APC) is intended to indicate the level of microorganism in a product. Detailed procedures for determining the APC of foods have been developed by the Association of Official Analytical Chemists (AOAC) (3) and the American Public Health Association (APHA) (1). The conventional plate count method for examining frozen, chilled, precooked, or prepared foods, outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 966.23, with one procedural change (966.23C). The suitable colony counting range (10) is 25-250. The automated spiral plate count method for the examination of foods and cosmetics (5), outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 977.27. For procedural details of the standard plate count, see ref. 2.Guidelines for calculating and reporting plate counts have been changed to conform with the anticipated changes in the 16th edition of *Standard Methods for the Examination of Dairy Products* (2) and the *International Dairy Federation* (IDF) procedures (6).

Conventional Plate Count Method

A. **Equipment and materials**

1. Work area, level table with ample surface in room that is clean, well-lighted (100 foot-candles at working surface) and well-ventilated, and reasonably free of dust and drafts. The microbial density of air in working area, measured in fallout pour plates taken during plating, should not exceed 15 colonies/plate during 15 min exposure.
2. Storage space, free of dust and insects and adequate for protection of equipment and supplies

3. Petri dishes, glass or plastic (at least 15 × 90 mm)
4. Pipets with pipet aids (no mouth pipetting) or pipettors, 1, 5, and 10 ml, graduated in 0.1 ml units
5. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps
6. Pipet and petri dish containers, adequate for protection
7. Circulating water bath, for tempering agar, thermostatically controlled to 45 ± 1°C
8. Incubator, 35 ± 1°C; milk, 32 ± 1°C
9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate
10. Tally register
11. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water (R11 (/food/laboratory-methods/bam-r11-butterfields-phosphate-buffered-dilution-water)); milk, 99 ± 2 ml
12. Plate count agar (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))
13. Refrigerator, to cool and maintain samples at 0-5°C; milk, 0-4.4°C
14. Freezer, to maintain frozen samples from -15 to -20°C
15. Thermometers (mercury) appropriate range; accuracy checked with a thermometer certified by the National Institute of Standards and Technology (NIST)

B. **Procedure for analysis of frozen, chilled, precooked, or prepared foods**

Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate, of food homogenate (**see** Chapter 1 (/food/laboratory-methods/bam-food-samplingpreparation-sample-homogenate) for sample preparation) by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 s. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 s if it stands more than 3 min before it is pipetted into petri dish. Add 12-15 ml plate count agar (cooled to 45 ± 1°C) to each plate within 15 min of original dilution. For milk samples, pour an agar control, pour a dilution water control and pipet water for a pipet control. Add agar to the latter two for each series of samples. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials, e.g., flour and starch. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium

thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify. Invert solidified petri dishes, and incubate promptly for 48 ± 2 h at 35°C. Do not stack plates when pouring agar or when agar is solidifying.

C. **Guidelines for calculating and reporting APCs in uncommon cases**

Official Methods of Analysis (3) does not provide guidelines for counting and reporting plate counts, whereas *Standard Methods for the Examination of Dairy Products*, 16th ed. (2) presents detailed guidelines; for uniformity, therefore, use APHA guidelines as modified (6,8). Report all aerobic plate counts (2) computed from duplicate plates. For milk samples, report all aerobic plate (2) counts computed from duplicate plates containing less than 25 colonies as less than 25 estimated count. Report all aerobic plate counts (2) computed from duplicate plates containing more than 250 colonies as estimated counts. Counts outside the normal 25-250 range may give erroneous indications of the actual bacterial composition of the sample. Dilution factors may exaggerate low counts (less than 25), and crowded plates (greater than 250) may be difficult to count or may inhibit the growth of some bacteria, resulting in a low count. Report counts less than 25 or more than 250 colonies as estimated aerobic plate counts (EAPC). Use the following guide:

1. Normal plates (25-250). Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size, on selected plate(s). Record dilution(s) used and total number of colonies counted.
2. Plates with more than 250 colonies. When number of CFU per plate exceeds 250, for all dilutions, record the counts as too numerous to count (TNTC) for all but the plate closest to 250, and count CFU in those portions of plate that are representative of colony distribution. See ref. 2 for detailed guidelines. Mark calculated APC with EAPC to denote that it was estimated from counts outside 25-250 per plate range (*see* D-3).
3. Spreaders. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth so that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreaders. When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the APC.

4. Plates with no CFU. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

D. **Computing and recording counts (see refs 6, 8)**

To avoid creating a fictitious impression of precision and accuracy when computing APC, report only the first two significant digits. Round off to two significant figures only at the time of conversion to SPC. For milk samples, when plates for all dilutions have no colonies, report APC as less than 25 colonies estimated count. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is odd and round down when the second digit is even.

Examples

Calculated Count	APC
12,700	13,000
12,400	12,000
15,500	16,000
14,500	14,000

1. Plates with 25-250 CFU.

$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$

a. Calculate the APC as follows:

$$\frac{(31+31)\ \text{colonies}}{0.0015\ \text{ml}} = 4.1 \times 10^4$$

= 537/0.022
= 24,409
≈ 24,000

b. When counts of duplicate plates fall within and without the 25-250 colony range, use only those counts that fall within this range.

2. All plates with fewer than 25 CFU. When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than 25 × 1/d when d is the dilution factor for the dilution from which the first counts were obtained.

Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
18	2	<>
0	0	<>

3. **All plates with more than 250 CFU**. When plates from both 2 dilutions yield more than 250 CFU each (but fewer than 100/cm^2), estimate the aerobic counts from the plates (EAPC) nearest 250 and multiply by the dilution.

   Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	640	640,000

   TNTC, too numerous to count.
   EAPC, estimated aerobic plate count.

4. All plates with spreaders and/or laboratory accident. Report respectively as Spreader (SPR), or Laboratory Accident (LA).
5. All plates with more than an average of 100 CFU per sq cm. Estimate the APC as greater than 100 times the highest dilution plated, times the area of the plate. The examples below have an average count of 110 per sq cm.

   Example

Colonies/Dilution		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	7,150[(a)]	>6,500,000 EAPC[(b)]
TNTC	6,490	>5,900,000 EAPC

[a] Based on plate area of 65 cm^2
[b] EAPC, estimated APC
[c] Based on plate area of 59 cm^2

Spiral Plate Method

The spiral plate count (SPLC) method for microorganisms in milk, foods, and cosmetics is an official method of the APHA (2) and the AOAC (3). In this method, a mechanical plater inoculates a rotating agar plate with liquid sample. The sample volume dispensed decreases as the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

A. **Equipment and materials**

1. Spiral plater (Spiral Systems Instruments, Inc., 7830 Old Georgetown Road, Bethesda, MD 20814)
2. Spiral colony counter (Spiral Systems) with special grid for relating deposited sample volumes to specific portions of petri dishes
3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)
4. Disposable micro beakers, 5 ml
5. Petri dishes, plastic or glass, 150 × 15 mm or 100 × 15 mm
6. Plate count agar (standard methods) (M124 (/food/laboratory-methods/bam-media-m124-plate-count-agar-standard-methods))
7. Calculator (optional), inexpensive electronic hand calculator is recommended
8. Polyethylene bags for storing prepared plates
9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)
10. Sterile dilution water
11. Syringe, with Luer tip for obstructions in stylus; capacity not critical
12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.
13. Sodium hypochlorite solution (5.25%). Available commercially.

B. **Preparation of agar plates.**

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for prepouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70°C into each petri dish. Let agar solidify on level surface with poured

plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°C. Bring prepoured plates to room temperature before inoculation.

C. **Preparation of samples.**

As described in Chapter 1, select that part of sample with smallest amount of connective tissues or fat globules.

D. **Description of spiral plater.**

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. Operator with minimum training can inoculate 50 plates per h. Within range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of prepoured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area. Studies have shown the method to be proficient not only with milk (4) but also with other foods (7,10).

E. **Plating procedure**

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope cover slip against face of stylus tip; if cover slip plane is parallel at about l mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for 48 ± 3 h at 35 ± 1°C.

F. **Sterility controls**

Check sterility of spiral plater for each series of samples by plating sterile dilution water. CAUTION: Prepoured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not

have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4°C for longer than l month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.

G. **Counting grid**

1. **Description.** Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by 4 arcs labeled l, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between 2 arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. Spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward center of plate.

2. **Calibration.** The volume of sample represented by various parts of the counting grid is shown in operator's manual that accompanies spiral plater. Grid area constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of 10^6-10^3 cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all Incubate both sets of plates for 48 ± 3 h at 35 ± 1°C. Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described in H, below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

$$\text{Volume (ml) for grid area} = \frac{\text{Spiral Colonies counted in area}}{\text{Baterial count/ml (APC)}}$$

$$\text{Volume (ml)} = \frac{31 + 30 \text{ colonies}}{4.1 \times 10^4 \text{ bateria/ml}} = 0.0015 \text{ ml}$$

To check total volume dispensed by spiral plater, weigh amount dispensed from stylus tip. Collect in tared 5 ml plastic beaker and weigh on analytical balance (± 0.2 mg).

 Fig. 1 10 cm plate

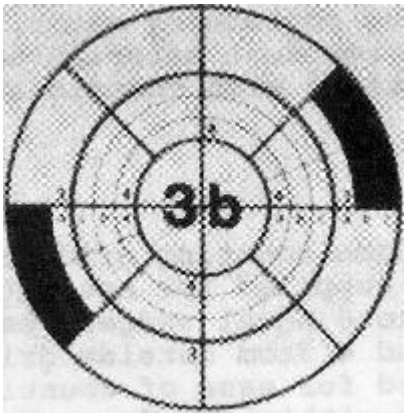

Figure 1. 10 cm plate, area (3b)

$$\frac{(31+31)\ \text{colonies}}{0.0015\,\text{ml}} = 4.1\times10^{4}$$

H. **Examination and reporting of spiral plate counts.**

Counting rule of 20. After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. In this counting procedure, numbers such as 3b, 4c (Fig. l) refer to area segments from outer edge of wedge to designated arc line. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Example of spirally inoculated plate (Fig. l) demonstrates method for determining microbial count. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted. See example under Fig. l.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas.

Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described in I-D, above. Report counts as SPLC or estimated SPLC per ml.

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AOAC Official Method 2015.01
Heavy Metals in Food
Inductively Coupled Plasma–Mass Spectrometry
First Action 2015

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

A. Principle

Food samples are thoroughly homogenized and then prepared by microwave digestion and the addition of dilute solutions of gold (Au) and lutetium (Lu). The Au is used to stabilize the Hg in the preparation, and the Lu is used to assess the potential loss of analyte during the microwave digestion process.

A prepared, diluted, aqueous sample digestate is pumped through a nebulizer, where the liquid forms an aerosol as it enters a spray chamber. The aerosol separates into a fine aerosol mist and larger aerosol droplets. The larger droplets exit the spray chamber while the fine mist is transported into the ICP torch.

Inside the ICP torch, the aerosol mist is transported into a high-temperature plasma, where it becomes atomized and ionized as it passes through an RF load coil. The ion stream is then focused by a single ion lens through a cylinder with a carefully controlled electrical field. For instruments equipped with dynamic reaction cell (DRC) or collision cell IRT, the focused ion stream is directed into the reaction/collision cell where, when operating with a pressurized cell, the ion beam will undergo chemical modifications and/or collisions to reduce elemental interferences. When not operating with a pressurized cell, the ion stream will remain focused as it passes through the cell with no chemical modification taking place.

The ion stream is then transported to the quadrupole mass filter, where only ions having a desired mass-to-charge ratio (m/z) are passed through at any moment in time. The ions exiting the mass filter are detected by a solid-state detector and the signal is processed by the data handling system.

B. Equipment

Perform routine preventative maintenance for the equipment used in this procedure.

An ultra-clean laboratory environment is critical for the successful production of quality data at ultra-low levels. All sample preparation must take place in a clean hood (Class 100). Metallic materials should be kept to a minimum in the laboratory and coated with an acrylic polymer gel where possible. Adhesive floor mats should be used at entrances to the laboratory and changed regularly to prevent the introduction of dust and dirt from the outside environment. Wear clean-room gloves and change whenever contact is made with anything non-ultra-clean. The laboratory floor should be wiped regularly to remove any particles without stirring up dust. *Note:* "Ultra-clean" (tested to be low in the analytes of interest) reagents, laboratory supplies, facilities, and sample handling techniques are required to minimize contamination in order to achieve the trace-level detection limits described herein.

(**a**) *Instrumentation*.—ICP-MS instrument, equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. The quadrupole mass spectrometer has a mass range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system achieves 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). *Note*: The procedure is written specifically for use with a PerkinElmer ELAN DRC II ICP-MS (www.perkinelmer.com). Equivalent procedures may be performed on any type of ICP-MS instrument with equivalent IRT if the analyst is fully trained in the interpretation of spectral and matrix interferences and procedures for their correction, including the optimization of IRT. For example, collision cell IRT can be used for arsenic determination using helium gas.

(**b**) *Gases*.—High-purity grade liquid argon (>99.996%). Additional gases are required for IRT (such as ultra-x grade, 99.9999% minimum purity oxygen, used for determination of As in DRC mode with some PerkinElmer ICP-MS instruments).

(**c**) *Analytical balance*.—Standard laboratory balance suitable for sample preparation and capable of measuring to 0.1 mg.

(**d**) *Clean-room gloves*.—Tested and certified to be low in the metals of interest.

(**e**) *Microwave digestion system*.—Laboratory microwave digestion system with temperature control and an adequate supply of chemically inert digestion vessels. The microwave should be appropriately vented and corrosion resistant.

(*1*) The microwave digestion system must sense the temperature to within ±2.5°C and automatically adjust the microwave field output power within 2 s of sensing. Temperature sensors should be accurate to ±2°C (including the final reaction temperature of 190°C). Temperature feedback control provides the primary control performance mechanism for the method.

(*2*) The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested food or beverage samples. These tests may require additional vessel requirements, such as increased pressure capabilities.

(**f**) *Autosampler cups*.—15 and 50 mL; vials are precleaned by soaking in 2–5% (v/v) HNO_3 overnight, rinsed three times with reagent water/deionized water (DIW), and dried in a laminar flow clean hood. For the 50 mL vials, as these are used to prepare standards and bring sample preparations to final volume, the bias and precision of the vials must be assessed and documented prior to use. The recommended procedure for this is as follows:

(*1*) For every case of vials from the same lot, remove 10 vials.

(*2*) Tare each vial on an analytical balance, and then add reagent water up to the 20 mL mark. Repeat procedure by adding reagent water up to the 50 mL mark.

(*3*) Measure and record the mass of reagent water added, and then calculate the mean and RSD of the 10 replicates at each volume.

(*4*) To evaluate bias, the mean of the measurements must be with ±3% of the nominal volume. To evaluate precision, the RSD of the measurements must be ≤3% using the stated value (20 or 50 mL) in place of the mean.

(**g**) *Spatulas*.—To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO_3 prior to use.

C. Reagents and Standards

Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use.

(**a**) *DIW*.—ASTM Type I; demonstrated to be free from the metals of interest and potentially interfering substances.

(**b**) *Nitric acid (HNO_3)*.—Concentrated; tested and certified to be low in the metals of interest.

(c) *Hydrogen peroxide (H_2O_2)*.—Optima grade or equivalent, 30–32% assay.

(**d**) *Stock standard solutions*.—Obtained from a reputable and professional commercial source.

(*1*) *Single-element standards*.—Obtained for each determined metal, as well as for any metals used as internal standards and interference checks.

(*2*) *Second source standard*.—Independent from the single-element standard; obtained for each determined metal.

(*3*) *Multi-element stock standard solution*.—Elements must be compatible and stable in solutions together. Stability is determined by the vendor; concentrations are then verified before use of the standard.

(**e**) *Internal standard solution*.—For analysis of As, Cd, Pb, and Hg in food matrices, an internal standard solution of 40 µg/L rhodium (Rh), indium (In), and thulium (Tm) is recommended. Rh is analyzed in DRC mode for correction of the As signal. In addition, the presence of high levels of elements, such as carbon and chlorine, in samples can increase the effective ionization of the plasma and cause a higher response factor for arsenic in specific samples. This potential interference is addressed by the on-line addition of acetic acid (or another carbon source, such as methanol), which greatly increases the effective ionization of incompletely ionized analytes, and decreases the potential increase caused by sample characteristics. The internal standard solution should be prepared in 20% acetic acid.

Table 2015.01A. Recommended concentrations for the calibration curve

Standard	As, µg/L	Cd, µg/L	Pb, µg/L	Hg, µg/L
0	0.00	0.00	0.000	0.00
1	0.01	0.01	0.005	0.01
2	0.02	0.02	0.010	0.05
3	0.10	0.10	0.050	0.10
4	0.50	0.50	0.250	0.50
5	5.00	5.00	2.500	2.00
6	20.00	20.00	10.000	5.00

(**f**) *Calibration standards*.—Fresh calibration standards should be prepared every day, or as needed.

(*1*) Dilute the multi-element stock standard solutions into 50 mL precleaned autosampler vials with 5% HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of quantitation (LOQ) when recalculated in units specific to the reported sample results.

(*2*) *See* Table **2015.01A** for recommended concentrations for the calibration curve.

(**g**) *Initial calibration verification (ICV) solution*.—Made up from second source standards in order to verify the validity of the calibration curve.

(**h**) *Calibration solutions*.—Daily optimization, tuning, and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer's suggestions.

(**i**) *Certified Reference Materials (CRMs)*.—CRMs should preferably match the food matrix type being analyzed and contain the elements of interest at certified concentrations above the LOQ. Recommended reference materials include NIST SRM 1568a (Rice Flour), NIST SRM 1548a (Typical Diet), NRCC CRM DORM-3 (Dogfish Muscle), and NIST SRM 2976 (Mussel Tissue).

(**j**) *Spiking solution*.—50 mg/L Au and Lu in 5% (v/v) HNO_3. Prepared from single-element standards.

D. Contamination and Interferences

(**a**) Well-homogenized samples and small reproducible aliquots help minimize interferences.

(**b**) *Contamination*.—(*1*) Contamination of the samples during sample handling is a great risk. Extreme care should be taken to avoid this. Potential sources of contamination during sample handling include using metallic or metal-containing homogenization equipment, laboratory ware, containers, and sampling equipment.

(*2*) Contamination of samples by airborne particulate matter is a concern. Sample containers must remain closed as much as possible. Container lids should only be removed briefly and in a

clean environment during sample preservation and processing, so that exposure to an uncontrolled environment is minimized.

(**c**) *Laboratory.*—(*1*) All laboratory ware (including pipet tips, ICP-MS autosampler vials, sample containers, extraction apparatus, and reagent bottles) should be tested for the presence of the metals of interest. If necessary, the laboratory ware should be acid-cleaned, rinsed with DIW, and dried in a Class 100 laminar flow clean hood.

(*2*) All autosampler vials should be cleaned by storing them in 2% (v/v) HNO_3 overnight and then rinsed three times with DIW. Then dry vials in a clean hood before use. Glass volumetric flasks should be soaked in about 5% HNO_3 overnight prior to use.

(*3*) All reagents used for analysis and sample preparation should be tested for the presence of the metals of interest prior to use in the laboratory. Due to the ultra-low detection limits of the method, it is imperative that all the reagents and gases be as low as possible in the metals of interest. It is often required to test several different sources of reagents until an acceptable source has been found. Metals contamination can vary greatly from lot to lot, even when ordering from the same manufacturer.

(*4*) Keep the facility free from all sources of contamination for the metals of interest. Replace laminar flow clean hood HEPA filters with new filters on a regular basis, typically once a year, to reduce airborne contaminants. Metal corrosion of any part of the facility should be addressed and replaced. Every piece of apparatus that is directly or indirectly used in the processing of samples should be free from contamination for the metals of interest.

(**d**) *Elemental interferences*.—Interference sources that may inhibit the accurate collection of ICP-MS data for trace elements are addressed below.

(*1*) *Isobaric elemental interferences*.—Isotopes of different elements that form singly or doubly charged ions of the same *m/z* and cannot be resolved by the mass spectrometer. Data obtained with isobaric overlap must be corrected for that interference.

(*2*) *Abundance sensitivity*.—Occurs when part of an elemental peak overlaps an adjacent peak. This often occurs when measuring a small *m/z* peak next to a large *m/z* peak. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Proper optimization of the resolution during tuning will minimize the potential for abundance sensitivity interferences.

(*3*) *Isobaric polyatomic interferences.*—Caused by ions, composed of multiple atoms, which have the same *m/z* as the isotope of interest, and which cannot be resolved by the mass spectrometer. These ions are commonly formed in the plasma or the interface system from the support gases or sample components. The objective of IRT is to remove these interferences, making the use of correction factors unnecessary when analyzing an element in DRC mode. Elements not determined in DRC mode can be corrected by using correction equations in the ICP-MS software.

(**e**) *Physical interferences.*—(*1*) Physical interferences occur when there are differences in the response of the instrument from the calibration standards and the samples. Physical interferences are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma-mass spectrometer interface.

(*2*) Physical interferences can be associated with the transfer of solution to the nebulizer at the point of nebulization, transport of aerosol to the plasma, or during excitation and ionization processes in the plasma. High levels of dissolved solids in a sample can result in physical interferences. Proper internal standardization (choosing internal standards that have analytical behavior similar to the associating elements) can compensate for many physical interferences.

Table 2015.01B. Recommended isotopes for analysis

Element	Isotope, amu	Isotopic abundance, %	Potential interferences
Cd	111	13	MoO^+
	114	29	MoO^+, Sn^+
Hg	200	23	WO^+
	202	30	WO^+
Pb[a]	Sum of 206, 207, and 208	99	OsO^+

[a] Allowance for isotopic variability of lead isotopes.

(**f**) *Resolution of interferences.*—(*1*) For elements that are subject to isobaric or polyatomic interferences (such as As), it is advantageous to use the DRC mode of the instrument. This section specifically describes a method of using IRT for interference removal for As using a PerkinElmer DRC II and oxygen as the reaction gas. Other forms of IRT may also be appropriate.

(*a*) Arsenic, which is monoisotopic, has an *m/z* of 75 and is prone to interferences from many sources, most notably from chloride (Cl), which is common in many foods (e.g., salt). Argon (Ar), used in the ICP-MS plasma, forms a polyatomic interference with Cl at *m/z* 75 [$^{35}Cl + {}^{40}Ar = {}^{75}(ArCl)$].

(*b*) When arsenic reacts with the oxygen in the DRC cell, $^{75}As^{16}O$ is formed and measured at *m/z* 91, which is free of most interferences. The potential ^{91}Zr interference is monitored for in the following ways: ^{90}Zr and ^{94}Zr are monitored for in each analytical run, and if a significant Zr presence is detected, then $^{75}As^{16}O$ measured at *m/z* 91 is evaluated against the ^{75}As result. If a significant discrepancy is present, then samples may require analysis using alternative IRT, such as collision cell technology (helium mode).

(*c*) Instrument settings used (for PerkinElmer DRC II): DRC settings for $^{91}(AsO)$ and ^{103}Rh include an RPq value of 0.7 and a cell gas flow rate of 0.6 L/min. Cell conditions, especially cell gas flow rates, may be optimized for specific analyte/matrix combinations, as needed. In such cases, the optimized methods will often have slightly different RPq and cell gas flow values.

(*2*) For multi-isotopic elements, more than one isotope should be measured to monitor for potential interferences. For reporting purposes, the most appropriate isotope should be selected based on review of data for matrix interferences and based on the sensitivity (or relative abundance) of each isotope. The table below lists the recommended isotopes to measure. Low abundance isotopes are not recommended for this method as it is specifically applicable for ultra-low level concentrations (8–10 ppb LOQs). *See* Table **2015.01B**.

(**g**) *Memory effects*.—Minimize carryover of elements in a previous sample in the sample tubing, cones, torch, spray chamber, connections, and autosampler probe by rinsing the instrument with a reagent blank after samples high in metals concentrations are analyzed. Memory effects for Hg can be minimized through the addition of Au to all standard, samples, and quality control (QC) samples.

Table 2015.01C. Digestion program for Berghof Speedwave 4 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	145	1	1
2	50	1	1
3	145	1	1
4	170	1	10
5	190	1	10

E. Sample Handling and Storage

(**a**) Food and beverage samples should be stored in their typical commercial storage conditions (either frozen, refrigerated, or at room temperature) until analysis. Samples should be analyzed within 6 months of preparation.

(**b**) If food or beverage samples are subsampled from their original storage containers, ensure that containers are free from contamination for the elements of concern.

F. Sample Preparation

(**a**) Weigh out sample aliquots (typically 0.25 g of as-received or wet sample) into microwave digestion vessels.

(**b**) Add 4 mL of concentrated HNO_3 and 1 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

(**c**) Add 0.1 mL of the 50 mg/L Au + Lu solution to each digestion vessel.

(**d**) Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave system according to the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

(**e**) Samples are digested at a minimum temperature of 190°C for a minimum time of 10 min. Appropriate ramp times and cool down times should be included in the microwave program, depending on the sample type and model of microwave digestion system. Microwave digestion is achieved using temperature feedback control. Microwave digestion programs will vary depending on the type of microwave digestion system used. When using this mechanism for achieving performance-based digestion targets, the number of samples that may be simultaneously digested may vary. The number will depend on the power of the unit, the number of vessels, and the heat loss characteristics of the vessels. It is essential to ensure that all vessels reach at least 190°C and be held at this temperature for at least 10 min. The monitoring of one vessel as a control for the batch/carousel may not accurately reflect the temperature in the other vessels, especially if the samples vary in composition and/or sample mass. Temperature measurement and control will depend on the particular microwave digestion system.

(*1*) *Note*: a predigestion scheme for samples that react vigorously to the addition of the acid may be required.

(*2*) The method performance data presented in this method was produced using a Berghof Speedwave 4 microwave digestion system, with the program listed in Table **2015.01C** (steps 1 and 2 are a predigestion step).

Table 2015.01D. Digestion program for CEM MARS 6 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	190	20	10
2	Cool down	NA	10

(*3*) Equivalent results were achieved using the program listed in Table **2015.01D** on a CEM MARS 6 microwave digestion system using the 40-position carousel and 55 mL Xpress digestion vessels.

(*4*) For infant formula samples, the program described in Table **2015.01E** has been shown to work effectively.

Table 2015.01E. Digestion program for infant formula

Step	Temp., °C	Ramp, min	Hold, min
1	180	20	20
2	Cool down	NA	20
3	200	20	20
4	Cool down	NA	20

(**f**) Allow vessels to cool to room temperature and slowly open. Open the vessels carefully, as residual pressure may remain and digestate spray is possible. Pour the contents of each vessel into an acid-cleaned 50 mL HDPE centrifuge tube and dilute with DIW to a final volume of 20 mL.

(**g**) Digestates are diluted at least 4x prior to analysis with the 1% (v/v) HNO_3 diluent. When the metals concentration of a sample is unknown, the samples may be further diluted or analyzed using a total quantification method prior to being analyzed with a comprehensive quantitative method. This protects the instrument and the sample introduction system from potential contamination and damage.

(**h**) Food samples high in calcium carbonate ($CaCO_3$) will not fully digest. In such cases, the CRM can be used as a gauge for an appropriate digestion time.

(**i**) QC samples to be prepared with the batch (a group of samples and QC samples that are prepared together) include a minimum of three method blanks, duplicate for every 10 samples, matrix spike/matrix spike duplicate (MS/MSD) for every 10 samples, blank spike, and any matrix-relevant CRMs that are available.

G. Procedure

(**a**) *Instrument startup*.—(*1*) Instrument startup routine and initial checks should be performed per manufacturer recommendations.

(*2*) Ignite the plasma and start the peristaltic pump. Allow plasma and system to stabilize for at least 30 min.

(**b**) *Optimizations*.—(*1*) Perform an optimization of the sample introduction system (e.g., *X*-*Y* and *Z* optimizations) to ensure maximum sensitivity.

(*2*) Perform an instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements, or monthly (at a minimum), to ensure the instrument's quadrupole mass filtering performance is adequate. Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications.

(*3*) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios.

(*4*) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. If the performance check is not satisfactory, additional optimizations (a "full optimization") may be necessary.

Table 2015.01F. Summary of quality control samples

QC sample	Measure	Minimum frequency	Acceptance criteria	Corrective action
Calibration standards	Linearity of the calibration curve	Analyzed once per analytical day	Correlation coefficient ≥0.995, 1st standard ≤MRL, low standard recovery = 75–125%, all other standard recoveries = 80–120%	Reanalyze suspect calibration standard. If criteria still not met, then re-prepare standards and recalibrate the instrument.
Internal standards	Variation in sample properties between samples and standards	Each standard, blank, and sample is spiked with internal standard	60–125% recovery compared to calibration blank	If the responses of the internal standards in the following CCB are within the limit, rerun the sample at an additional 2x dilution. If not, then samples must be reanalyzed with a new calibration.
Lu digestion check spike	Assessment of potential loss during digestion	Added to every digested samples	Recovery ≥75%	Re-prepare the sample
Initial calibration verification (ICV)	Independent check of system performance	One following instrument calibration	Recovery = 90–110%	Correct problem prior to continuing analysis. Recalibrate if necessary.
Continuing calibration verification (CCV)	Accuracy	At beginning and end of analysis and one per 10 injections	Recovery = 85–115%	Halt analysis, correct problem, recalibrate, and reanalyze affected samples
Method blanks (MB)	Contamination from reagents, lab ware, etc.	Minimum of three per batch	Mean ≤ MRL; SD ≤ MDL or MBs <1/10th sample result	Determine and eliminate cause of contamination. Affected samples must be re-prepared and reanalyzed.
Method duplicates (MD)	Method precision within a given matrix	Minimum of one per 10 samples	RPD ≤ 30% or ±2x LOQ if results ≤5x LOQ	If RPD criteria not met, then sample may be re-prepared and reanalyzed, but this is not required. Sample matrix may be inhomogeneous. A post-digestion duplicate (PDD) can be analyzed to evaluate instrument precision.
Matrix spikes/matrix spike duplicates (MS/MSD)	Method accuracy and precision within a given matrix	Minimum of one per 10 samples	Recovery = 70–130% and RPD ≤ 30%	If RPD >30%, results must be qualified
Post-preparation spike (PS)	Check for matrix interference	When required (samples spiked too low/high, dilution test fails, etc.)	Recovery = 75–125%	Analyze samples using MSA or results flagged accordingly
Laboratory fortified blank (LFB) or blank spike (BS)	Method accuracy	Minimum of one per batch	Recovery = 75–125%	If LFB recovery is outside of the control limit, then batch must be re-prepared and reanalyzed
Certified Reference Material (CRM)	Method accuracy	Must be matrix-matched to samples; minimum of one per batch	Recovery = 75–125% unless limits set by CRM manufacturer are greater or element/CRM specific limits have been established	If CRM true value is ≥5x the LOQ and recovery is outside of the control limit, then batch must be re-prepared and reanalyzed

(**c**) *Internal standardization and calibration*.—(*1*) Following precalibration optimizations, prepare and analyze the calibration standards prepared as described in **C**(**e**).

(*2*) Use internal standardization in all analyses to correct for instrument drift and physical interferences. Refer to **D**(**e**)(*2*). Internal standards must be present in all samples, standards, and blanks at identical concentrations. Internal standards can be added using a second channel of the peristaltic pump to produce a responses that is clear of the pulse-to-analog detector interface.

(*3*) Multiple isotopes for some analytes may be measured, with only the most appropriate isotope (as determined by the analyst) being reported.

(*4*) Use IRT for the quantification of As using the Rh internal standard.

(**d**) *Sample analysis*.—(*1*) Create a method file for the ICP-MS.

(*2*) Enter sample and calibration curve information into the ICP-MS software.

(*3*) Calibrate the instrument and ensure the resulting standard recoveries and correlation coefficients meet specifications (**H**).

(*4*) Start the analysis of the samples.

(*5*) Immediately following the calibration, an initial calibration blank (ICB) should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination.

(*6*) Immediately following the ICB, an ICV should be analyzed. This standard must be prepared from a different source than the calibration standards.

(*7*) A minimum of three reagent/instrument blanks should be analyzed following the ICV. These instrument blanks can be used to assess the background and variability of the system.

(*8*) A continuing calibration verification (CCV) standard should be analyzed after every 10 injections and at the end of the run. The CCV standard should be a mid-range calibration standard.

(*9*) An instrument blank should be analyzed after each CCV (called a continuing calibration blank, or CCB) to demonstrate that there is no carryover and that the analytical system is free from contamination.

(*10*) Method of Standard Additions (MSA) calibration curves may be used any time matrix interferences are suspected.

(*11*) Post-preparation spikes (PS) should be prepared and analyzed whenever there is an issue with the MS recoveries.

(**e**) Export and process instrument data.

H. Quality Control

(**a**) The correlation coefficients of the weighted-linear calibration curves for each element must be ≥0.995 to proceed with sample analysis.

(**b**) The percent recovery of the ICV standard should be 90–110% for each element being determined.

(**c**) Perform instrument rinses after any samples suspected to be high in metals, and before any method blanks, to ensure baseline sensitivity has been achieved. Run these rinses between all samples in the batch to ensure a consistent sampling method.

(**d**) Each analytical or digestion batch must have at least three preparation (or method) blanks associated with it if method blank correction is to be performed. The blanks are treated the same as the samples and must go through all of the preparative steps. If method blank correction is being used, all of the samples in the batch should be corrected using the mean concentration of these blanks. The estimated method detection limit (EMDL) for the batch is equal to 3 times the standard deviation (SD) of these blanks.

(**e**) For every 10 samples (not including quality control samples), a matrix duplicate (MD) sample should be analyzed. This is a duplicate of a sample that is subject to all of the same preparation and analysis steps as the original sample. Generally, the relative percent difference (RPD) for the replicate should be ≤30% for all food samples if the sample concentrations are greater than 5 times the LOQ. RPD is calculated as shown below. An MSD may be substituted for the MD, with the same control limits.

$$RPD = 200 \times \frac{|S1 - S2|}{S1 + S2}$$

where S1 = concentration in the first sample and S2 = concentration in the duplicate.

(**f**) For every 10 samples (not including quality control samples), an MS and MSD should be performed. The percent recovery of the spikes should be 70–130% with an RPD ≤30% for all food samples.

(*1*) If the spike recovery is outside of the control limits, an MSA curve that has been prepared and analyzed may be used to correct for the matrix effect. Samples may be corrected by the slope of the MSA curve if the correlation coefficient of the MSA curve is ≥0.995.

(*a*) The MSA technique involves adding known amounts of standard to one or more aliquots of the processed sample solution. This technique attempts to compensate for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interferences which cause a baseline shift.

(*b*) The best MSA results can be obtained by using a series of standard additions. To equal volumes of the sample are added a series of standard solutions containing different known quantities of the analyte(s), and all solutions are diluted to the same final volume. For example, addition 1 should be prepared so that the resulting concentration is approximately 50% of the expected concentration of the native sample. Additions 2 and 3 should be prepared so that the concentrations are approximately 100% and 150%, respectively, of the expected native sample concentration. Determine the concentration of each solution and then plot on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated to zero absorbance, the point of interception of the abscissa is calculated MSA-corrected concentration of the analyte in the sample. A linear regression program may be used to obtain the intercept concentration.

(*c*) For results of the MSA technique to be valid, take into consideration the following limitations:

(*i*) The apparent concentrations from the calibration curve must be linear (0.995 or greater) over the concentration range of concern.

(*ii*) The effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes, and the MSA curve should respond in a similar manner as the analyte.

(*2*) If the sample concentration levels are sufficiently high, the sample may be diluted to reduce the matrix effect. Samples should be diluted with the 1% (v/v) HNO_3 diluent. For example, to dilute a sample by a 10x dilution factor, pipette 1 mL of the digested sample into an autosampler vial, and add 9 mL of the 1% (v/v) HNO_3 diluent. MS/MSD sets should be performed at the same dilution factor as the native sample.

(*3*) Spike at 1–10 times the level of a historical sample of the same matrix type, or, if unknown, spike at 1–5 times a typical value for the matrix. Spiking levels should be no lower than 10 times the LOQ.

Table 2015.01G. Method blank results and LOD/LOQ, µg/kg

Method blanks	^{91}(AsO)	^{111}Cd	^{114}Cd	Pb	^{200}Hg	^{202}Hg
MB-01	2.83	0.229	0.270	1.90	1.61	0.95
MB-02	1.48	–0.088	0.270	0.14	1.48	1.13
MB-03	1.80	0.007	0.115	0.13	0.76	0.25
MB-04	1.03	0.154	0.288	0.12	1.46	0.33
MB-05	1.43	0.010	0.259	1.84	1.28	0.27
MB-06	1.07	0.105	0.096	3.02	0.87	0.76
MB-07	2.31	–0.002	0.297	2.67	0.89	0.44
MB-08	1.20	0.285	0.200	4.24	0.55	0.28
MB-09	1.05	0.002	0.182	0.09	0.96	0.25
MB-10	2.12	0.047	0.150	0.19	0.71	0.02
MB-11	2.09	–0.145	0.226	0.12	0.64	0.57
MB-12	1.44	0.037	0.165	0.18	0.45	0.50
MB-13	0.70	–0.122	0.160	0.17	0.81	0.19
MB-14	1.12	–0.001	0.074	0.14	0.85	0.21
MB-15	2.33	0.097	0.207	0.11	0.18	0.17
MB-16	1.53	–0.117	0.146	0.16	1.33	1.09
MB-17	1.79	–0.070	0.180	0.03	3.46	2.19
MB-18	1.90	0.049	0.115	0.06	3.30	2.36
MB-19	1.18	0.043	0.224	0.39	4.01	2.78
MB-20	1.24	–0.060	0.199	0.07	0.99	0.56
MB-21	0.92	0.165	0.120	0.03	0.73	0.33
MB-22	1.69	0.005	0.186	0.09	0.60	0.25
MB-23	2.13	0.171	0.152	0.08	0.41	–0.23
SD	0.54	0.113	0.063	1.18	1.01	0.77
LOD	1.6	0.50[a]	0.50[a]	3.5	3.0	2.3
LOQ	3.3	1.60[a]	1.60[a]	7.1	6.0	4.6

[a] Adjusted to conform to lowest calibration point.

(**g**) Percent recoveries of the CRMs should be 75–125% of their certified value.

(**h**) Percent recoveries of the CCV standards should be within 85–115%. Sample results may be CCV-corrected using the mean recovery of the bracketing CCVs. This should only be done after careful evaluation of the data. The instrument should show a trending drift of CCV recoveries and not just a few anomalous outliers.

(**i**) CCBs should be monitored for the effects of carryover and for possible system contamination. If carryover of the analyte at levels greater than 10 times the MDL is observed, the sample results may not be reportable.

(**j**) Absolute response of any one internal standard should not vary from the original response in the calibration blank by more than 60–125%. Some analytical samples, such as those containing concentrations of the internal standard and tissue digestates, can have a serious effect on the internal standard intensities, but this does not necessarily mean that the analytical system is out of control. In some situations, it is appropriate to reprocess the samples using a different internal standard monitored in the analysis. The data should be carefully evaluated before doing this.

Table 2015.01H. Sample-specific LOQs

Sample	LOQ, µg/kg (as received)			
	As	Cd	Pb	Hg
Infant formula	2	1	4	3
Chocolate	4	2	8	6
Rice flour	4	2	8	6
Fruit juice	1	1	2	2

(**k**) The recovery of the Lu that was spiked into the sample preparation prior to digestion should be evaluated to assess any potential loss of analyte during the process. The concentration of Lu in the sample preparation is 0.25 mg/L, and for samples diluted 4x at the instrument, this is equivalent to 62.5 µg/L at the instrument (if samples are diluted more than 4x, this must be taken into account). The Lu recovery should be no less than 75% of the original spiked concentration.

(**l**) Refer to Table **2015.01F** for a summary of all recommended quality control samples, minimum frequency at which they are to be analyzed, acceptance criteria for each, and appropriate corrective action if the acceptance criteria are not met.

I. Method Performance

(**a**) Limit of detection (LOD) and LOQ were determined through the analysis of 23 method blanks (*see* Table **2015.01G**). LOD was calculated as 3 times the SD of the results of the blanks, and LOQ was calculated as 2 times the value of the LOD, except where the resulting LOQ would be less than the lowest calibration point, in which case LOQ was elevated and set at the lowest calibration point and LOD was calculated as 1/3 of the LOQ. All LOQs achieved are ≤10 µg/kg for all food matrices and ≤8 µg/kg for liquid matrices, such as infant formula.

(**b**) Sample-specific LOQs for several matrices, based on LOQs determined by the default method, and adjusted for changes in sample mass for particular samples, are shown in Table **2015.01H**. Values have been rounded up to the nearest part-per-billion.

(**c**) Numerous relevant CRMs were analyzed to establish method accuracy. Example percent recoveries are provided in Table **2015.01I** (recoveries have been omitted for CRMs that do not provide a certified value or if the certified value is less than the LOQ).

Table 2015.01I. Recoveries for numerous relevant CRMs

Certified Reference Material	As, %	Cd, %	Pb, %	Hg, %
DOLT-4 Dogfish Liver	104	97	87	114
DORM-3 Fish Protein	105	109	94	114
DORM-4 Fish Protein	105	91	91	81
NIST 1548a Typical Diet	103	95	113	NA
NIST 1568a Rice Flour	98	99	NA	NA
NIST 1946 Lake Superior Fish Tissue	119	NA	NA	101
TORT-2 Lobster Hepatopancreas	109	104	95	116
TORT-3 Lobster Hepatopancreas	113	89	86	86

Table 2015.01J. AOAC SMPR 2012.007 (ref. 1)

Concn range, µg/kg	Repeatability, %	Reproducibility, %	Recovery, %
LOQ–100	15	32	60–115
100–1000	11	16	80–115
>1000	7.3	8	80–115

(**d**) *Standard Method Performance Requirements* (AOAC SMPR® 2012.007; 1) for repeatability, reproducibility, and recovery for the method are shown in the Table **2015.01J**. *See* Appendix A (available on the *J. AOAC Int*. website as supplemental material, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac) for detailed method performance information supporting acceptance of the method.

(**e**) *See* Appendix A for detailed method performance information supporting acceptance of the method. Method validation samples were prepared and analyzed for all applicable matrices. In general, all SMPR criteria were met for As, Cd, Hg, and Pb in the matrices apple juice, infant formula, cocoa powder, and rice flour.

References: (1) AOAC SMPR 2012.007
J. AOAC Int. **96**, 704(2013)
DOI: 10.5740/jaoac.int.2012.007

J. AOAC Int. **98**, 1113(2015)
DOI: 10.5740/jaoac.int.2015.01

Posted: September 9, 2015

Method: **Determination of Heavy Metals by ICP-MS**

Reference: **AOAC Method 2015.01**

Approved: (b) (4)

Date: 4/25/19

1. Purpose

This method is to describe the steps for preparation of samples and standards to perform quantitative determination of metal impurities by microwave digestion and analysis by ICP-MS.

2. Scope

This method is applicable for the detection of metal impurities by ICP-MS. This method is suitable for a range of elements to be quantified; however, the elements of primary concern are arsenic, cadmium, lead and mercury.

3. Background

This method should be used by analysts familiar with trace element analysis and ICP-MS.

4. Responsibilities

4.1 Laboratory Co-Director authorized to assign and approve subject analysis is responsible for

- Approving Method Folder content
- Assuring the sample is fit for use
- Resolving analytical issues and deficiencies with subject analysis

4.2 Section Supervisor authorized to conduct subject analysis is responsible for

- Approving assigned analyst work
- Assuring the Method Folder is up to date including content and appendices
- Discussing any deviations with the Laboratory Co-Director

4.3 Analyst authorized to conduct this analysis is responsible for

- Reviewing Method Folder instructions prior to initiating analysis, especially for matrix applicability
- Analyzing the sample according to documented instructions
- Assessing method and instrument performance both real time and at reporting
- Addressing any deviation from instructions or specifications with the Section Supervisor
- Updating Method Folder performance data

5.0 References

5.1 Method

- AOAC INTERNATIONAL. Official Methods of Analysis, 20th ed., Method 2015.01 – Heavy Metals in Food – Inductively Coupled Plasma-Mass Spectrometry.
- FDA EAM (Elemental Analysis Manual) 4.7 Vesrion 1.1 (March 2015), P. Gray, W. Midak, J. Cheng – “Inductively Coupled Plasma-Mass Spectrometric Determination of Arsenic,

Cadmium, chromium, Lead, Mercury and Other Elements in Food Using Microwave Assisted Digestion"

- Perkin Elmer – "Determination of Elemental Impurities in Cannabis and Related Materials by Indirect Closed-Vessel Microwave Digestion and ICP-MS Analysis"

5.2 Instrumentation

- Perkin Elmer NexION 1000/2000 ICP-MS

6.0 Method Folder

6.1 Instrumentation

The analyst authorized to perform this test method must be deemed knowledgeable in the operation of the instrumentation cited in **5.2 Instrumentation**

6.2 Safety

This method does not address all safety issues associated with its use. The analyst must establish appropriate safety and health practice prior to initiating analysis. The analyst must be familiar with (b) (4) hazardous waste plan.

Reagents should be regarded as potential health hazards and exposure to these compounds should be limited.

6.3 Definitions

Analytical sample – sample, prepared by the laboratory (by homogenization, grinding, blending, etc.), from which analytical portions (aliquots) are removed for analysis.

Analytical portion – quantity of material removed from the analytical sample.

Analytical solution – solution prepared by decomposing an analytical portion and diluting to volume.

Batch – a group of analytical portions processed in a continuous sequence under relatively stable conditions. Specifically:

- Method is constant
- Instrument and its conditions (i.e. pertinent operating parameters) are constant
- Standardization is constant

Dilution Factor (DF) – factor by which concentration in a diluted solution (e.g. diluted analytical solution) is multiplied to obtain concentration in the initial solution (e.g. analytical solution).

Method Blank (MBK) – solution that is prepared using all reagents and exposed to all laboratory ware, apparatus, equipment, digestion process and analyses in the same manner as if it were an analytical portion being analyzed without the sample. The MBK is analyzed to ensure analytes have not significantly been added to the analytical portion from materials and laboratory environment.

Reagent Blank (RB) – solution that is prepared using the same labware, acids, and dilution as calibration standards, prepare a solution as if it were a calibration standard without added sample.

Reference material (RM) – food related materials developed for analytical quality control, which have reference value concentration for the element of interest.

Independent calibration verification (ICV) – solution of method analytes of known concentration obtained from a source external to the laboratory and different from the source used for instrument standardization. The ICV is used to ensure a valid standardization and to check laboratory performance.

Continuous calibration verification (CCV) – verification of one of the calibration standard points. It is used to verify the calibration accuracy during the analysis of the analytical batch.

Matrix Spike (SP) – analytical portion fortified (spiking) with the analyte before digestion. Measurement of the final concentration of the analyte is made according to the analytical method. The purpose of the spike is to determine if the preparation procedure or sample matrix contribute bias to the results.

Blank Spike (BS) – solution that is spiked with known concentration analytes and prepared using the same labware, acids, dilutions and exposed to the same digestion process as the Method Blank. The purpose is to determine the spiked analyte recoveries to determine the accuracy.

Internal Standards Solution (ISS) – non analyte solution that is added to all calibration standards, quality control and analyzed samples, which uses the isotope ratio to correct for the instrument drift and matrix interferences.

Stock standard solution – a solution containing a high concentration of the analyte purchased from a reputable commercial source. Stock standard solutions are used to prepare standard solutions and other needed analyte solutions.

Intermediate standard solution – a solution containing one or more analytes prepared in the laboratory by diluting an aliquot of stock solution.

Standard solution – a solution prepared from the dilution of stock standard or intermediate standard solutions. Standard solutions are used to standardize instrument response (absorbance) to analyte concentration.

Analytical solution detection limit (ASDL) – an estimate of the lowest concentration of the analyte element in a MBK according to the statistics of hypothesis with a 95% confidence.

Limit of detection (LOD) – an estimate of the element concentration a method can detect in an analytical portion according to the statistics of hypothesis testing with a 95% confidence.

Limit of Quantitation (LOQ) – the minimum concentration of an analyte in a specific matrix that can be reliably quantified while also meeting predefined goals for bias and imprecision.

7.0 Method Work Level Instructions

7.1 Equipment and materials

(a) Analytical Balance – capable of weighing to the nearest 0.001 gram.
(b) Digestion vials – disposable glass tubes
(c) Microwave Digestor – Milestone UltraWave
(d) ICP-MS – Perkin Elmer

7.2 Reagents and Standards

All reagents may contain impurities that may affect the integrity of the analytical results. Due to the high sensitivity of the ICP-MS, high-purity reagents, water, acids, glassware and sample tubes that are suitable for trace metal analysis must be used at all time.

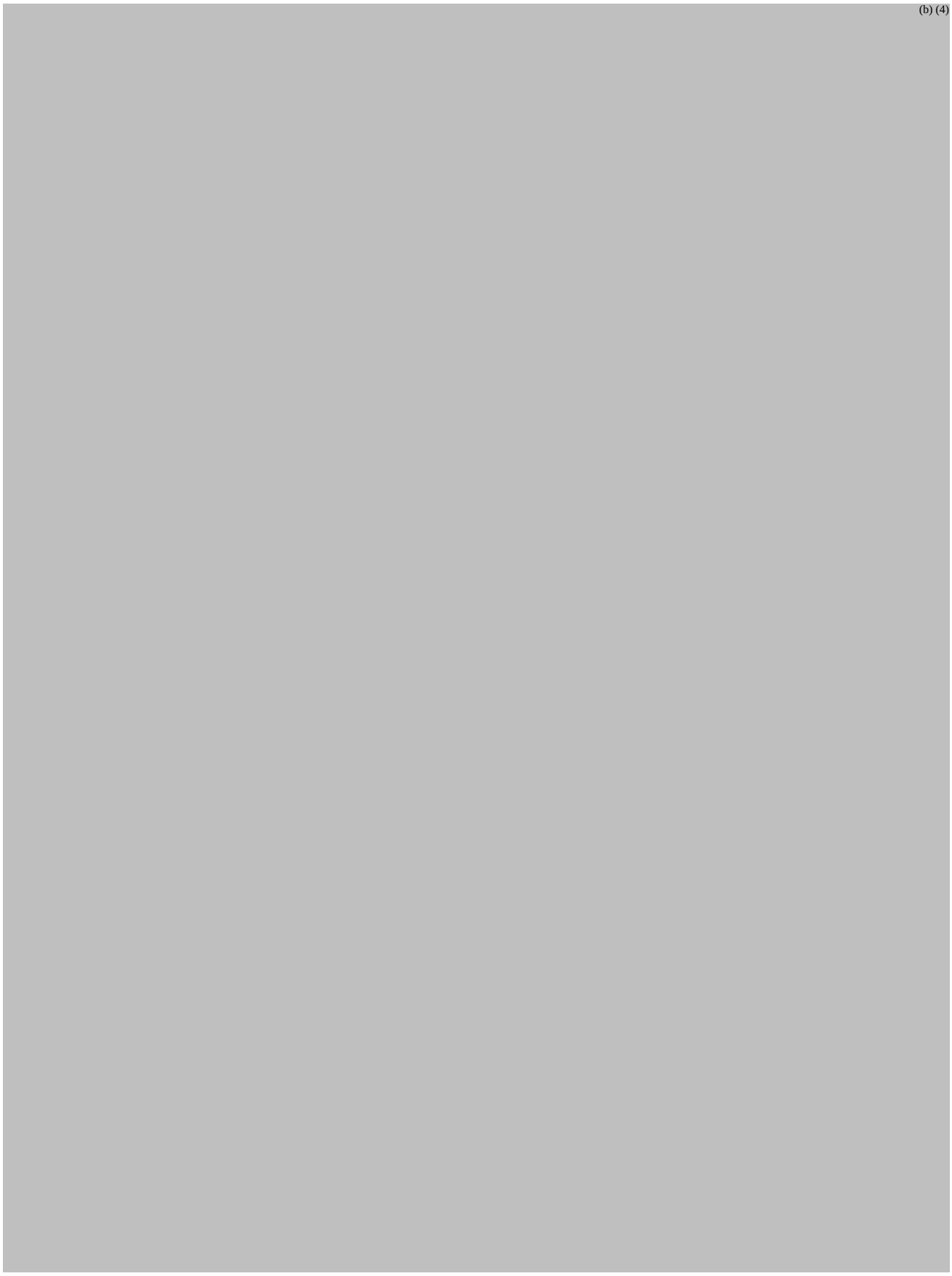
(a) 100 mg/L (ppm) Gold (Au) Stock Standard
(b) 1000 mg/L (ppm) Arsenic (As) Stock Standard
(c) 1000 mg/L (ppm) Cadmium (Cd) Stock Standard
(d) 1000 mg/L (ppm) Lead (Pb) Stock Standard
(e) 1000 mg/L (ppm) Mercury (Hg) Stock Standard
(f) Nitric Acid (HNO_3) – Concentrated (sp gr 1.41), trace metal grade
(g) Hydrochloric Acid (HCl) – Concentrated, trace element grade
(h) Internal Standard Solution – 50 mg/L Germanium (Ge), 20 mg/L Gallium (Ga), 1 mg/L Indium (In), 1 mg/L Terbium (Tb)
(i) Deionized water (DI H_2O)

7.2.1 Working solutions

Please always use safety precautions when preparing solutions. Always add acid to water! Shake each solution after all the reagents are combined.

(b) (4)

(b) (4)



7.3 Test Sample Treatment

Milestone UltraWave microwave is used to digest in order to prepare the analytical batch.

7.3.1 Sample Preparation:

(b) (4)

(b) (4)

7.4 Instrumentation Set up

(b) (4)

7.4.3 Running Samples:

(b) (4)

(b) (4)

(b) (4)

7.4.4 While Running:

(b) (4)

7.4.5 Data Processing:

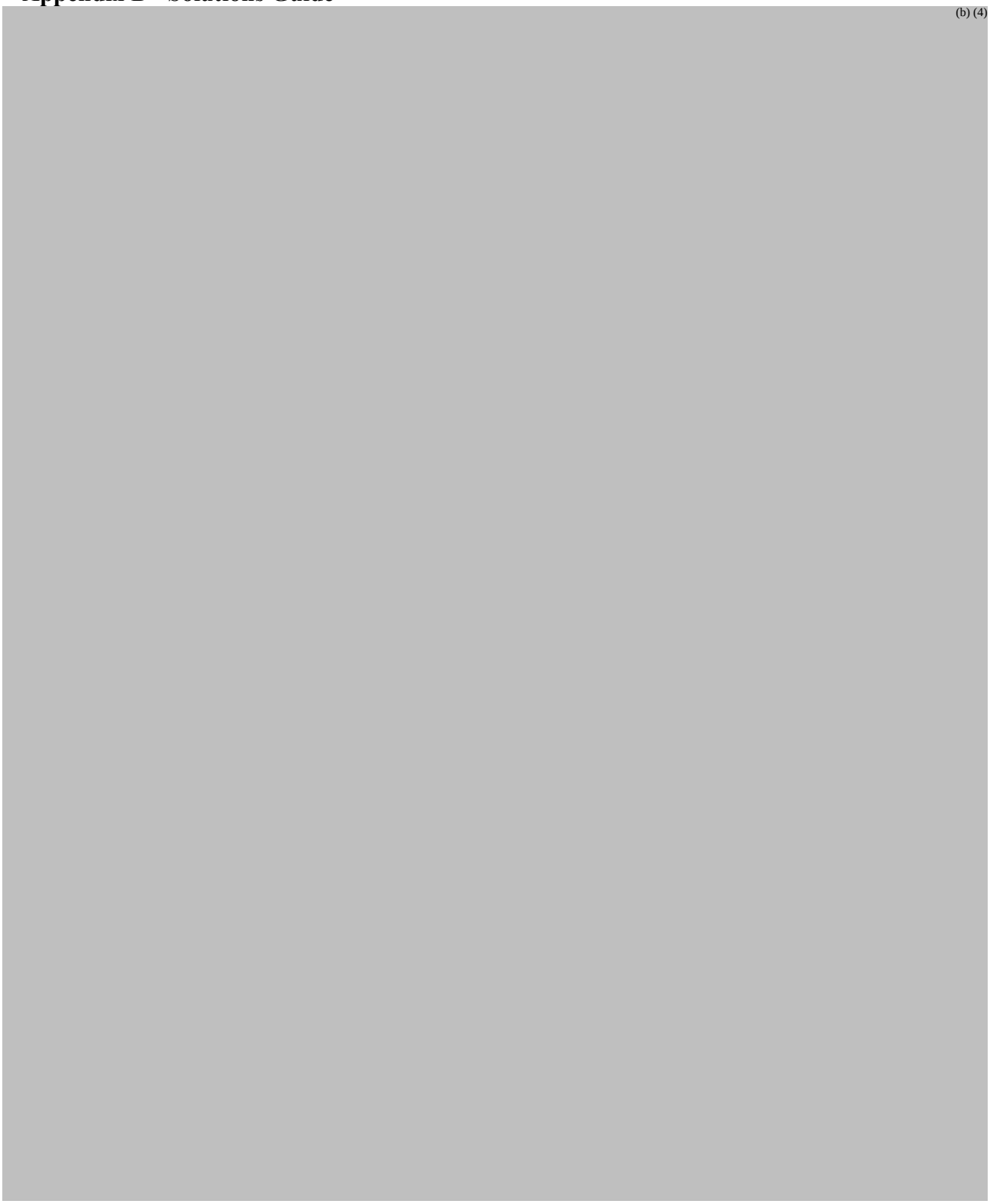
(b) (4)

Appendix A - Calibration Concentrations

Calibrations

(b) (4)

Appendix B - Solutions Guide



AOAC Official Method 2013.01
Salmonella in a Variety of Foods
VIDAS® UP *Salmonella* (SPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Salmonella* in raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates (30 mL), and stainless steel, plastic, and ceramic environmental surfaces.]

See Tables **2013.01A** and **B** for a summary of results of the interlaboratory study. For detailed results of the interlaboratory study, *see* Tables A–F in Appendix 1 on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The VIDAS SPT method is for use on the automated VIDAS instrument for the detection of *Salmonella* receptors using the enzyme-linked fluorescent assay. The solid-phase receptacle (SPR) serves as the solid phase, as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Salmonella* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. The instrument performs all the assay steps automatically. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Salmonella* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Salmonella* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS SPT assay kit from bioMérieux Inc., Hazelwood, MO.

(**a**) *VIDAS or miniVIDAS automated immunoassay system.*

(**b**) *SPT reagent strips.*—60 polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.01C**.

(**c**) *SPR.*—60 SPRs coated with proteins specific for *Salmonella* receptors.

(**d**) *Standard.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**f**) *Negative control solution.*—One vial (6 mL). Contains Tris-buffered saline (150 mmol/L)–Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test.

(**h**) *Package insert*.

(**i**) *Disposable pipet to dispense appropriate volumes.*

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath (95–100°C) or equivalent system.*

(**l**) *Stomacher®-type bag with filter.*

(**m**) *Stomacher.*—Stomacher Lab Blender 400, available from Seward Medical (London, UK); Smasher, bioMérieux, Inc., or equivalent.

(**n**) *BPW.*—Available from bioMérieux, Inc.

(**o**) *Salmonella supplement.*—Available from bioMérieux, Inc.

(**p**) *Incubators.*—Capable of maintaining 42 ± 1°C and 35 ± 1°C.

(**q**) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* **967.27** (*see* 17.9.03).

(**r**) *IBISA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**s**) *ASAP chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Vancomycin.*—Available from bioMérieux, Inc.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS SPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Mix standard, controls, and heated test portions well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS SPT package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion in BPW using filter Stomacher bags to initiate growth of *Salmonella.* For 25 g test portions, add 225 mL BPW to each test portion and homogenize thoroughly for 2 min. For 375 g test portions, prewarm BPW to 42 ± 1°C, add 1125 mL to each test portion, and homogenize thoroughly for 2 min.

(**b**) After homogenization add *Salmonella* supplement to each test portion. For 25 g test portions, add 1 mL of *Salmonella* supplement, mix samples manually, and incubate for 18–24 h at 42 ± 1°C. For 375 g test portions, add 5 mL of *Salmonella* supplement, mix samples manually, and incubate for 22–26 h at 42 ± 1°C.

(**c**) After incubation, homogenize samples manually. If a water bath is used, transfer 2–3 mL enrichment broth into a tube. Seal the tube. Heat for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS SPT reagent strip. If the VIDAS Heat and Go is used, transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS SPT reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's

Table 2013.01A. Summary of results for the detection of *Salmonella* spp. in raw ground beef (25 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/144	144/144	144/144	0/144	144/144	144/144	0/144	144/144	144/144
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_L[e]	0.00 (0.00 +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_R[f]	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)
P-value	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Candidate confirmed positive/total samples analyzed	0/144	143/144	144/144	0/144	143/144	144/144	0/144	143/144	144/144
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)
s_r	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)
s_R	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)
P-value	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000
Positive reference samples/total samples analyzed	0/144	84/144	138/144	0/144	84/144	138/144	0/144	84/144	138/144
Reference POD	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)
s_R	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)
P-value	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179
dLPOD (candidate vs reference)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)
dLPOD (candidate presumptive vs candidate confirmed)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01B. Summary of results for the detection of *Salmonella* spp. in raw ground beef (375 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/132	58/131	130/132	0/132	58/131	130/132	0/132	57/131	130/132
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.33, +0.54)	0.98 (+0.965, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44. +0.52)	0.12 (+0.11, +0.16)
s_L[e]	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.26)	0.00 (0.00, +0.05)
s_R[f]	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.1551	0.5190	1.0000	0.1906	0.5190
Candidate confirmed positive/total samples analyzed	0/132	58/131	130/132	0/132	59/131	130/132	0/132	58/131	130/132
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.45 (+0.35, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)
s_r	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)
s_L	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.25)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.2060	0.5190	1.0000	0.1551	0.5190
Positive reference samples/total samples analyzed	0/132	57/132	132/132	0/132	57/132	132/132	0/132	54/132	131/132
Reference POD	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.41 (+0.32, +0.50)	0.99 (+0.96, +1.00)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.05 (0.00, +0.22)	0.00 (0.00, +0.04)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.10)
P-value	1.0000	0.6261	1.0000	1.0000	0.6261	1.0000	1.0000	0.3313	0.4338
dLPOD (C vs R)	0.00 (–0.03, +0.03)	0.01 (–0.12, +0.15)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.02 (–0.18, +0.22)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.03 (–0.18, +0.24)	–0.01 (–0.05, +0.03)
dLPOD (CP vs CC)	0.00 (–0.03, +0.03)	0.00 (–0.15, +0.15)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.15, +0.14)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.21, +0.23)	0.00 (–0.04, +0.04)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01C. Reagents included in 10-well reagent strip

Wells	Reagents (SPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): Buffer pH 7.8 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-buffered saline (150 mmol/L) – Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Salmonella* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent; *see* VIDAS SPT package insert for more information.

Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature.

(**c**) Use one VIDAS SPT reagent strip and one VIDAS SPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "SPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the SPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

Table 2013.01D. Interpretation of test

Test value threshold	Interpretation
<0.25	Negative
≥0.25	Positive

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, test sample identification, date and time, lot number, and expiration date of the reagent kit being used, each sample's RFV**,** test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A negative result has a test value less than the threshold (0.25) and indicates that the sample does not contain *Salmonella* spp. or contains *Salmonella* spp. at a concentration below the detection limit. A positive result has a test value equal to or greater than the threshold (≥0.25) and indicates that the sample may be contaminated with *Salmonella* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2012.01D**).

G. Confirmation

All positive VIDAS SPT results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth stored between 2 and 8°C, and should be initiated within 72 h after the end of incubation at 42 ± 1°C. Presumptive positive results may be confirmed by isolating on selective agar plates such as IBISA or ASAP, or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in **967.27** (*see* 17.9.03). As an alternative to the conventional tube system for *Salmonella*, any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in **978.24** (*see* 17.9.04), **989.12** (*see* 17.9.05), **991.13** (*see* 17.9.06), and **2011.17** (*see* 17.15.01).

Reference: *J. AOAC Int*. **96**, 808(2013)
DOI: 10.5740/jaoacint.CS2013_01

AOAC Official Method 2013.10
Listeria species in a Variety of Foods and Environmental Surfaces
VIDAS® UP *Listeria* (LPT) Method
First Action 2013
Final Action 2016

[Applicable to detection of *Listeria* in deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic and concrete environmental surfaces.]

See Tables **2013.10A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is, therefore, recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

VIDAS® UP *Listeria* (LPT) method is for use on the automated VIDAS instrument for the detection of *Listeria* antigens using the enzyme-linked fluorescent assay (ELFA) method. The assay also incorporates phage proteins allowing an increase in sensitivity and specificity compared to traditional immunoassay. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Listeria* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Listeria* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument, which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS UP *Listeria* (LPT) assay kit from bioMérieux (Hazelwood, MO, USA).

(**a**) *VIDAS or miniVIDAS automated immunoassay system*.

(**b**) *LPT reagent strips*.—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents shown in Table **2013.10C**.

(**c**) *SPR*.—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard*.—One vial (1 × 6 mL). Ready-to-use. Contains purified and inactivated *Listeria* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution*.—1 × 6 mL. Contains purified and inactivated *Listeria monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution*.—1 × 6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master Lot Entry (MLE) card*.—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert*.

(**i**) *Disposable pipet*.—To dispense appropriate volumes.

(**j**) *VIDAS Heat and Go*.—Available from bioMérieux, Inc.

(**k**) *Water bath*.—95–100°C, or equivalent.

(**l**) *Bag with filter*.

(**m**) *Smasher™ Blender/Homogenizer*.—Available from bioMérieux, Inc., or equivalent.

(**n**) *LPT broth*.—bioMérieux, Inc.

(**o**) *Incubators*.—Capable of maintaining 30 ± 1°C and 35 ± 1°C.

(**p**) *Diagnostic reagents*.—Necessary for culture confirmation of assays.

(**q**) *ALOA chromogenic agar*.—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**r**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls, and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS LPT package insert (Warnings and Precautions and Waste Disposal).

(**i**) *See* Centers for Disease Control recommendations in handling pathogens. http:/www.cdc.gov/biosafety/publications/bmb15/index.htm/

Table 2013.10A. Summary of results for the detection of *Listeria* spp. in queso fresco (25 g)[a]

	VIDAS LPT with OXA			VIDAS LPT with ALOA		
	Inoculation level					
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. samples analyzed	1/156	80/156	156/156	1/156	80/156	156/156
Candidate presumptive POD (CP)	0.01	0.51	1.00	0.01	0.51	1.00
	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)
s_r[b]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)
s_R[d]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)
P value[e]	0.4395	0.9210	1.0000	0.4395	0.9210	1.0000
Candidate confirmed positive/ total No. samples analyzed	0/156	78/156	156/156	0/156	78/156	156/156
Candidate confirmed POD (CC)	0.00	0.50	1.00	0.00	0.50	1.00
	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.9161	1.0000	1.0000	0.9161	1.0000
Positive reference samples/ total No. samples analyzed	0/156	76/156	156/156	0/156	76/156	156/156
Reference POD	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)
s_r	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)
s_R	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value	1.0000	0.9937	1.0000	1.0000	0.9937	1.0000
dLPOD (candidate vs reference)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)
dLPOD (candidate presumptive vs candidate confirmed)	0.01	0.01	0.00	0.01	0.01	0.00
	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

Table 2013.10B. Summary of results for the detection of *Listeria* spp. in queso fresco (125 g)[a]

	VIDAS LPT with OXA			VIDAS LPT with ALOA		
	Inoculation level					
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/ total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/ total No. of samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

Table 2013.10C. Reagents included in 10-well reagent strip

Wells	Reagents (LPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Listeria* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA) (0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion using filter Stomacher type bags to initiate growth of *Listeria.* For 25 g test portions, add 225 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min. For cantaloupe melons, soak entire melon in approximately 1 L prewarmed (18–25°C) LPT broth. For 125 g test portions, add 375 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min.

(**b**) *Test portions.—(1) 25 g test portions/cantaloupe melons rinses.*—After homogenization, incubate for 26–30 h at 30 ± 1°C.

(2) 125 g test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

From the primary enrichment broth, transfer a 1 mL aliquot into 10 mL prewarmed (18–25°C) LPT broth and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually. Follow appropriate instructions based on heating method.

(*1*) *Boiling.*—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS LPT reagent strip. Perform the VIDAS test.

(2) Heat and Go.—Transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS LPT reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and let them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LPT reagent strip and one VIDAS LPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Table 2013.10D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *Listeria* spp. or contains *Listeria* spp. at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *Listeria* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.10D**).

G. Confirmation

All positive VIDAS LPT results must be culturally confirmed. Confirmation should be performed using the nonheated enrichment broth stored between 2–8°C and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *Listeria*, **2012.02** VITEK 2 GP Biochemical Identification or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *Listeria*.

Reference: *J. AOAC Int*. **97**, 431(2014)
DOI: 10.5740/jaoacint.13-372

Posted: May 2014, February 2016

Appendix 008

[redacted]

Name: Ascus Biosciences Inc.
Customer: Martin Mayhew
Address: 6450 Lusk Blvd. Suites E109/209
San Diego, CA
92121
USA
877-696-8945

Order ID: [redacted]
Report ID: [redacted]
Date Received: 12/11/2020 11:17:20
Reported: 12/16/2020 16:24:21
P.O. #: N/A
Page: 1 of 1

Report of Results

[redacted] **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-10 Lot: 1801.2034

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-10 Lot: 1801.2036

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-10 Lot: 1801.2038

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-19 Lot: 1801.2033

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-19 Lot: 1801.2035

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted] **Analysis Date:** 2020/12/11 **Receiving Temperature:** 4.4C **Sample Condition:** Okay

Description: Dairy-19 Lot: 1801.2037

Test:	Result:	Units:	Method:	Reference:	Comment:
C.botulinum Toxin	Negative	/2g	FDA BAM	ed. 8, ch. 17	

[redacted]

CERTIFICATE OF ANALYSIS

Customer: (b) (4)

Product: Ammonium Hydroxide 29%

Sales Order #: (b) (4)

Purchase Order #: (b) (4)

Shipment Date: 6/24/2019

Lot #: 05-02-19-01

Analysis

Ammonia, wt.%:	29.9
Specific Gravity @ 60°F, g/mL:	0.896
Appearance:	Clear, Colorless

(b) (4)

Maximum Usage Level:
10 mg/L

This document was produced electronically and no signature is required.

(b) (4)

Certificate Of Analysis

Item Number	C1473	Lot Number	1JD0338
Item	Cysteine Hydrochloride, Monohydrate, USP		
CAS Number	7048-04-6		
Molecular Formula	$C_3H_7NO_2S.HCl.H_2O$	Molecular Weight	175.64

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.5	101.5 %	99.5 %
SPECIFIC ROTATION $[a]_D$	+5.7° to +6.8°		+5.95°
LOSS ON DRYING	8.0	12.0 %	10.12 %
RELATED COMPOUNDS:			.
INDIVIDUAL IMPURITY		0.5 %	<0.5 %
TOTAL IMPURITIES		2.0 %	<2.0 %
RESIDUE ON IGNITION		0.4 %	0.01 %
SULFATE		0.03 %	<0.03 %
IRON		30 ppm	<30 ppm
ELEMENTAL IMPURITIES	AS REPORTED		NO ELEMENTAL IMPURITIES PRESENT
IDENTIFICATION (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE CRYSTALS
EXPIRATION DATE			06-OCT-2021
DATE OF MANUFACTURE			07-OCT-2019
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

(b) (4)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(b) (4)

Certificate Of Analysis

Item Number	S1303	Lot Number	1GA0557
Item	Sodium Hydroxide, Pellets, FCC		
CAS Number	1310-73-2		
Molecular Formula	NaOH	Molecular Weight	40.00

Test	Specification		Result
	min	max	
ASSAY (TOTAL ALKALI as NaOH)	95.0 - 100.5 %		98.05 %
ARSENIC (As)		3 mg/kg	<3 mg/kg
CARBONATE (as Na_2CO_3)		3.0 %	0.55 %
INSOLUBLE SUBSTANCES & ORGANIC MATTER		TO PASS TEST	PASSES TEST
LEAD (Pb)		2 mg/kg	<2 mg/kg
MERCURY		0.1 mg/kg	<0.1 mg/kg
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED HALAL			HALAL
EXPIRATION DATE			26-APR-2021
DATE OF MANUFACTURE			27-APR-2016
APPEARANCE			WHITE PELLET

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

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All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	FE110	Lot Number	2IA0400
Item	Ferrous Sulfate, Heptahydrate, Granular, USP		
CAS Number	7782-63-0		
Molecular Formula	$FeSO_4.7H_2O$	Molecular Weight	278.02

Test	Specification		Result
	min	max	
ASSAY (as HEPTAHYDRATE)	99.5	104.5 %	100.0 %
ARSENIC		3 ppm	<3 ppm
LEAD		10 ppm	<1 ppm
MERCURY		3 µg/g	<1 µg/g
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
IDENTIFICATION	POSITIVE FOR IRON, FERROUS SALTS AND SULFATE		POSITIVE FOR IRON, FERROUS SALTS AND SULFATE
EXPIRATION DATE			01-JUN-2021
DATE OF MANUFACTURE			01-JUN-2018
APPEARANCE			PALE BLUE GREEN CRYSTALS
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED

(b) (4)

Certificate of Analysis Results Certified by:

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Quality Control Manager

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All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

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Certificate Of Analysis

Item Number	MA135	Lot Number	1IJ0734
Item	Magnesium Sulfate, Heptahydrate, USP, EP, BP		
CAS Number	10034-99-8		
Molecular Formula	$MgSO_4.7H_2O$	Molecular Weight	246.48

Test	Specification		Result
	min	max	
ASSAY ($MgSO_4$; ANHYDROUS BASIS)	99.0	100.5 %	99.92 %
pH OF A 5% SOLUTION @ 25°C	5.0	9.2	8.33
LOSS ON IGNITION	48.0	52.0 %	51.0 %
APPEARANCE OF SOLUTION	TO PASS TEST		PASSES TEST
IDENTIFICATION	TO PASS TEST		PASSES TEST
CHLORIDE		0.014 %	0.009 %
IRON (Fe)		20 µg/g	< 20 µg/g
ELEMENTAL IMPURITIES	AS REPORTED		.
SELENIUM		30 µg/g	< 30 µg/g
ALKALINITY OR ACIDITY	TO PASS TEST		PASSES TEST
ARSENIC		2 ppm	< 2 ppm
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			06-JUN-2022
DATE OF MANUFACTURE			06-JUN-2019
APPEARANCE			WHITE CRYSTALS
RESIDUAL SOLVENTS	TO PASS TEST		NO RESIUDUAL SOLVENTS USED

(b) (4)

Certificate of Analysis Results Certified by:

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Quality Control Manager

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All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

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Certificate Of Analysis

Item Number	P1382	Lot Number	1JI0296
Item	Potassium Phosphate Monobasic, FCC		
CAS Number	7778-77-0		
Molecular Formula	KH_2PO_4	Molecular Weight	136.09

Test	Specification		Result
	min	max	
ASSAY (KH_2PO_4; DRIED BASIS)	98.0 %		101.0 %
ARSENIC (As)		3 mg/kg	0.01 mg/kg
FLUORIDE		10 mg/kg	1.2 mg/kg
INSOLUBLE SUBSTANCES		0.2 %	0.00 %
LEAD (Pb)		2 mg/kg	0.01 mg/kg
LOSS ON DRYING		1 %	0.04 %
IDENTIFICATION	TO PASS TEST		PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			30-APR-2023
DATE OF MANUFACTURE			01-APR-2020
APPEARANCE			WHITE CRYSTALLINE POWDER
MONOGRAPH EDITION			(FCC) 11

(b) (4)

Certificate of Analysis Results Certified by:

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Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

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Certificate Of Analysis

Item Number	SO104	Lot Number	1JH0059
Item	Sodium Acetate, Anhydrous, USP	Manufacturer Lot	4350064-A
CAS Number	127-09-3	Manufacturer Code	14941
Molecular Formula	$C_2H_3NaO_2$	Molecular Weight	82.03

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	99.0	101.0 %	100.1 %
pH OF A 3% SOLUTION @ 25°C	7.5	9.2	8.5
LOSS ON DRYING		1.0 %	0.03 %
INSOLUBLE MATTER		0.05 %	0.001 %
CHLORIDE (Cl)		350 ppm	< 350 ppm
SULFATES (SO_4)		50 ppm	< 50 ppm
CALCIUM AND MAGNESIUM	NO TURBIDITY		NO TURBIDITY
POTASSIUM (K)	NO PRECIPITATE		NO PRECIPITATE
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
IDENTIFICATION (A)	POSITIVE FOR SODIUM		POSITIVE FOR SODIUM
IDENTIFICATION (B)	POSITIVE FOR ACETATE		POSITIVE FOR ACETATE
EXPIRATION DATE			30-NOV-2021
DATE OF MANUFACTURE			01-MAY-2020
APPEARANCE			WHITE GRANULAR
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

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(b) (4)

Certificate Of Analysis

Item Number	SO155	Lot Number	1JI0681
Item	Sodium Chloride, Granular, USP	Manufacturer Lot	RI20191040
CAS Number	7647-14-5	Manufacturer Code	12349
Molecular Formula	NaCl	Molecular Weight	58.44

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	99.0	100.5 %	99.5 %
APPEARANCE OF SOLUTION	CLEAR COLORLESS		CLEAR COLORLESS
ACIDITY OR ALKALINITY		0.5 ml	<0.5 ml
LOSS ON DRYING		0.5%	0.1 %
ALUMINUM		0.2 ppm	<0.05 ppm
BROMIDES		100 ppm	<100 ppm
PHOSPHATES		25 ppm	<25 ppm
POTASSIUM		500 ppm	32 ppm
IODIDES	NO BLUE COLOR		NO BLUE COLOR
MAGNESIUM AND ALKALINE-EARTH METALS (as Ca)		100 ppm	4 ppm
ARSENIC (As)		1 ppm	<1 ppm
IRON (Fe)		2 ppm	<1 ppm
BARIUM (Ba)	OPALESCENCE LESS THAN REFERENCE		OPALESCENCE LESS THAN REFERENCE
FERROCYANIDES	NO BLUE COLOR		NO BLUE COLOR
SULFATE (SO_4)		200 ppm	<200 ppm
NITRITES		0.01	0.00
BACTERIAL ENDOTOXINS		5 IU/g	<2.5 IU/g
ELEMENTAL IMPURITIES	AS REPORTED		NO ELEMENTAL IMPURITIES PRESENT
IDENTIFICATION (A)	POSITIVE FOR SODIUM		POSITIVE FOR SODIUM
IDENTIFICATION (B)	PRECIPITATE DISSOLVES		PRECIPITATE DISSOLVES
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE GRANULES
RETEST DATE			09-JUL-2023
DATE OF MANUFACTURE			09-JUL-2020
RESIDUAL SOLVENTS	-AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

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Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

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All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

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27 Stearine

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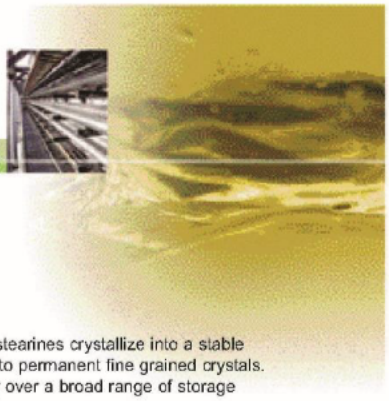
27 Stearine™



Product Data Sheet

Product Description:
27 Stearine is a highly functional hardened palm oil. Palm stearines crystallize into a stable betaprime configuration. Beta-prime hard fats crystallize into permanent fine grained crystals. This allows for maximum oil stabilization as well as stability over a broad range of storage conditions.

Typical data suggests that it may be used for stabilizing peanut butter, as well as a melt point adjuster for many types of processed foods. The user is advised to fully evaluate the functionality and shelf life of the shortening in their intended finished product at their own facilities, as performance may be affected by varying formulations and process conditions.

Ingredient Statement:
Hydrogenated Palm Oil. Kosher. (US)

Typical Data:

Capillary Melting Point	.....58-62°C/136-144°F
Color (5 1/4") Lovibond	.....5 R max
Free Fatty Acid (% as oleic)	.....0.10% max
Iodine Value	.....4 max

The typical data provided here is valid at the point of shipment from our manufacturing facility.

Packaging:
27 Stearine is available in 50 lb. beaded poly-lined cartons and in bulk liquid.

Storage and Handling:
27 Stearine needs no refrigeration, however, like all fats, it will absorb odors and should be stored between 40-80°F in a dry place away from odor-producing substances. Bulk liquid product can be stored at 150-160°F for 30 days. Based on the typical data a shelf-life of 180 days is suggested for packaged product stored at 40-80°F. *

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* Actual shelf-life may vary and is dependant upon several factors including the type of application, interaction with other components of the finished product, process conditions used in the preparation of the finished product and conditions of subsequent storage and shipping of the finished product. The user is advised to carry out a full evaluation of the shortening to determine its suitability in their finished product.

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KFO-402 Antifoam

(b) (4)

KFO™ 402

Product Type	**FOOD GRADE – GENERAL PURPOSE PROCESS AID DEFOAMER**
Product Description	KFO™ 402 is a defoamer designated to control foam in many processes. KFO™ 402 is especially effective when used in fermentation processes where a certain degree of foam control is needed without affecting oxygen transfer for optimum product yield. This product is made with food grade ingredients under our Good Manufacturing Practices Program. The components of KFO™ 402 meet FDA requirement for use in egg washing, potato processing defoamers as a dispersing aid for mineral oil at a limit of 10 ppm in the processing water followed by a potable water rinse. This product also contains ingredients for which the FDA has provided the Enzyme Technical Association with a "no objection" letter acknowledging that they are used as defoaming agents in the manufacture of enzyme preparations used in food in accordance with the principles of GMPs. Other uses in the processing and manufacture of food ingredients may also qualify for GRAS status. KFO™ 402 also is composed of ingredients that meet the current requirements of the FDA for food contact applications when used in accordance with the requirements and limitations of 21CFR 176.210(d)(3). Consideration for other FDA permitted uses would require further evaluation.

Typical Properties		
	Appearance	Clear Liquid
	Viscosity @ 100°F, Kinematic	185 – 210 Cst
	Odor	Sweet
	Weight per gallon	8.5 Lbs
	Flash Point (°C)	> 216°C PMCC (Min)
	Specific Gravity	1.02

Typical Applications	Typical applications for KFO™ 402 include: • Fermentation • Egg washing
Incorporation	KFO™ 402 should be added, as received, early in the processing to prevent foam before it forms. KFO™ 402 should be evaluated in the process to determine the optimum dosage and legal limits allowed.
Shelf Life	2 years from date of manufacture when properly stored in the original container following proper storage and handling.
Storage & Handling	Keep from freezing. Store product between 40 and 100°F. Keep containers tightly closed when not in use.
Responsible Care	For complete safety, health, personnel protection and first aid information, refer to the Safety Data Sheet (SDS) that can be ordered through the numbers below.

Updated January 16, 2017

(b) (4)

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

Mr. Gary Yingling
Kirkpatrick & Lockhart LLP
1800 Massachusetts Avenue, NW
Second Floor
Washington, DC 20036-1221

Dear Mr. Yingling:

You requested, on behalf of the Enzyme Technical Association, that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between ETA members and OFAS representatives, facilitated telephone contacts with technical experts from ETA member companies, and responded to numerous requests for clarification. We appreciate your and ETA's cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition

Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food

Enzyme Preparations

Most enzymes currently used in food are derived from microorganisms. The manufacturing process of such enzymes includes three major steps: fermentation, enzyme recovery, and enzyme formulation. The formulated products are generally referred to as enzyme preparations. In addition to the enzymes of interest, enzyme preparations contain added substances such as diluents, preservatives, and stabilizers. They may also contain metabolites derived from the production microorganism and the residues of substances used in the manufacturing process, such as components of the fermentation medium or defoaming and flocculating agents used during fermentation and recovery. When FDA reviews safety data on enzyme preparations, it considers all components of the preparation.

Defoaming Agents

Defoaming agents (defoamers) are used by enzyme manufacturers to reduce or prevent foaming during fermentation and recovery. They are formulated with ancillary ingredients such as surface-active agents or carriers. Defoamers currently used in the manufacture of food enzymes are listed in Table 1. The Table includes five major defoamers that are identified by a double asterisk and several compounds that are used either as secondary defoamers or ancillary ingredients in defoamer formulations.

The major defoamers are added to the fermentation broth at levels within the range of 0.05-1% on a weight basis. Some of these defoamers, for example, polyoxyethylene-polyoxypropylene block copolymer, may contain trace levels of ethylene oxide, propylene oxide, and 1,4-dioxane which are known to cause cancer in laboratory animals. The Office of Food Additive Safety (OFAS) has evaluated the use of defoamers listed in Table 1 and determined that human exposure to the residues of these defoamers in enzyme preparations does not present human safety concern.

Flocculating Agents

Flocculating agents (flocculants) are used in the enzyme recovery step to separate microbial cells and cell debris from the fermentation broth containing the dissolved enzyme. The flocculation typically consists of two steps - primary flocculation and secondary flocculation. In the primary flocculation, inorganic salts (such as calcium chloride or aluminum sulfate) or "low molecular weight" polymers (such as polyamines) are used to agglomerate the cellular debris. The primary flocculation is usually followed by the secondary flocculation in which "high molecular weight" polymers are used to aid the formation of larger agglomerates that are subsequently removed by centrifugation or filtration. The polymers used as flocculants can be either cationic or anionic. The cationic polymers are added to the fermentation broth at levels not higher than 1% on a

weight basis. The anionic polymers are used at levels at or below 0.025%.

The flocculants used in the manufacture of food enzymes are listed in Table 2. They include inorganic salts, polyamines, and polyacrylamides. Several of these compounds are regulated in 21 CFR either as food additives or GRAS substances. Certain polyamines may contain traces of epichlorohydrin and 1,3-dichloro-2-propanol. Polyacrylamides usually contain very low levels of acrylamide. These contaminants of polyamines and polyacrylamides are known to cause cancer in laboratory animals. OFAS has evaluated all polymers included in Table 2 and determined that human exposure to the residues of these flocculants in enzyme preparations does not present human safety concern.

Sources of Information on Defoamers and Flocculants

OFAS compiled data on defoamers and flocculants listed in Tables 1 and 2 using information voluntarily submitted by the Enzyme Technical Association. OFAS also relied on the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. Other sources of information included published articles, computer searches, and Material Safety Data Sheets issued by manufacturers of defoamers and flocculants.

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Polypropylene glycol**	25322-69-4	Average MW: 2000
Polyglycerol polyethylene-polypropylene glycol ether oleate**	78041-14-2	
Polyoxyethylene-polyoxypropylene block copolymer**	9003-11-6	Average MW: 2000
Polypropylene glycol monobutyl ether**	9003-13-8	
Polydimethylsiloxane**	63148-62-9 68083-18-1	
Silica	7631-86-9 63231-67-4	
Stearic acid	57-11-4	
Sorbitan sesquioleate	8007-43-0	
Glycerol monostearate	123-94-4	
Polysorbates (polyoxyethylene sorbitan fatty acid esters)		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
Rape oil mono- and diglycerides	93763-31-6	
White mineral oil	64742-47-8	

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Dimethylamine-epichlorohydrin copolymer	25988-97-0	Cationic polyamine
Methylamine-epichlorohydrin copolymer	31568-35-1	Cationic polyamine
Dimethylamine-epichlorohydrin-ethylenediamine terpolymer	42751-79-1	Cationic polyamine
Polyacrylamide modified by condensation with formaldehyde and dimethylamine	67953-80-4	Cationic polyacrylamide
Acrylamide-acryloxyethyl-trimethyl-ammonium chloride copolymer	69418-26-4	Cationic polyacrylamide
Acrylamide-acrylic acid copolymer	25987-30-8 9003-06-9	Anionic polyacrylamide
Aluminum sulfate	10043-01-3	
Calcium chloride	10035-04-8 10043-52-4	

(b) (4)

Certificate Of Analysis

Item Number	AS102	Lot Number	2JI0075
Item	Ascorbic Acid, USP		
CAS Number	50-81-7		
Molecular Formula	$C_6H_8O_6$	Molecular Weight	176.13

Test	Specification		Result
	min	max	
ASSAY	99.0	100.5 %	99.8 %
SPECIFIC ROTATION $[a]_D$	+20.5 to+21.5		+21.0 °
RESIDUE ON IGNITION		0.1 %	0.03 %
ELEMENTAL IMPURITIES:			.
CADMIUM (Cd)		AS REPORTED	< 0.01 g/g
LEAD (Pb)		AS REPORTED	< 2 g/g
ARSENIC (As)		AS REPORTED	< 3 g/g
MERCURY (Hg)		AS REPORTED	< 1 g/g
IDENTIFICATION A (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
IDENTIFICATION (B)	REDUCES ALKALINE CUPRIC TARTRATE TS		REDUCES ALKALINE CUPRIC TARTRATE TS
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			29-MAR-2022
DATE OF MANUFACTURE			30-MAR-2019
APPEARANCE			WHITE CRYSTALLINE POWDER
RESIDUAL SOLVENTS		AS REPORTED	.
CLASS 2 (SOLVENT) / METHANOL			< 3000 ppm

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

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(b) (4)

Certificate Of Analysis

Item Number	MA164	Lot Number	2FF0011
Item	Manganese Sulfate, Monohydrate, Powder, FCC, BP		
CAS Number	10034-96-5		
Molecular Formula	$MnSO_4.H_2O$	Molecular Weight	169.02

Test	Specification		Result
	min	max	
ASSAY ($MnSO_4.H_2O$)	98.0	102.0%	99.95 %
ASSAY (IGNITED)	99.0	101.0%	100.49 %
LOSS ON HEATING	10.0	12.0%	11.15 %
APPEARANCE OF SOLUTION	TO PASS TEST		PASSES TEST
ARSENIC (As)		3 mg/kg	<3 mg/kg
LEAD (Pb)		4 mg/kg	0.6 mg/kg
SELENIUM (Se)		0.003%	<0.003 %
HEAVY METALS		20 ppm	<20 ppm
ELEMENTAL IMPURITIES		AS REPORTED	COMPLIES WITH STANDARD
IRON		10 ppm	<10 ppm
ZINC (Zn)		50 ppm	6 ppm
CHLORIDE (Cl)		100 ppm	<50 ppm
IDENTIFICATION		TO PASS TEST	PASSES TEST
RETEST DATE			15-APR-2021
DATE OF MANUFACTURE			16-APR-2016
APPEARANCE			PINK CRYSTALLINE POWDER
RESIDUAL SOLVENTS		TO PASS TEST	NO RESIDUAL SOLVENTS USED

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

(b) (4)

Specifications for Sodium Sulfate

Ingredient:	Sodium Sulfate
Chemical Nomenclature:	$NaSO_4$
Specifications:	Feed Grade
Moisture:	≤ 1% by LOD
Purity:	≥ 98%

(b) (4)

Specification for Ammonium Chloride, Granular, FCC (A1167)

Item Number	A1167
Item	Ammonium Chloride, Granular, FCC
CAS Number	12125-02-9
Molecular Formula	NH_4Cl
Molecular Weight	53.49
MDL Number	
Synonyms	

Test	Specification	
	Min	Max
ASSAY (DRIED BASIS)	99.0 %	
LEAD (Pb)		4 mg/kg
LOSS ON DRYING		0.5 %
IDENTIFICATION	TO PASS TEST	
RETEST DATE		

(b) (4)

Cerelose® Dextrose M Non-GMO

technical specification

(b) (4)

CERELOSE® Dextrose M NON-GMO IP 02001090

CERELOSE® Dextrose 02001090 is a general purpose crystalline monohydrate dextrose suitable for most food, beverage, and industrial uses. This product is produced under (b) (4) for non-GM products.

Chemical and Physical Properties

	Min.	Max.
Moisture %	8.0	9.0
Dextrose Equivalent	99.5	-
SO_2, ppm	-	<10
Dextrose, % d.b.	99.5	-
Ash, % d.b.	-	0.1
Solution Color		Passes test
Apparent Starch		Passes test

Physical Appearance

	Typical
Color	White
Form	Powder

Screen Test

	Typical
On USS 20 mesh, %	<1
On USS 100 mesh, %	<60

Microbiological Limits

	Max.
Standard Plate Count, cfu/g	100
Yeast, cfu/g	25
Mold, cfu/g	25
Salmonella/10 g	Negative
Coliforms, MPN/g	3

Nutritional Data/ 100g

	Typical
Calories	362
Calories from Fat	0
Total Fat, g	0
Cholesterol, mg	0
Sodium, mg	0
Total Carbohydrate, g	90.5
Dietary Fiber, g	0
Total Sugars**, g	90.5
Added Sugars, g	0
Other Carbohydrate, g	0
Protein, g	0
Vitamin D, mcg	0
Calcium mg	0
Iron, mg	0
Potassium, mg	0
Ash, g	<0.1*

* Not present at level of quantification.
** "Total Sugars" in this product may contribute to "Added Sugars" for nutrition labelling purposes in the final consumer product.

Certification

Kosher Pareve
Halal

Packaging and Storage

Bags
Product should be stored in a clean, dry area, not exposed to prolonged high (> 90°F / 32°C) temperature.

Shelf Life

3 years, provided product is stored the original container, well-closed in a cool, dried place free from humidity, dust, or foreign contamination.

Regulatory Data

Source	Corn (IP-TrueTrace™)
CAS No.	50-99-7

United States

Meets FCC (Food Chemical Codex) requirements.

Standard of Identity	21 CFR 168.111
GRAS Affirmation	21 CFR 184.1857
Labeling	Dextrose or Dextrose monohydrate

Canada

Standard Food	CFDA Regulation
Standard of Identity	B.18.015
Labeling	Dextrose or Dextrose monohydrate

Features and Benefits

TrueTrace™ certified non-GM.
Dry crystalline powder, Free flowing.
Mild sweetness
Bulking, Carrying
Highly fermentable

(b) (4)

(b) (4)

SPECIFICATIONS

Ref: B31-003A40

SOLULYS® 095K

DEFINITION :

Spray-dried Corn Steep Liquor
CAS no.: 66071-94-1
EINECS : 266-113-4

SPECIFICATIONS :

LOSS ON DRYING (%)	5.5 max.
REDUCING SUGARS (% d.b. Bertrand)	1.5 max.
pH	3.5 - 4.5
ASH (% d.b.)	22.0 max.
PROTEIN (% d.b.)	43.0 - 54.0
NITROGEN (% d.b.)	7.0 - 8.5
AMINO NITROGEN (% d.b.)	1.5 - 3.5
ACIDITY as LACTIC ACID (% d.b.)	14.0 min.
PHOSPHOROUS (total, % d.b.)	2.4 min.

COMMENTS :

SOLULYS 095K is a spray-dried version of the Roquette SOLULYS 048K corn steep liquor. SOLULYS is a high quality corn steep liquor that is produced to a very consistent quality from batch to batch. It may be used effectively as a nutrient source in a wide variety of fermentations.

(b) (6)

QUALITY ASSURANCE / INDUSTRY

February 10, 2016

(b) (4)

Mannitol

(b) (4)

Specification for Mannitol, Powder, USP (MA165)

Item Number	MA165
Item	Mannitol, Powder, USP
CAS Number	69-65-8
Molecular Formula	$C_6H_{14}O_6$
Molecular Weight	182.17
MDL Number	
Synonyms	Cordycepic Acid ; 1,2,3,4,5,6-Hexanehexol ; Mannite ; D-Mannitol

Test	Specification	
	Min	Max
ASSAY (DRIED BASIS)	97.0	102.0 %
MELTING RANGE	165° -170°C	
APPEARANCE OF SOLUTION	TO PASS TEST	
LOSS ON DRYING		0.5 %
NICKEL		1 µg/g
CONDUCTIVITY @ 25 C		20 µS/cm
RELATED SUBSTANCES	TO PASS TEST	
REDUCING SUGARS	TO PASS TEST	
MICROBIAL LIMITS:		
TOTAL AEROBIC MICROBIAL COUNT		10^3 cfu/g
TOTAL COMBINED MOLDS AND YEASTS COUNT		10^2 cfu/g
ESCHERICHIA COLI		Negative
ELEMENTAL IMPURITIES	AS REPORTED	
IDENTIFICATION	TO PASS TEST	
EXPIRATION DATE		
DATE OF MANUFACTURE		
APPEARANCE		
RESIDUAL SOLVENTS	TO PASS TEST	

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	SU103	Lot Number	1JG0452
Item	Sucrose, Crystal, NF		
CAS Number	57-50-1		
Molecular Formula	$C_{12}H_{22}O_{11}$	Molecular Weight	342.30

Test	Specification		Result
	min	max	
APPEARANCE OF SOLUTION	NO MORE OPALESCENCE THAN STANDARD		NO MORE OPALESCENCE THAN STANDARD
SPECIFIC ROTATION $[a]_D20$	+66.3 to +67.0°		+66.6°
CONDUCTIVITY @ 20 C		35 µS/cm	10 µS/cm
COLOR VALUE		75	52
LOSS ON DRYING		0.1 %	0.03 %
SULFITE		10 PPM	< 10 PPM
REDUCING SUGARS	BLUE COLOR DOES NOT DISAPPEAR COMPLETELY		BLUE COLOR DOES NOT DISAPPEAR COMPLETELY
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES TO STANDARD
IDENTIFICATION (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
CERTIFIED HALAL			CERTIFIED HALAL
RETEST DATE			28-FEB-2022
DATE OF MANUFACTURE			29-FEB-2020
APPEARANCE			WHITE CRYSTALS
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(NF) 37

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

(b) (4)


(b) (4)

Date: 07/08/20
Time: 14:56:17
Page 0 of 0

[redacted]

Certificate of Analysis

[redacted]

Sold To: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: [redacted]
Customer Order No.:
Item No.: AX1003-40-AG AMBEREX 1003 AG 40 LB BAG
40 LB BAG
Customer Item:
Lot No.: 2964284 1.000000 BG
Manufacture Date: 12/04/18
Lot Expiration Date: 12/03/20

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	30.0	100.0	32.5
Ash %	AOAC 930.30	0	16.0	14.8
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	1.0
pH (5% solution)	pH Meter	5.3	6.3	5.7
Protein (N x 6.25) %	AOAC 990.03	55.0	100.0	64.8
Salmonella /750g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE
Salt as Chlorides %	AOAC 971.27	0	1.50	0.6
Standard Plate Count cfu/g	AOAC 990.12	0	10000	100.0
Yeast and Mold cfu/g	AOAC 121301	0	100	0.0

[redacted]

[redacted]

*ND = NOT DETECTED

Quality Assurance Manager

Date: 06/18/19
Time: 14:32:38
Page 0 of 0

[redacted] (b) (4)

[redacted] (b) (4)

Certificate of Analysis

Sold To:

Customer PO No.:
Customer Order No.:
Item No.: SN2000025737 AMBERFERM 7020 AG
18.14 KG/40 LB BAG
Customer Item:
Lot No.: 3117600
Manufacture Date: 03/12/19
Lot Expiration Date: 03/11/21

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	6.0	100.0	9.1
Ash %	AOAC 930.30	0	15.0	10.1
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	3.6
pH (5% solution)	pH Meter	5.2	6.2	5.8
Protein (N x 6.25) %	AOAC 990.03	70.0	100.0	74.7
Salt as Chlorides %	AOAC 971.27	0	2.00	0.27
Standard Plate Count cfu/g	AOAC 990.12	0	10000	10.0
Yeast and Mold cfu/g	AOAC 121301	0	100	0.0
Salmonella /375g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE

[redacted] (b) (4)

*ND = NOT DETECTED

[redacted] (b) (4)
Quality Assurance Manager

Date: 01/25/19
Time: 15:28:53

[redacted] (b) (4)

[redacted] (b) (4)

Certificate of Analysis

[redacted] (b) (4)

d To:

Customer PO No.:
Customer Order No.: 157431
Item No.: SN2000027196

Amberferm 4210
50 LB Carton w/ Liner

Customer Item:
Lot No.: 3022424
Manufacture Date: 01/15/19
Lot Expiration Date: 01/15/21

300.000000 CT

Test Identification	Method	Min Value	Max Value	Test Value
MOISTURE METTLER POWDER		0	6.0	3.8
PH (10% SOLUTION)		.5	5.5	4.8
SALT AS CHLORIDES %		0	2.5	1.1
AMINO NITROGEN/TOTAL NITROGEN		50.0	100.0	79.5
ASH		0	12.0	7.6
% EQUIV. PROTEIN (NX6.25)		74.0	100.0	78.6
FLAVOR		PASS	PASS	PASS
APPEAR		PASS	PASS	PASS
ODOR		PASS	PASS	PASS
AEROBIC PLATE COUNT (CFU/G)		<10000 /G	<10000 /G	<10000 /G
COLIFORM (CFU /G)		<10 /G	<10 /G	<10 /G
YEAST & MOLD (CFU/G)		<100 /G	<100 /G	<100 /G
SALMONELLA ELFA METHOD 375G		ND	ND	ND
E. COLI MPN/g		ND	ND	ND

*ND = NOT DETECTED

[redacted] (b) (4)

Quality Assurance Manager

Date: 05/01/20
Time: 18:29:59

[redacted]

Certificate of Analysis

[redacted]

Sold To: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: [redacted]
Customer Order No.:
Item No.: SN2000041472 SENSIFERM GROW 605 40 LB BAG
40 LB BAG

Customer Item:
Lot No.: 2835511 1.000000 BG
Manufacture Date: 09/11/18
Lot Expiration Date: 09/10/21

Test Identification	Method	Min Value	Max Value	Test Value
Amino Nitrogen/Total Nitrogen%	PPC 12th Edition	5.0	100.0	7.0
Ash %	AOAC 930.30	0	20.0	16.9
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	0.0
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	3.7
pH (5% solution)	pH Meter	5.5	6.5	6.1
Salt as Chlorides %	AOAC 971.27	0	1.00	0.66
Standard Plate Count cfu/g	AOAC 990.12	0	10000	0.0
Yeast and Mold cfu/g	AOAC 121301	0	50	0.0
Salmonella /375g	AOAC OMA 2003.09	NEGATIVE	NEGATIVE	NEGATIVE
Protein (N x 6.25) %	AOAC 990.03	50.0	100.0	55.2

[redacted]

[redacted]

*ND = NOT DETECTED

Quality Assurance Manager

(b) (4)

Certificate Of Analysis

Item Number	HY106	Lot Number	1ID0491
Item	Hydrochloric Acid, 37 Percent, FCC		
CAS Number	7647-01-0		
Molecular Formula	HCl	Molecular Weight	36.46

Test	Specification		Result
	min	max	
ASSAY	36.0 - 38.0 %		37.5 %
COLOR		TO PASS TEST	PASSES TEST
SPECIFIC GRAVITY		TO PASS TEST	PASSES TEST
IRON (Fe)		5 mg/kg	<0.01 mg/kg
LEAD (Pb)		1 mg/kg	<0.01 mg/kg
MERCURY		0.10 mg/kg	<0.001 mg/kg
NONVOLATILE RESIDUE		0.5 %	<0.0005 %
ORGANIC COMPOUNDS		TO PASS TEST	PASSES TEST
OXIDIZING SUBSTANCES (as Cl2)		0.003 %	<0.0001 %
REDUCING SUBSTANCES (as SO_3)		0.007 %	<0.007 %
SULFATE		0.5 %	<0.00001 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
EXPIRATION DATE			28-FEB-2021
DATE OF MANUFACTURE			28-FEB-2019
APPEARANCE			CLEAR COLORLESS LIQUID

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	P1097	Lot Number	1IH1295
Item	Phosphoric Acid, 85 Percent, FCC		
CAS Number	7664-38-2		
Molecular Formula	H_3PO_4	Molecular Weight	98.00

Test	Specification		Result
	min	max	
ASSAY	85.0 %		85.2 %
ARSENIC (As)		3 mg/kg	< 0.2 mg/kg
CADMIUM (Cd)		3 mg/kg	< 2 mg/kg
FLUORIDE		10 mg/kg	< 5 mg/kg
LEAD (Pb)		3 mg/kg	< 3 mg/kg
IDENTIFICATION	POSITIVE FOR PHOSPHATE		POSITIVE FOR PHOSPHATE
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			12-JUN-2021
DATE OF MANUFACTURE			13-JUN-2019
APPEARANCE			CLEAR COLORLESS LIQUID

(b) (4)

Certificate of Analysis Results Certified by:

(b) (4)

Quality Control Manager

(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia.

(b) (4)

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19

Confidential Manufacturing Information

The raw materials used in the manufacture of *B. fibrisolvens* ASCUSDY19 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009U.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *B. fibrisolvens* ASCUSDY19

Material	Function	Regulatory Status	Grade
Ammonium Hydroxide	Seed Medium and Fermentation Medium	21 CFR 184.1139	FCC
L-Cysteine Hydrochloride	Seed Medium and Fermentation Medium	21 CFR 582.5271	USP
Sodium Hydroxide	Seed Medium and Fermentation Medium	21 CFR 582.1763	FCC
Iron (Ferrous) Sulfate Heptahydrate	Seed Medium and Fermentation Medium	AAFCO 57.83; 21 CFR 582.5315	USP
Magnesium Sulfate Heptahydrate	Seed Medium and Fermentation Medium	AAFCO 57.88; 21 CFR 582.5443; IFN 6-02-758	USP
Monopotassium Phosphate	Seed Medium and Fermentation Medium	21 CFR 172.892; Common ingredient	FCC
Sodium Acetate, Anhydrous	Seed Medium and Fermentation Medium	21 CFR 582.1721	USP
Sodium Chloride	Seed Medium and Fermentation Medium	AAFCO 57.31	USP
Hydrogenated Glycerides	Fat Encapsulation	AAFCO 33.19	Feed grade

Table continued on next page.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *B. fibrisolvens* ASCUSDY19 (cont'd)

Material	Function	Regulatory Status	Grade
Polyoxyethylene polyoxypropylene block copolymer	Seed Medium and Fermentation Medium	21 CFR 176.210; FDA-ETA Letter, 2003	Specific product specified. Allowed for Food/feed production
Ascorbic Acid, Vitamin C	DSP and Freeze Drying Processing Aid	IFN 7-00-433; 21 CFR 582.5013	USP or FCC
Manganese Sulfate, Monohydrate	Seed Medium and Fermentation Medium	AAFCO 57.96; 21 CFR.5461	USP
Sodium Sulfate	Fat Encapsulation	AAFCO 57.109	FCC, Moisture: ≤ 1% by LOD, Purity: ≥ 98%
Ammonium Chloride	Seed Medium and Fermentation Medium	AAFCO 57.265	USP
Dextrose Monohydrate	Seed Medium and Fermentation Medium	21 CFR 168.111; 21 CFR 184.1857	FCC
Solulys 095K Corn Steep Powder	Seed Medium and Fermentation Medium	21 CFR 582.1778; 21 CFR 582.5778	Feed Grade
Mannitol	Fermentation Medium and Freeze Drying	21 CFR 582.5470	USP
Sucrose	Freeze Drying	21 CFR 184.1854	NF
Amberex 1003 AG Yeast Extract	Seed Medium and Fermentation Medium	AAFCO 96.11	Specific food grade product specified.
Hydrochloric Acid	Seed Medium and Fermentation Medium	21 CFR 582.1057	FCC
Phosphoric Acid	Seed Medium and Fermentation Medium	AAFCO 57.19; IFN 6-03-707	FCC

Abbreviations: AAFCO – Association of American Feed Control Officials; IFN – International Feed Identification Number; FCC – Food Chemicals Codex; USP – United States Pharmacopoeia; NF – National Formulary

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19

1 Overview

Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19 is produced through a series of processes: Fermentation, Preservation by Vaporization, Milling and Fat Encapsulation. A process diagram of the production of Fat Encapsulated *B. fibrisolvens* ASCUSDY19 is below (Appendix A). The strain (*B. fibrisolvens* ASCUSDY19) is a strictly anaerobic non-spore-forming *B. fibrisolvens* bacterium, that is produced by glucose fed-batch anaerobic fermentation.

Once the fermentation is complete, the biomass (b) (4)

2 Master Cell Bank / Working Cell Bank

(b) (4)

3 Fermentation

(b) (4)

(b) (4)

4 Biomass Harvest by Centrifugation

(b) (4)

5 Preservation Mixture Formulation

(b) (4)

(b) (4)

6 Freeze Drying

(b) (4)

Table 2. Freeze Dryer Profile

(b) (4)

(b) (4)

7 Milling

(b) (4)

8 Fat Encapsulation

(b) (4)

(b) (4)

(b) (4)

Appendix A. Process Diagram of the Production of Fat Encapsulated *B. fibrisolvens* ASCUSDY19

08 Dec 2020

Appendix 011

Comparison of Physical Properties of Fat Encapsulated Powder *B. fibrisolvens* ASCUSDY19 to recent prior submission (AGRN 38) *Pichia kudriavzevii* ASCUSDY21

Physical Attribute	*B. fibrisolvens* ASCUSDY19	(AGRN 38) *P. kudriavzevii* ASCUSDY21	Method
Organism concentration	(b) (4)		Internal Method (Appendices 012C & 012F)
Particle size (d_{50})			Laser Diffraction
Particle size (d_{90})			Laser Diffraction
Milled foam dried organism composition (g/kg in in final formula)			By addition
Sodium Sulfate composition (g/kg in final formula)			By addition
Hydrogenated glycerides composition (g/kg in final formula)			By addition
Moisture content			Internal Method (Appendix 012D)

Method Validation Protocol, Version 1

Method Title and Versions

Title	DY19 Solid Intermediate Microbe Enumeration
Version	01

Lab Performing the Validation: Native Microbials Inc.

Pre-Execution Approval:

Printed Name & Title	Signature
Martin Mayhew – VP-Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF49... 11/13/2020
Patricia A. Williams – Quality	DocuSigned by: Patricia A. Williams 11/13/2020

Post Execution Approval:

Printed Name & Title	Signature
Martin Mayhew – VP-Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
Patricia A. Williams – Quality	DocuSigned by: Patricia A. Williams 5B301285A10643D... 12/1/2020

Personnel Executing the Validation:
Your signature indicates that you have read and understand this protocol.

Printed Name	Signature	Tasks Performed
[redacted] (b) (4), (b) (6)		Analyst 1
		Analyst 2

Purpose:
This validation will demonstrate that the DY19 Solid Intermediate Microbe Enumeration method can quantify the amount of DY19 (*Butyrivibrio fibrisolvens*) in solid intermediates, such as preservation by vaporization (PBV), milled preservation by vaporization (MPBV), and lipid encapsulated intermediates samples. The following parameters will be tested in this validation:

- Repeatability – closeness of results obtained on the same sample when assayed multiple times by the same person with the same reagents and equipment.
- Robustness – reliability of the method to withstand small variations such as different technicians and reagent preparations.
- Linearity – the assay produces reliable results over a range of concentrations.

Background:

DY19 (*Butyrivibrio fibrisolvens)* solid intermediates are produced by freeze drying the preservation mixture to product PBV material. The PBV is milled to produce MPBV, then coated with wax to produce the lipid encapsulated material. Samples from any of the three steps may be tested. The lipid encapsulated material is used in the production of the finished product.

The growth conditions (media, time, and temperature) for each organism were selected based on standard lab practices for these organisms, development studies, and similar approved methods. All reagents are known to be stable for the duration of the validation activities.

Method Overview:

(b) (4)

Sample Preparation:

(b) (4)

Primary Dilution Preparation

Sample #	Sample Type	Sample Lot Number/ID	Approximate Viability
1	Lot A, normal concentration	787C-2042-A001	~5E8 CFU/g
2	Lot A, 5x lower concentration	787C-2042-A001	~5E8 CFU/g
3	Lot A, 10x lower concentration	787C-2042-A001	~5E8 CFU/g
4	Lot B, normal concentration	19-0202-007-P56	~5E8 CFU/g

Validation Approach:

Version 1 of the DY19 Solid Intermediate Microbe Enumeration method will be followed. The method is retained here:

(b) (4)

Sample 1 will be assayed three times by analyst 1 to demonstrate repeatability of the assay.

Samples 2 – 4 will be assayed one time by analyst 1.

A second analyst will assay samples 1 – 4.

Each analyst will use different batches of reagents and plates.

The closeness of results between analysts will be assessed to determine the robustness of the assay. Graphs of the data from samples 1-3 will be generated to demonstrate assay linearity.

All equipment calibrations are recorded in lab documentation. Raw data will be recorded directly in the protocol.

Data Analysis:

The calculation for converting the raw colony numbers to the CFU/g is listed in the method. The CV and Standard Deviation calculations are also listed in the method.

Acceptance Criteria:

- The assay yields comparable results when the same sample is assayed multiple times by one analyst (repeatability).
- The assay is robust when the same sample is assayed by different personnel with different reagents.
- The assay is linear.
- Coefficient of Variation (CV%) is +/- 75% for results on the same sample.

Summary and Conclusions:

A summary report will be prepared based on the validation results. Post-approval of the executed protocol and the summary report will occur simultaneously. The summary will include the following information:

- Changes to the original protocol
- Deviations from the protocol
- Statistical analysis of the data
- Conclusions developed from the data, including if the acceptance criteria were met
- Statement as to the method validation status
- Location of all raw data (if not recorded in the protocol).

Data Collection – Analyst 1 Name [redacted]

Sample ID	Replicate	Dilution	Colonies	Initial/Date	Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-1	[redacted]			[redacted] 23Nov20	1-2	[redacted]			[redacted] 23Nov20

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-3	(b) (4)			(b) (4), (b) (6) 23Nov20

Sample ID	Replicate	Dilution	Colonies	Initial/Date
2	(b) (4)			(b) (4) 23Nov20

Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	(b) (4)			(b) (4), (b) (6) 23NOV20

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	(b) (4)			(b) (4), (b) (6) 23NOV20

CFU Results

Sample		[redacted] (b) (4), (b) (6)	Initial/Date		Sample		[redacted] (b) (4), (b) (6)	Initial/Date
1-1	Final Result (CFU/mL)		23Nov20		1-3	Final Result (CFU/mL)		23Nov20
	Standard Deviation		23Nov20			Standard Deviation		23Nov20
1-2	Final Result (CFU/mL)		23Nov20		2	Final Result (CFU/mL)		23Nov20
	Standard Deviation		23Nov20			Standard Deviation		23Nov20

CFU Results			
Sample			**Initial/Date**
3	Final Result (CFU/mL)	(b) (4), (b) (6)	23Nov20
	Standard Deviation		23Nov20
4	Final Result (CFU/mL)		23Nov20
	Standard Deviation		23Nov20

Data Collection – Analyst 2 . Name Sean Gilmore

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1	(b) (4)	[redacted]	[redacted]	(b) (4), (b) (6) 13NOV2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
2	[redacted]	[redacted]	(b) (4)	(b) (4), (b) (5) 13NOV2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	[redacted]	[redacted]	[redacted]	(b) (4), (b) (6) 23NOV2020

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	[redacted]	[redacted]	[redacted]	(b) (4), (b) (6) 23NOV2020

CFU Results

Sample			Initial/Date		Sample			Initial/Date
1	Final Result (CFU/~~mL~~) CY	(b) (4), (b) (6)	3/30/20		3	Final Result (CFU/mL)	(b) (4), (b) (6)	3/30/20
	Standard Deviation		3/30/20			Standard Deviation		3/30/20
2	Final Result (CFU/mL)		3/30/20		4	Final Result (CFU/mL)		3/30/20
	Standard Deviation		3/30/20			Standard Deviation		3/30/20

Method Validation Summary Report

Method

Dairy-19 Solid Intermediate Microbe Enumeration, V1

Objective

The objective of this validation was to demonstrate that the DY19 Solid Intermediate Microbe Enumeration method can quantify the amount of DY19 (*Butyrivibrio fibrisolvens* ASCUSDY19) in solid forms such as the *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate final product. The method was evaluated for repeatability, robustness, and linearity.

Repeatability was assessed through the closeness of results obtained on the same sample (787C-2042-A001) when assayed multiple times by the same person with the same reagents and equipment.

Robustness was assessed through the closeness of results obtained on the same set of samples (787C-2042-A001 and 19-0202-007-P56) across multiple analysts and reagent preparations.

Linearity was assessed by enumerating the same sample at a concentration of 20% and 10% of the original sample (787C-2042-A001).

Results

Repeatability

The average of samples 1-1, 1-2, and 1-3 is 3.75E+08 CFU/g with a standard deviation of 1.26E+08 CFU/g. The coefficient of variation from these samples is 33%. The low CV resulting from repeated measurements of the same sample demonstrates the repeatability of the assay.

Table 1: Summary table of DY19 solid enumeration method validation results

		Average CFU/g	STDEV	CV
Analyst 1	Sample 1-1	(b) (4)		
	Sample 1-2			
	Sample 1-3			
	Sample 2			
	Sample 3			
	Sample 4			
Analyst 2	Sample 1			
	Sample 2			
	Sample 3			
	Sample 4			

Robustness

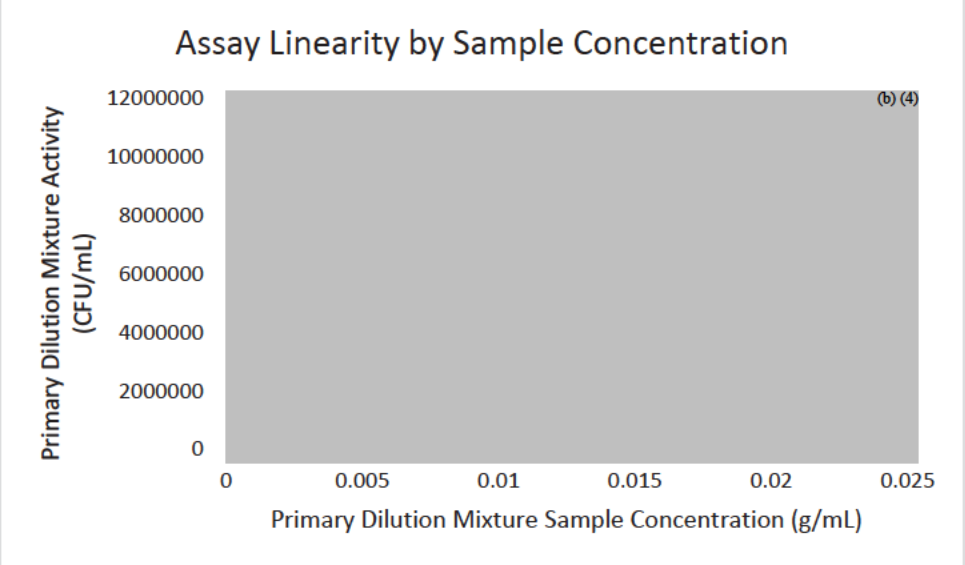
Samples 1-4 were enumerated by two independent analysts. All measurements yielded a CV less than 41% within an analyst's measurements and a CV less than 43% for combined measurements of both analysts, demonstrating that the assay is robust to different analysts and reagent preparations.

Table 2: Summary of Repeatability, Linearity, and Robustness

Repeatability			
Average Sample 1	(b) (4)		
3.75E+08			
Linearity			
$R^2 = 0.89$			
Robustness across analysts			
	Average	STDEV	CV
Sample 1	(b) (4)		
Sample 2			
Sample 3			
Sample 4			

Linearity

Sample 2 was prepared by diluting Sample 1 to 20%, and Sample 3 was prepared by diluting Sample 1 to 10% in the primary dilution mixture. The activity (CFU/mL) of the resulting primary dilution mixtures were plotted against the sample concentration (g/mL). The resulting linear regression had an R^2 value of 0.89, suggesting linearity between the two parameters.



Conclusion

The Dairy-19 Solid Intermediate Microbe Enumeration assay is valid, demonstrated by the repeatability, robustness, and linearity of the assay. The protocol was executed as written with no deviations or changes during execution.

Raw data and analysis can be found on the company (b) (4) (b) (4)

Approval

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
Patricia A. Williams Quality	DocuSigned by: Patricia A. Williams 5B301285A10643D... 12/1/2020

Method

Title	Dairy-19 Solid Intermediate Microbe Enumeration
Version	01
Effective Date	13Nov2020
Author	Sean Gilmore
Approver (Signature & Date)	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 11/10/2020 Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Butyrivibrio fibrisolvens* in Dairy-19 solid intermediates in samples from:

- Preservation by Vaporization (PBV) or milled PBV (mPBV) intermediate
- Lipid Encapsulated intermedate

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with liquid nitrogen and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

1000 µL pipette tips, sterile, anaerobic
200 µL pipette tips, sterile, anaerobic
20 µL pipette tips, sterile, anaerobic
96-well (8x12 well) 200 µL plate, sterile, anaerobic
Reagent reservoir, sterile, anaerobic
1.5 mL microcentrifuge tubes, sterile, anaerobic
Liquid Nitrogen
10% Bleach
>70% Ethanol or Isopropanol

Equipment

Autoclave
Laboratory Vortexer
Mortar and Pestle
Anaerobic Chamber
Dissection microscope or magnifying glass
1000 µL Pipette
200 µL Pipette
200 µL Multi-channel Pipette
20 µL Multi-channel Pipette

Media & Reagents

TSB+FAC plates

Anaerobic Phosphate Buffered Saline (PBS)

(recipes can be found here:

(b) (4)

Method

1. De-encapsulation of DY19 Lipid Encapsulate

(b) (4)

2. Prepare the Primary Dilution Mix

(b) (4)

3. DY19 Solid Intermediate Anaerobic Plating

(b) (4)

(b) (4)

4. Spot Plating

(b) (4)

5. Enumeration and Colony Forming Unit Determination

(b) (4)

(b) (4)

Re

(b) (4)

ASCUS BIOSCIENCES

Method

Title	**Moisture Analysis**
Version	**01**
Effective Date	**15Dec2019**
Author	**Adam Taylor**
Approver (Signature & Date)	DocuSigned by: Martin Maynew D1605F1B4C3E49A... 12/3/2019 **Martin Maynew – VP – Process Development & Manufacturing**

Scope

This method is used to determine the moisture content of solid samples such as Galaxis 100, Altius 5, DY20 SDP, and DY21 POE.

Safety

Wear safety goggles, lab coat, and gloves when handling samples.
Use caution when removing the sample as the sample, chamber, and draft shield may be extremely hot.

Materials

None

Equipment

Ohaus Moisture Analyzer (multiple models may be used)

Media and Reagents

None

Method

(b) (4)

(b) (4)

Reasons for Revision

(b) (4)

Method

Title	Dairy-19 Liquid Intermediate Microbe Enumeration
Version	02
Effective Date	09Nov2020
Author	Sean Gilmore
Approver (Signature & Date)	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 11/3/2020 Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Butyrivibrio fibrisolvens* in Dairy-19 liquid intermediates samples from:

- End of Fermentation
- Cell Concentrate
- Preservation Mixture

Safety

Consult the Safety Data Sheet for all reagents prior to handling.

Materials

1000 µL pipette tips, sterile, anaerobic
200 µL pipette tips, sterile, anaerobic
20 µL pipette tips, sterile, anaerobic
96-well (8x12 well) 200 µL plate, sterile, anaerobic
Reagent reservoir, sterile, anaerobic
1.5 mL microcentrifuge tubes, sterile, anaerobic

Equipment

Autoclave
Laboratory Vortexer
Anaerobic Chamber
Dissection microscope or magnifying glass
1000 µL Pipette
200 µL Pipette
200 µL Multi-channel Pipette
20 µL Multi-channel Pipette

Media & Reagents

TSB+FAC plates
Anaerobic Phosphate Buffered Saline (PBS)

(b) (4)

Method

(b) (4)

(b) (4)

3. Spot Plating

(b) (4)

4. Enumeration and Colony Forming Unit Determination

(b) (4)

Cont

(b) (4)

Reasons for Revision

(b) (4)

Method

Title	DY21-POE Microbe Enumeration
Version	05
Effective Date	15May2020
Author	Miranda Striluk
Approver (Signature & Date)	DocuSigned by: Martin Maylew 5/8/2020 Martin Maylew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of Dairy-21 in Dairy-21 Palm Oil Encapsulate by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath, hot liquids, liquid nitrogen, and extremely cold material. Liquid nitrogen can cause cold burns, frostbite, and permanent eye damage from brief exposure. Avoid skin and eye contact with liquid nitrogen and wear appropriate personal protective equipment (safety glasses and gloves) at all times. Analyst should be trained on liquid nitrogen handling before continuing this method.

Materials

Corning® 15mL Polypropylene Centrifuge Tubes (Corning 430052)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Mortar and Pestle
Magnetic Stir Plate

Media & Reagents

YPD Plates
Growcells 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (Growcells MRGF-6235)
Growcells 1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (Growcells MRGF-6275)
Reagent grade 95% Ethanol
70% Ethanol
10% Bleach
Liquid Nitrogen
1N Hydrochloric Acid
1N Sodium Hydroxide

DY21-POE Microbe Enumeration

Method

1. Preparation of sterile 1X Phosphate Buffered Saline (PBS), pH 7.4

(b) (4)

2. De-encapsulation of Spray Congealed DY21-POE

(b) (4)

3

(b) (4)

4. DY21-POE Aerobic Plating

(b) (4)

DY21-POE Microbe Enumeration

5. Negative Control Plating

6.

DY21-POE Microbe Enumeration

Reasons for Revision

Product Certificate of Analysis

Product Name	*B. fibrisolvens* ASCUSDY19 Fat Encapsulated Product
Batch Number	1801.2033
Date of Manufacture	24Nov2020
Expiration Date	N/A
Retest Date	24Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count		(b) (4)
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

DocuSigned by:
Kelly Mercier
7BD513E026E94C0...

12/28/2020

Kelly Mercier
Quality

Product Certificate of Analysis

Product Name	*B. fibrisolvens* ASCUSDY19 Fat Encapsulated Product
Batch Number	1801.2035
Date of Manufacture	23Nov2020
Expiration Date	N/A
Retest Date	23Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count		(b) (4)
Coliform		
E. coli		
Salmonella		
Listeria		

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

DocuSigned by:
Kelly Mercier
7BD513E026E94C0...

12/28/2020

Kelly Mercier
Quality

Product Certificate of Analysis

Product Name	*B. fibrisolvens* ASCUSDY19 Fat Encapsulated Product
Batch Number	1801.2037
Date of Manufacture	24Nov2020
Expiration Date	N/A
Retest Date	24Nov2021
Storage Conditions	2 - 10°C

Analytical Property	**Specification**	**Result**
Viable cell count	>2.0 E+07 CFU/g	5.48 E+08 CFU/g
Coliform	<10 CFU/g	<10 CFU/g
E. coli	<10 CFU/g	<10 CFU/g
Salmonella	Negative/25g	Negative
Listeria	Negative/25g	Negative

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

DocuSigned by:
Kelly Mercier
7BD513E026E94C0...

12/28/2020

Kelly Mercier
Quality

Stability Protocol Title:	DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 50°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	24hr	48hr	72hr	96hr
DY19 Solid Intermediate Microbe Enumeration method	(b) (4)				

Protocol Approvals:

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F... 12/1/2020
Kelly Mercier Quality	DocuSigned by: kelly Mercier 7BD513E026E94C0... 12/1/2020

Product Certificate of Analysis

Product Name	*B. fibrisolvens* ASCUSDY19 Freeze-dried Powder
Batch Number	1801.2035
Date of Manufacture	26Oct2020
Expiration Date	N/A
Retest Date	26Oct2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count		(b) (4)

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

DocuSigned by:
Kelly Mercier
7BD513E026E94C0...

12/28/2020

Kelly Mercier
Quality

Product Certificate of Analysis

Product Name	*B. fibrisolvens* ASCUSDY19 Freeze-dried Powder
Batch Number	1801.2037
Date of Manufacture	03Nov2020
Expiration Date	N/A
Retest Date	03Nov2021
Storage Conditions	2 - 10°C

Analytical Property	Specification	Result
Viable cell count		(b) (4)

Approval (Name, Title, Signature, and Date)

This batch was manufactured and tested according to the product registration and regulatory agency requirements.

DocuSigned by:
Kelly Mercier
7BD513E026E94C0...

12/28/2020

Kelly Mercier
Quality

Analysis of *Butyrivibrio fibrisolvens* ASCUSDY19 POE (DY19) for Heavy Metals & Microbial Contamination

Approvers:

DocuSigned by:
Martin Mayhew
CA3DAF452B8A47C...

12/18/2020

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
Kelly Mercier
7BD513E026E94C0...

12/18/2020

Kelly Mercier
Quality

Date

DocuSigned by:
Kevin Korth
1C2149273B2345F...

12/18/2020

Kevin Korth
Regulatory

Date

Prepared by
Native Microbials, Inc
San Diego, CA

December 2020

Analysis of *Butyrivibrio fibrisolvens* ASCUSDY19 POE for Heavy Metals & Microbial Contamination

Three lots of *Butyrivibrio fibrisolvens* ASCUSDY19 POE were sent for heavy metal and microbial contamination analysis at (b) (4) (b) (4) Note: *B. fibrisolvens* Dairy-19 Fat Encapsulate which was internal name used by Native Microbials, Inc.)

The ICP-MS/AOAC 2015.01 method was used for the heavy metal analysis of the samples and results are summarized in the following table.

Table 1. Heavy Metal Analysis of Three Lots of *Butyrivibrio fibrisolvens* ASCUSDY19 POE

Lot Number	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
Detection Limit	0.004	0.0008	0.001	0.001
DY19 1801.2033	ND	ND	ND	ND
DY19 1801.2035	0.013	ND	ND	ND
DY19 1801.2037	0.015	ND	0.003	ND

ND – None Detected

The methods used for analysis were AOAC 2018.13 for Coliforms/E. coli, AOAC 2013.01 for Salmonella, and AOAC 2013.10 for Listeria. Results are summarized in the following table.

Table 2. Microbial Contamination Testing for *Butyrivibrio fibrisolvens* ASCUSDY19 POE

Lot Number	Coliform, CFU/g	E. coli, CFU/g	Salmonella, per 25g	Listeria, per 25g
Requirement	<10	<10	Negative	Negative
DY19 1801.2033	<10	<10	Negative	Negative
DY19 1801.2035	<10	<10	Negative	Negative
DY19 1801.2037	<10	<10	Negative	Negative

B. fibrisolvens ASCUSDY19 POE is intended to be fed as part of the product mixed in a grain premix then further diluted in a total mixed ration or grain supplement. Given the low inclusion rate in the grain mix (5 g/cow/day) and further dilution in the total mixed ration, no heavy metal specification is needed. However, all lots will be tested for microbial contamination at the end of the production of *B. fibrisolvens* ASCUSDY19 POE.

Attachment 1. Certificate of Analysis – Heavy Metal Analysis (b) (4) Sample No. 1065824)

[redacted]

Certificate of Analysis

December 09, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No (b) (4)
Sample No. 1065824

SAMPLE INFORMATION

Description	Dairy-19 Fat Encapsulate
Lot Number	1801.2033
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 08, 2020 to December 09, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	None detected
Cadmium	0.0008/0.0008	None detected
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	None detected

Reported by
(b) (4)
[redacted]
December 09, 2020

ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

(b) (4)
[redacted]

Attachment 2. Certificate of Analysis – Heavy Metal Analysis (b) (4) Sample No. 1065825)

(b) (4)

Certificate of Analysis

December 09, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No. (b) (4)
Sample No. 1065825

SAMPLE INFORMATION

Description	Dairy-19 Fat Encapsulate
Lot Number	1801.2035
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 08, 2020 to December 09, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	0.013
Cadmium	0.0008/0.0008	None detected
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	None detected

Reported by
(b) (4)
December 09, 2020

ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

(b) (4)

Attachment 3. Certificate of Analysis –Heavy Metal Analysis (b) (4) Sample No. 1065826)

(b) (4)

Certificate of Analysis

December 09, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No (b) (4)
Sample No. 1065826

SAMPLE INFORMATION

Description	Dairy-19 Fat Encapsulate
Lot Number	1801.2037
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 08, 2020 to December 09, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	0.015
Cadmium	0.0008/0.0008	None detected
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	0.003

Reported by
(b) (4)
Lab Co Director
December 09, 2020

ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

(b) (4)

Attachment 4. Certificate of Analysis – Microbial Contamination Testing (b) (4) Sample No. 1065824)

(b) (4)

Certificate of Analysis

December 15, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

Order No (b) (4)
Sample No. 1065824

SAMPLE INFORMATION

Description	Dairy-19 Fat Encapsulate
Lot Number	1801.2033
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis Date December 08, 2020 to December 15, 2020

Findings

Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

Reported by
(b) (4)

(b) (4)

Attachment 5. Certificate of Analysis – Microbial Contamination Testing (b) (4) Sample No. 1065825)

(b) (4)

Certificate of Analysis

December 15, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

(b) (4)
Order No.
Sample No. 1065825

SAMPLE INFORMATION

Description	Dairy-19 Fat Encapsulate
Lot Number	1801.2035
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis Date December 08, 2020 to December 15, 2020

Findings

Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

Reported by
(b) (4)

(b) (4)

Attachment 6. Certificate of Analysis – Microbial Contamination Testing (b) (4) ample No. 1065826)

(b) (4)

Certificate of Analysis

December 15, 2020

NATIVE MICROBIALS, INC.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

(b) (4)
Order No
Sample No. 1065826

SAMPLE INFORMATION

Description	Dairy-19 Fat Encapsulate
Lot Number	1801.2037
Received	December 08, 2020

ANALYTICAL RESULTS

Analysis Date December 08, 2020 to December 15, 2020

Findings

Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

Reported by
(b) (4)

(b) (4)

Suggested Decision Tree for determining the safety of microbial cultures for consumption by humans and animals (Pariza et al, 2015)

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?
(If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).

2. Has the strain genome been sequenced?
(If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?
(If YES, go to 4. If NO, go to 15.)

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?
(If YES, go to 5. If NO, go to 15.)

5. Does the strain produce antimicrobial substances?
(If NO, go to 6. If YES, go to 15.)

6. Has the strain been genetically modified using rDNA techniques?
(If YES, go to 7a or 7b. If NO, go to 8a or 8b.)

7a For strains to be used in human food: Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food?
(If YES, go to 8a. If NO, the expressed product(s) must be shown to be safe before proceeding to 8a.)

7b For strains to be used in animal feed: Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in feed for the target animal species?
(If YES, go to 8b. If NO, the expressed product(s) must be shown to be safe for the target animal species before proceeding to 8b.)

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')?
(If YES, go to 9a. If NO, go to 13a.)

8b For strains to be used in animal feeds: Was the strain isolated from a feed (for example, silage) that has a history of safe consumption by target animals, for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?
(If YES, go to 9b. If NO, go to 13b.)

9a For strains to be used in human food: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts?
(If YES, go to 10a. If NO, go to 13a.)

9b For strains to be used in animal feeds: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for feed use by an authoritative group of qualified scientific experts?
(If YES, go to 10b. If NO, go to 13b.)

10a For strains to be used in human food: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food?
(If YES, go to 11a. If NO, go to 13a.)

10b For strains to be used in animal feeds: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9b continue to support the conclusion that the species, to which the strain belongs, is safe for use in feed?
(If YES, go to 11b. If NO, go to 13b.)

11a For strains to be used in human food: Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in “traditional” food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)?
(If NO, go to 12a. If YES, go to 13a.)

11b For strains to be used in animal feeds: Will the intended use of the strain expand exposure to the species beyond the target animals that typically consume the species in “traditional” feed(s) in which it is typically found (for example, will a strain that was isolated from silage be used in swine feed)?
(If NO, go to 12b. If YES, go to 13b.)

12a For strains to be used in human food: Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)?
(If NO, go to 14a. If YES, go to 13a.)

12b For strains to be used in animal feeds: Will the intended use of the strain expand intake of the species (for example, increasing the number of feeds beyond the traditional feeds in which the species is typically found, or using the strain as a probiotic rather than as a silage starter culture)?
(If NO, go to 14b. If YES, go to 13b.)

13a For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
(If yes, go to 15. If no, go to 14a.)

13b For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?
(If yes, go to 15. If no, go to 14b.)

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

14b The strain is deemed to be safe for use in the manufacture of feeds, probiotics, and dietary supplements for animal consumption.

15. The strain is NOT APPROPRIATE for human or animal consumption.

Pariza Decision Tree as applied to *Butyrivibrio fibrisolvens* ASCUSDY19

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?

Yes, go to 2.

2. Has the strain genome been sequenced?

Yes, go to 3.

3. Is the strain free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?

Yes, go to 4.

4. Is the strain genome free of functional transferable antibiotic resistance gene DNA?

Yes, go to 5.

5. Does the strain produce antimicrobial substances?

No, go to 6.

6. Has the strain been genetically modified using rDNA techniques?

No, go to 8b.

8b. For strains to be used in animal feeds: Was the strain isolated from a feed (for example, silage) that has a history of safe consumption by target animals, for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')?

No, go to 13b.

13b For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?

No, go to 14b.

14b The strain is deemed to be safe for use in the manufacture of feeds, probiotics, and dietary supplements for animal consumption.

Safety is based on (a) natural occurrence and prevalence of B. *fibrisolvens* ASCUSDY19 in the rumen of ruminants; and (b) characterization of the strain to indicate absence of any anticipated virulence factors for pathogenicity or anti-microbial resistance of concern.

A literature search was conducted on December 22, 2020 in order to identify potential information related to the safety and utility of *Butyrivibrio fibrisolvens* as a direct fed microbial (DFM) strain for cattle. The overall search strategy is described in Table 1. The Web of Science database was searched using the keyword/search terms listed in Table 2. The search was verified by reviewing the primary hits from a Google Scholar search.

Considering the number of articles identified (>500), the search results were reviewed to identify articles representative of the body of available data relating to the safety of the species. In particular, the review focused on identifying comprehensive reviews, widely cited articles and recent articles of relevance.

Nomenclature

The NCBI database was reviewed as well as the published literature to identify all recognized taxonomic classification of the species. This species only has one classified name: *Butyrivibrio fibrisolvens.*

Table 1: Literature Search and Selection Strategy		
Step 1	Records identified using selected literature databases	Web of Science
	Total records (titles/abstracts) identified through electronic search	
Step 2	Screen titles/abstracts and exclude obviously irrelevant records	
Step 3	Review full texts and assess for relevance and eligibility for inclusion	

Table 2: Topic Specific Search Terms using Species			
Search strategy for safety of species [Safety Search]	Keywords/search terms [Database: Google Scholar]	Term 1	*Butyrivibrio fibrisolvens*
		Term 2	Toxi*(n=37) Pathogen* (n=1,080) Safe*(n=954) Infection (n=1,470) Disease (n=2,990) Mortal* (n=30)
Search strategy for safety of *Butyrivibrio fibrisolvens* for cattle [Target Animal Search]	Keywords/search terms [Database: Google Scholar]	Term 1	*Butyrivibrio fibrisolvens*
		Term 2	Cattle (n=4,130) Cow* (n=2,790) Bovine (n=3,750) Ruminant* (n=4,240) Calf (n=691) Calves (n=1,170) Bull* (n=854)

			Heifer* (n=207)
Search strategy for history of use of *Butyrivibrio fibrisolvens* for use in food and feed [History of Use Search]	Keywords/search terms [Database: Google Scholar]	Term 1	*Butyrivibrio fibrisolvens*
		Term 2	Food* (n=6,000) Feed* (n=5,030)

Search: Term 1 in combination with one or more of Term 2; Boolean search techniques were applied.

Microbiome Safety for *Butyrivibrio fibrisolvens* ASCUSDY19

1 Objectives

The objective of this work was to:

- Identify the typical microbial composition of the rumen microbial community of dairy cows using:
    a. Internal datasets (e.g. data and analyses created by Native Microbials)
    b. External datasets (e.g. data published in peer reviewed manuscripts)

2 Materials and Methods

2.1 Native Microbials Animal Experiments

A series of experiments were analyzed in order to obtain a representative sampling of the rumen microbiome composition. These samples were used to determine the typical ranges of abundances of rumen microorganisms under normal, farm-like conditions.

2.1.1 First Survey Experiment Summary

The first survey experiment identified the rumen composition of 8 Holstein dairy cows and 8 Jersey dairy cows. The survey took place in Tulare, CA, and utilized the following diet:

Ingredient	g/100 g dry matter
Alfalfa hay	7.79
Alfalfa green chop	5.98
Hay cubes	4.53
Corn silage	4.08
Wheat Silage	9.51
Almond Hulls	13.58
Citrus pulp	1.36
Wheat straw	0.89
Dry distiller's grains	10.41
Steamed rolled corn	22.54
Canola	5.41
Cottonseed	5.33
Millrun	5.88
Salt	0.46
Molasses + Mineral and vitamin mix	2.26

Chemical analysis	
Crude protein	17.26
Neutral detergent fiber	33.13
Acid detergent fiber	21.12

Animals were also induced into a milk fat depressed state by increasing the amount of concentrate in the diet. Although this report focuses on the microbial composition of healthy animals, this information has been included since independent research has also studied the bacterial composition of acidotic animals.

All animals were cannulated, and rumen samples were a composite sample comprised of rumen content collected from the dorsal, ventral, central, anterior, and posterior regions of the rumen. Samples were collected on Days 0, 1, 3, 6, 9, 10, 11, 14, 19, 22, and 28. Cows were observed daily for overall clinical health throughout the study.

2.1.2 Second Survey Experiment Summary

The second survey experiment identified the rumen composition of 8 Holstein dairy cows. The survey took place in Clemson, South Carolina, and utilized the following diet:

Ingredient	**g/100 g dry matter**
Corn silage	37.0
Alfalfa haylage	17.3
Ground corn	9.2
Matrix corn	—
Roasted soybeans/SBM	5.2
Canola meal	9.4
Cookie meal	5.8
Grass hay/straw	5.4
Sugar cane molasses	2.3
Optigen / Urea	0.5
Cottonseed hulls	5.4
Mineral and vitamin mix	2.5
Chemical composition	**% DM**
CP	16.9
NDF	36.1
ADF	20.8
Starch	23.0

Animals were also induced into a milk fat depressed state by increasing the amount of concentrate in the diet. Although this report focuses on the microbial composition of healthy animals, this information has been included since independent research has also studied the bacterial composition of acidotic animals.

All animals were cannulated, and rumen samples were a composite sample comprised of rumen content collected from the dorsal, ventral, central, anterior, and posterior regions of the rumen. Samples were

collected on Days 0, 3, 6, 9, 10, 16, 19, 22, and 28. Cows were observed daily for overall clinical health throughout the study.

2.2 Sample Collection

Samples were collected by tube or fistula from each cow. Samples were added to a 15-mL conical containing 3 mL stop solution consisting of 95% molecular grade 200 proof ethyl alcohol (Sigma-Aldrich, ST. Louis, MO, USA) and 5% TRI-Reagent (Sigma-Aldrich, St. Louis, MO, USA) and shaken to mix. Samples were stored on site at -80°C and shipped the following Monday overnight on ice to Native Microbials. Upon arrival, 0.5 g of each sample was aliquoted for DNA and RNA extraction and the remaining sample was stored at -80°C.

2.3 DNA/RNA Extraction and Amplification

Rumen samples were centrifuged at 4,000 x g for 15 min, the supernatant was decanted and removed. Approximately 0.5 mL of resultant pellet was aliquoted for DNA extraction using the PowerViral® Environmental RNA/DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The 16S rRNA gene was amplified using 27F and 534R (Lane, et al. 1991, Muyzer, et al. 1993) primers modified for Illumina sequencing, following standard protocols Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA). Following amplification, PCR products were verified with a standard 2% agarose gel electrophoresis and purified using AMPure XP bead (Beckman Coulter, Brea, CA, USA). The purified amplicon library was quantified and sequenced on the MiSeq Platform (Illumina, San Diego, CA, USA) according to standard protocols using a 2x300 v3, 600-cycle kit. Raw fastq reads were de-multiplexed on the MiSeq Platform (Illumina, San Diego, CA, USA). All samples were sequenced at a depth such that each sample file contained at least 10,000 sequences after processing.

2.4 Analysis Method

All raw sequencing data was trimmed of adapter sequences and phred33 quality filtered at a cutoff of 20 using Trim Galore (Krueger, et al. 2015). All remaining sequences were then filtered for PhiX, low complexity reads, and cross-talk. 16S rRNA taxonomic sequence clustering and classification was performed with the USEARCH's UNOISE and SINTAX (v10.0.240) (Edgar, et al. 2015 and 2016b) with the RDA 16S rRNA database (Cole, et al. 2014). Relative abundance was calculated by taking the number sequences matched and the total sequences in each file and dividing them.

2.5 Animal Experiments from Peer-Reviewed Literature

Peer reviewed manuscripts describing the bacterial rumen community using high-throughput, comprehensive bacterial community analyses were collected for further comparative analysis to establish the composition of the "typical" rumen and prevalence of *B. fibrisolvens*. Several bacterial analyses conducted by academic institutions were found for dairy and beef cattle: Jewell, et al. 2015, AlZahal, et al. 2017, Noel et al. 2017, Ribeiro et al. 2017, Petri et al. 2013. These manuscripts were selected based on the marker selected for microbiome analysis (e.g. to maintain compatibility and consistency to internal analyses) and the breadth of diets represented in the analyses:

- Jewell, et al. studied fourteen Holstein dairy cows across two lactation cycles. The major TMR components were corn silage, alfalfa haylage, high-moisture corn, dry corn, and roasted soybeans.

- AlZahal, et al. investigated the role of dietary yeast on the rumen microbial community of 16 multiparous, lactating Holstein cows. The microbiome was characterized while the animals were fed both a high-forage and high-grain diet. The rumen solids, rumen fluids, and epimural microbial communities were analyzed.
- Noel, et al. monitored the rumen microbiome of dairy cows grazing a rye-grass and clover pasture over 5 years.
- Ribeiro, et al. transferred the rumen content of bison to 16 Angus x Hereford heifers to determine if the rumen microbiome could be altered. Heifers were fed a barley straw diet consisting of 70:30 forage-to-concentrate. Although both pre- and post-rumen trasnfer microbiome composition are reported in the manuscript, only the pre-transfer results are presented here.
- Petri, el al. studied the rumen microbiome of 8 Angus heifers undergoing an acidosis challenge. Animals were fed a forage diet, a mixed forage diet, a high grain diet, a challenge diet, and a recovery diet. The microbiome was profiled for each diet.

McCann et al., 2016, McCabe et al., 2015, Meale et al. 2016, and Martinez-Fernandez et al. 2016 were also utilized to determine the abundance of *B. fibrisolvens* in cattle. Although their microbiome analyses were not robust enough to include in the analysis here, the raw reads used for their analyses were publicly available and thus could be used in internal analysis.

3 Results

The rumen microbial community composition is constantly in flux. The microbial population has been shown to change over time in response to a variety of factors, including diet composition, time after feeding, season, and stage of lactation. Additionally, there are groups of microorganisms that are unique to particular breeds of cow, regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Despite this variability, there is a core microbiome that appears in majority of animals. This core has been investigated at Native Microbials, as well as in independent academic studies. Although the results are variable at times, there are several phyla that tend to appear across all dairy cows.

The rumen microbiome is very plastic and highly responsive to external variables. Because of this, defining a “normal healthy” rumen is challenging. High-throughput bacterial community analyses were found for cattle and dairy cows fed a variety of diets (Jewell, et al. 2015, AlZahal, et al. 2017, Noel et al. 2017, Ribeiro et al. 2017, Petri et al. 2013). These manuscripts were further investigated to determine prevalence of *B. fibrisolvens* in cattle and the overall bacterial taxonomic composition of the typical rumen microbiome.

Table 1: Abundance of bacterial phyla in the rumen from independent studies, reported as a percent. Empty cells indicate that data was not reported for the phylum.

** "Rumen core" values reported in Petri, et al 2013 were sourced from Jouany 1991

Study / Phylum	Jewell (TMR)	Noel (Pasture)	Ribeiro (Barley straw)	Petri (Rumen Core*)	Petri (Forage)	Petri (High grain)	Petri (Acidotic)	Petri (Recovery)
Actinobacteria			1.78			1.6		
Bacteroidetes	49.42	11.8	20.29	32.8	25.7	40.3	40	31.5
Fibrobacteres		2.4	25.04		7.1			
Firmicutes	39.32	82.1	40.53	43.2	55.2	37	33.6	43.7
Lentisphaerae			1.35					
Proteobacteria	5.67		1.64	14.3	4.7	17.9	16.5	15.2
Spirochaetes			6.13		2.8			
Tenericutes	2.17							
Unclassified		1.5						
Other (low abundance)		2.2 (16 phyla)	0.08					

Several manuscripts describing the composition of the rumen bacterial community were found. Cumulatively, these independent studies investigated the microbial community across a variety of breeds, diets, and feed management regimes. Lactating and non-lactating animals are also both represented. Table 1 summarizes the findings from Jewell, et al. 2015, Noel et al. 2017, Ribeiro et al. 2017, and Petri et al. 2013 at the phylum level. Overall, Bacteroidetes and Firmicutes tended to dominate the rumen bacterial community, with the exception of the Ribeiro study in which *Fibrobacteres* also represented a substantial portion of the community. As can be seen from this data, there is a broad range of abundances for each phyla across all experiments. Noel, et al. 2017, for example, reported an abundance of 11.8% for Bacteroidetes, and 82.1% for Firmicutes. At the other end of the spectrum, the rumen microbiome composition of acidotic animals in Petri, et al. 2013 consisted of 40% Bacteroidetes and 33.6% Firmicutes. The primary source of variation is likely the diversity of diets fed to the animals. In general, acidotic animals and animals on high concentrate diets exhibited much higher Bacteroidetes abundances than animals primarily receiving forage. On average, these animals had a Bacteroidetes abundance of 44% while the animals receiving forage had an average Bacteroidetes abundance of 22%. Despite the high variability in abundance, there does seem to be a typical range for the most predominant phyla. Overall, the observed abundance of Bacteroides within this group of healthy animals ranged from 11.8%-49.49%, while the observed abundance of Firmicutes ranged from 33.6%-82.1%. Other phyla did appear, but often represented less than 10% of the total bacterial population

Table 2. Abundance of bacterial phyla in the rumen of control animals from AlZahal, et al. 2017, reported as a percent.

Diet	**High Forage**			**High Grain**		
Rumen Sampling Location	**Solids**	**Fluid**	**Epimural**	**Solids**	**Fluid**	**Epimural**
Phylum						
Bacteroidetes	29.3	38	30	44.2	50.5	39
Firmicutes	15.4	13.5	21.9	27.3	23.3	22
Unclassified	18.8	15.8	23.6	13.1	11.6	17
Fibrobacteres	19	12.3	5.4	7.6	4.1	1.1
Proteobacteria	2.1	4.8	7.2	1.1	2.4	12.7
Tenericutes	6.2	3.9	3.5	1	0.8	0.7
Cyanobacteria	1.8	4.1	1.5	1.4	3	1.3
SR1	1.8	2	1.4	0.2	0.8	1.3
Spirochaetes	2.5	2	1.4	1.5	0.7	1

AlZahal, et al. provided the most comprehensive analysis of the rumen bacterial microbial community. In their experiment, the bacterial community was profiled in 16 multiparous Holstein cows using Illumina amplicon sequencing of the 16S rRNA region. Cows received a high forage or high grain diet, with or without yeast supplementation. The rumen was sampled from three different locations (solids, fluid, and epimural), and the rumen bacterial population was reported for animals within each treatment group for each sampling location (Tables 2 and 3). Again, Bacteroides and Firmicutes were found the dominate the rumen bacterial community. However, in this study, both Bacteroides and Firmicutes abundances increased in the high grain diet. Despite this, the observed abundances of Bacteroides was consistent with the previously described studies-- Bacteroides percent abundance ranged from 28.6%-50.5%. Firmicutes abundance was slightly lower, as it ranged from 13.5%-27.3%. The reads for this study are unavailable, limiting further analysis of this discrepancy (e.g. determining if the "Unclassified" reads are sequencing error or novel phyla).

Table 3. Abundance of bacterial phyla in the rumen of animals supplemented with yeast from AlZahal, et al. 2017, reported as a percent.

Diet	**High Forage**			**High Grain**		
Rumen Sampling Location	**Solids**	**Fluid**	**Epimural**	**Solids**	**Fluid**	**Epimural**
Phylum						
Bacteroidetes	28.6	38.5	33.1	42.8	47.1	37.1
Firmicutes	16.6	13.2	22	21.2	21.8	21.4
Unclassified	18.4	14.9	22.1	13.9	13.7	20
Fibrobacteres	17.8	11.8	5.1	11.4	4.7	1.5
Proteobacteria	2.1	5.6	7.3	1.2	2.7	9.6
Tenericutes	6.2	3.9	3.3	1.4	1.3	1.1
Cyanobacteria	2	4.5	1.7	1.7	3.8	1.8
SR1	2.5	3	1.8	0.5	1.1	2
Spirochaetes	2.3	1.5	1.5	1.9	1	1.3

Table 4. The abundance of *B. fibrisolvens* in the rumen based on independent research, reported as a percent.

Study	**Animal Type**	**Average Abundance (%)**	**Low (%)**	**High (%)**
McCann, 2016	**Holstein cows**	N/A	0.001	0.82
Meale, 2016	**Holstein calves (female)**	N/A	1.27	3.39
Jewell, 2015	**Holstein cows**	2.38	0.038	3.0
Petri, 2013	**Holstein heifers**	2.3	1.9	2.7

The abundance of *B. fibrisolvens,* specifically, was also investigated in the studies in which raw sequencing reads could be obtained. Table 4 lists the results of this analysis. As can be seen from the table, *B. fibrisolvens* was found in all studies analyzed. The average abundance of this organism ranged from 0.0011%-0.32%. The highest observed value occurred in Meale, et al. with a value of 3.39%.

The Native Microbials studies reported here include two different general survey experiments. The first survey experiment determined rumen microbial compositions of 16 mid-lactation Holstein and Jersey cows fed a typical California farm TMR diet. The average rumen bacterial phyla abundances are shown in Table 5. The second survey experiment was a general survey of 16 mid-lactation Holstein cows production animals receiving a TMR diet in South Carolina. In all of these experiments, the abundances of the most predominant phyla were comparable to the ranges observed in the independent literature studies. The typical abundance of *B. fibrisolvens*, specifically, in the rumen of a dairy cow based on Native Microbials studies was found to be ~0.0001%-1% of rumen bacterial population.

Table 5. Abundance of Major Rumen Bacterial Phyla in the Rumen from Native Microbials Survey Experiments, Reported as a Percent

Study Phylum	NM Experiment 1 – TMR	NM Experiment 1– Acidotic	NM Experiment 2 – TMR	NM Experiment 2– Acidotic
Acidobacteria	0.0046	0.0016	0.0018	0.0025
Actinobacteria	0.88	1.52	1.53	1.871
Armatimonadetes	0.027	0.0050	0.0010	0.0024
Bacteroidetes	36.67	36.339	24.75	44.35
Chloroflexi	0.16	0.026	0.16	0.19
Elusimicrobia	0.035	0.27	0.077	0.033
Fibrobacteres	1.53	0.49	3.71	1.15
Firmicutes	46.82	48.41	61.85	46.98
Fusobacteria	0.0001	0.0014	0.00048	0.0045
Lentisphaerae	0.16	0.078	0.045	0.028
Planctomycetes	0.028	0.024	0.083	0.024
Proteobacteria	5.49	11.20	3.630	3.36
Spirochaetes	2.72	0.66	1.70	0.55
Synergistetes	0.11	0.30	0.22	0.08
Tenericutes	1.26	0.43	1.20	0.70
Verrucomicrobia	0.068	0.015	0.029	0.008
Cyanobacteria/Chloroplast	0.20	0.21	0.30	0.22
SR1	0.51	0.0012	0.16	0.04
TM7	2.41	0.0044	0.54	0.40

4 Conclusion

The rumen bacterial population composition was investigated using internal animal survey experiments as well as external, peer-reviewed experiments. Typical ranges of the native bacteria population as well as the abundance of the native population of *B. fibrisolvens* were identified.

Signed: (b) (6) _____ Date: _____

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Stability Protocol Title:	DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 40°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	Day 3	Day 7	Day 14	Day 21
DY19 Solid Intermediate Microbe Enumeration method	(b) (4)				

Protocol Approvals:

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F... 12/1/2020
Kelly Mercier Quality	DocuSigned by: kelly Mercier 7BD513E026E94C0... 12/1/2020

Stability Protocol Title:	DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 50°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	24hr	48hr	72hr	96hr
DY19 Solid Intermediate Microbe Enumeration method	(b) (4)				

Protocol Approvals:

Name & Title	Signature & Date
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Kelly Mercier Quality	DocuSigned by: kelly Mercier 7BD513E026E94C0... 12/1/2020

Stability Protocol Title:	DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 60°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	4hr	8hr	24hr	48hr
DY19 Solid Intermediate Microbe Enumeration method	(b) (4)				

Protocol Approvals:

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F... 12/1/2020
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Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19 Accelerated Stability Report

Approvers:

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12/30/2020

Sean Gilmore
Scientist

Date

Prepared by
Native Microbials, Inc
San Diego, CA

December 2020

Objective

The objective of this analysis was to utilize accelerated stability data obtained from *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate Lots 1801.2033, 1801.2035, and 1801.2037 to establish a shelf life under normal storage conditions (2-10°C).

The Arrhenius equation can be used to predict decay rates at various temperatures according to the following equation:

$$k = Ae^{\frac{-Ea}{RT}}$$

where k represents the rate of decay, A is the pre-exponential factor, E_a is the activation energy of the decay reaction, R is the universal gas constant, and T is the temperature of the reaction. When rearranged, the equation can take a linear form:

$$ln\,(k) = ln\,(A) + \frac{-Ea}{R}\left(\frac{1}{T}\right)$$

Results

Samples from each lot were placed at 40°C, 50°C, and 60°C and analyzed over time for viable cell count. The results are shown in Tables 1-3 and plotted in Figure 1.

Table 1: Stability at 40°C

	Lot 1801.2033	Lot 1801.2035	Lot 1801.2037
Time (hr)	(b) (4)		
0			
72			
168			
336			
504			

Table 2: Stability at 50°C

	Lot 1801.2033		Lot 1801.2035		Lot 1801.2037	
Time (hr)	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV
0	(b) (4)					
8						
24						
48						
96						

Table 3: Stability at 60°C

	Lot 1801.2033		Lot 1801.2035		Lot 1801.2037	
Time (hr)	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV
0	(b) (4)					
4						
8						
24						
48						

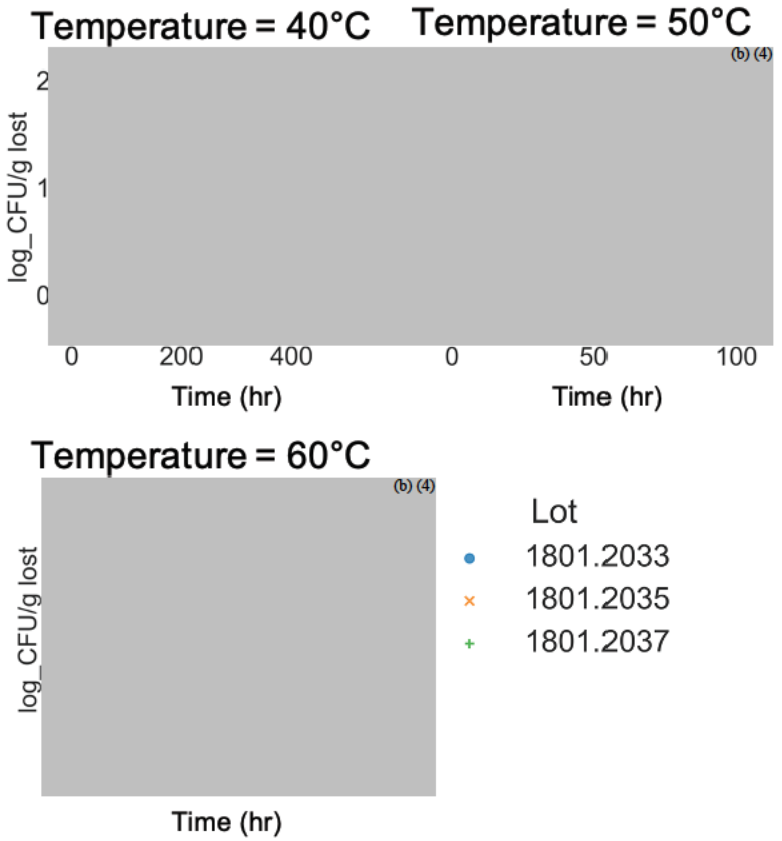

Figure 1: Rate of Decay at 40°C, 50°C, and 60°C. The decay over time is plotted for each lot at each temperature. A rate of decay was calculated from the slope of the regression, displayed as a dark line. The light shaded area represents the 95% confidence interval for the regression.

Rates of decay for each lot at each temperature were calculated from the slope of decay over time. As shown in Figure 2, the probability distributions of predicted rates of decay for the 3 lots at 40°C were not overlapping. Therefore, independent shelf life analysis of each lot was required and the rate data from all 3 lots could not be pooled for a combined analysis.

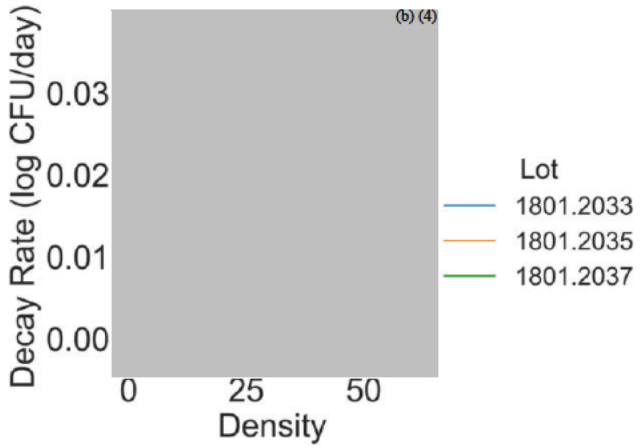

Figure 2: Probability Distributions for the Rate of Decay at 40°C. The probability distribution of decay rates for the three lots are represented. The distributions are non-overlapping, demonstrating that the rate of decay for the three lots are significantly different at 40°C. Therefore, independent analysis of each lot is required.

The rates of decay for each lot were fit to the linear form of the Arrhenius equation in order to provide a prediction for the rate of decay at 10°C, given storage temperatures of 2-10°C. The linear Arrhenius regressions for the 3 lots are shown in Figure 3.

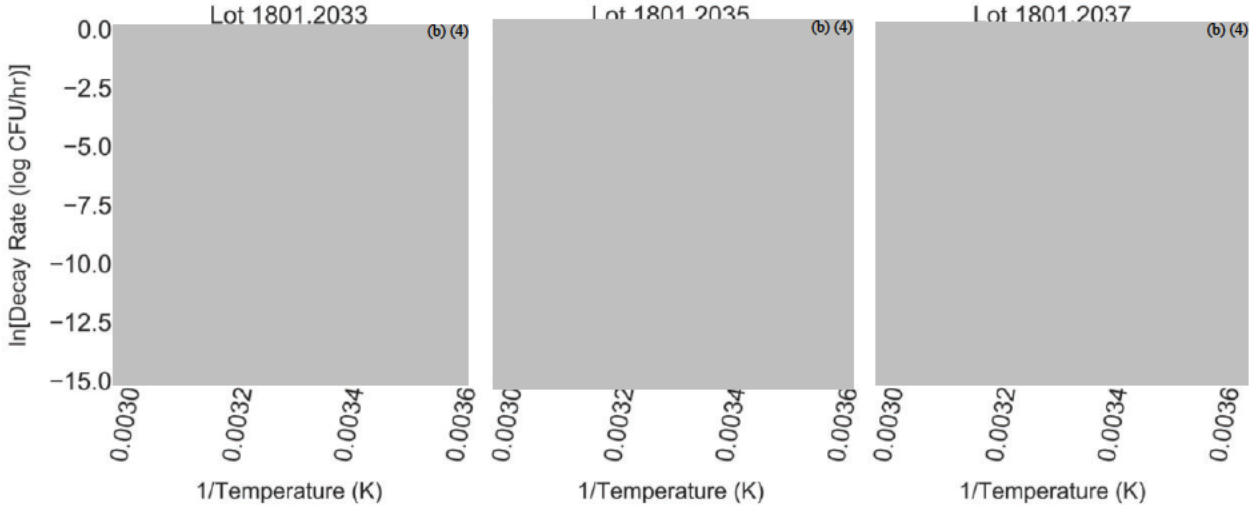

Figure 3: Linear Arrhenius Regression. Decay rate and temperature were transformed to fit the linear form of the Arrhenius equation. The linear regression of the decay rate probability distributions for each lot are displayed. From the linear regression, a rate of decay at 10°C was predicted for each lot, which was then used to determine the shelf life. 10°C corresponds to a value of 0.00353 on the x-axis.

From the linear regression, the upper-tailed 95% confidence interval for decay rate at 10°C was (b) (4) (b) (4) which resulted in the shortest predicted shelf life at 10°C of 41,366 days.

Table 4: Predicted Decay Rate and Shelf Life at 10°C

Lot	Initial CFU/g	Log CFU/g to minimum	ln[Decay Rate (log CFU/hr)]	Predicted Decay Rate (Log CFU/day)	Predicted Shelf Life (days)
1801.2033	(b) (4)				
1801.2035					
1801.2037					

Conclusion

Accelerated stability analysis using the Arrhenius equation leads to a minimum predicted time of 41,366 days at 10°C until the minimum label claim is reached. The analysis was conducted on three representative lots of manufactured product and justifies a shelf life of 12 months under 2-10°C storage conditions.

Data Availability

Stability data, Arrhenius analysis, and the original protocol can be found on the company drive under (b) (4)

From: Kristi Smedley
To: Animalfood-premarket
Cc: Kevin Korth; Howard Green
Subject: RE: [EXTERNAL] RE: GRAS Notice AGRN 42
Date: Thursday, November 4, 2021 9:54:51 PM
Attachments: image001.png
CFR-FDA Cover letter AGRN amendment Nov 4 2021.pdf
AGRN 42-Native Microbials Amendment-Response (2).pdf
Attachments_R2-AGRN 42 Butyrivibrio fibrisolvens ASCUSDY19.pdf

In response to the request from the agency for additional information to support AGRN 42 -*Butyrivibrio fibrisolvens* ASCUSDY19 for use as a viable microorganism in dairy cattle feed, we are providing the attached (narrative and attachments). The follow-up email will include copies of the new reference materials.

Should you have issues receiving the information or have additional concerns please contact us.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
(b) (6)
Fax 703-580-8637

From: Animalfood-premarket [mailto:Animalfood-premarket@fda.hhs.gov]
Sent: Thursday, October 21, 2021 3:39 PM
To: Kristi Smedley
Cc: Animalfood-premarket; Howard Green
Subject: RE: [EXTERNAL] RE: GRAS Notice AGRN 42

Dr. Smedley:

Please see the attached letter and minutes regarding the teleconference from October 14, 2021.

Sincerely,

Carissa Adams, MPH
Animal Scientist, Division of Animal Feeds (DAF)

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration

RECEIVED DATE
NOV 8, 2021

Tel: 240-402-6283
Personal e-mail address: carissa.adams@fda.hhs.gov
To schedule a meeting with DAF, please e-mail: animalfood-premarket@fda.hhs.gov

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Thursday, October 14, 2021 4:38 PM
To: Adams, Carissa <Carissa.Adams@fda.hhs.gov>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Howard Green <howard@nativemicrobials.com>
Subject: [EXTERNAL] RE: GRAS Notice AGRN 42

Ms. Adams:

Thank you for organizing this meeting.

We did add a participant, it was Martin Mayhew.

Please provide us the notes of today's meeting via email. I have cc'd Howard Green on this email, and I would apologize if you would provide Howard and I the notes of meetings, when they are sent.

Thank you,

Kristi

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
(b) (6)
Fax 703-580-8637

From: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]

Sent: Tuesday, October 05, 2021 2:11 PM
To: Kristi Smedley
Cc: Animalfood-premarket
Subject: GRAS Notice AGRN 42

Good afternoon,

CVM is currently in the process of evaluating GRAS Notice #42 – *Butyrivibrio fibrisolvens* ASCUSDY19 for use as a viable microorganism in dairy cattle feed.

We would like to schedule a 1 hour teleconference to offer an update on the status of this GRAS Notice.

We are available at the following dates and times;

Tuesday, October 12th from 11:00 - 12:00 pm or 3:00 – 4:00 pm US EST
Thursday, October 14th from 1:00 - 2:00 pm or 3:00 – 4:00 pm US EST
Friday, October 15th from 1:00 – 2:00 pm US EST

Please let me know if one of these times works for you and your client or if I will need to look at other dates. Additionally, we request a list of those you expect to be in attendance at this meeting.

We look forward to speaking with you.

Sincerely,

Carissa Adams, MPH
Animal Scientist, Division of Animal Feeds (DAF)

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration
Tel: 240-402-6283
Personal e-mail address: carissa.adams@fda.hhs.gov
To schedule a meeting with DAF, please e-mail: animalfood-premarket@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

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5200 Wolf Run Shoals Road
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703 590 7337 (Fax 703 580 8837)
Smedley@cfr-services.com

Since 1973
consultants to the regulated industry

David Edwards, Director
Division of Animal Feeds (HFV- 220)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855

November 4, 2021

Subject: Amendment Animal GRAS Notice 42
Butyrivibrio fibrisolvens ASCUSDY19
For Dairy Cattle
Notifier: Native Microbials, Inc.
10255 Science Center Dr . Suite C2
San Diego, California 92121

Dear Dr. Edwards:

On behalf of Native Microbials, I am providing a response to questions raised during the Division of Animal Feeds review of AGRN 42 --Butyrivibrio fibrisolvens ASCUSDY19 -for Dairy Cattle. These questions were discussed in a teleconference on October 14, 2021, and later provided in writing by a letter and notes of teleconference as dated and provided by email on October 21, 2021. The notes of meeting concluded with a statement that the response should be emailed no later than November 5, 2021.

We have addressed all the concerns raised in the notes of the teleconference. Should you have any questions on the filing, please contact me directly.

Sincerely,

(b) (6)

/Kristi O. Smedley, Ph.D.
Consultant to Native Microbials, Inc.

Cc: Mallory Embree, Native Microbials, Inc.

ATTACHMENTS:

Narrative Response to Comments (including appendices)
Complete References to Support GRAS Addendum

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

In the October 14, 2021 teleconference between Native Microbials, Inc. and FDA-CVM it was communicated that in regards to the GRAS Notification AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19 (as a viable microorganism in the diets of dairy cattle with an intended use rate of 1 x 10^8 CFU/cow/day) a review was conducted by FDA-CVM and several items were noted that could be addressed in an amendment. The teleconference was followed-up by an FDA-CVM memorandum, sent and received Oct 21, 2021 restating in writing those items to be addressed.

Native Microbials, Inc. continues to conclude that *B. fibrisolvens* ASCUSDY19 is generally recognized as safe as a direct fed microbial in dairy cattle at the intended rate of inclusion.

Below represents each of the issues presented by FDA-CVM in the meeting and through memorandum and the corresponding response from Native Microbials, Inc.

Identity

1. The 16S rRNA (in short 16S) analysis used for strain identification is unclear. Only a portion of the 16S sequence of the *B. fibrisolvens* ASCUSDY19 strain was used to perform the alignment. The notifier indicates ASCUSDY19 exhibits only 95.2% identity to the B. fibrisolvens type strain ATCC 19171 (DSM3071) for the partial 16S alignment. This value is lower than the commonly accepted cut-off value (98.7%) for species differentiation. However, the notifier indicates ASCUSDY19 shares almost identical 16S sequence with one of its comparator strains *B. fibrisolvens* InBov1 (99.7% identity), so it is expected that the comparator strain InBov1 and ASCUSDY19 would share similar 16S sequence homologies to the type strain ATCC 19171. In CVM's analysis, using the NCBI accession numbers provided in the notice, reveals that the comparator strain InBov1 shares a considerably higher 16S identity (98.38%) with the type strain ATCC 19171 for a full length 16S alignment. Due to the discrepancy, the notifier should provide the information about the full length 16S sequence alignment between ASCUSDY19 and the type strain ATCC 19171. If the difference in homology (95.2% vs. 98.38%) is still observed, the notifier should provide an explanation in the narrative addressing why ASCUSDY19 and InBov1 exhibit different levels of 16S sequence homology to the type strain ATCC 19171, as well as how the identification of the ASCUSDY19 strain can be reliable with such a low level of 16S sequence identity to the B. fibrisolvens type strain.

   Response: We acknowledge that the partial 16S alignment did not supply percent identity above the generally accepted 98.7% cutoff to the *B. fibrisolvens* type strain. We have amended the analysis in the Section 2 narrative (included as Attachment 2) to include commentary on the full length 16S comparison between *B. fibrisolvens* ASCUSDY19 and the *B. fibrisolvens* type strain. After using the full length 16S sequence the percent identity between *B. fibrisolvens* ASCUSDY19 and the *B. fibrisolvens* type strain is above the 98.7% cutoff.

2. The Guanine-Cytosine (GC) percentage of the genome is reported as (b) (4) The notifier should address this discrepancy.

(b) (4)

Molecular Biology

1. The notifier's cut-off setting is too stringent and would not allow identification of toxin homologs with reasonable similarities. The notifier should apply the cut-off setting commonly used in the published literature, e.g., e-values, when conducting its database searches and revise its narrative as appropriate. If an alternative cut-off setting is used, the notifier needs to provide a narrative and literature reference to support the selected cut-off setting. CVM recommends the notifier search the original databases, including VFDB and Victors for toxins and virulence factors, instead of solely relying on the data integrated in the PATRIC database, e.g., VFDB (PATRIC) and Victors (PATRIC), because pertinent data/entries in the original databases may not have been completely integrated into the PATRIC database. CVM has questions regarding the notifier's conclusion that IslandViewer 4 web server did not identify any pathogenicity islands in *B. fibrisolvens* ASCUSDY19, e.g., how many genomic islands are predicted for the B. fibrisolvens ASCUSDY19 genome using IslandViewer 4? Were some genomic islands excluded by the notifier in its analysis for pathogenicity islands? If so, what were the criteria for exclusion? How did the notifier determine those excluded genomic islands in *B. fibrisolvens* ASCUSDY19 did not raise safety concerns, e.g., being associated with pathogenicity? The notifier should address these questions in a revised narrative.

   Response: We have edited the narrative in Section 2.1.6-2.1.8 (included within Attachment 2) to contain the information as requested by the FDA. Below is a summary of the edits made to the narrative in Section 2.

   Per comments from the FDA, the thresholds used for querying databases at the amino acid level were re-evaluated. This re-evaluation has led to two different analyses (both presented below):

   i. At the whole genome level, the 80% identity and 70% coverage initially presented in the dossier is appropriate for identifying virulence factors and antimicrobial genes. Additional sources supporting this threshold are provided in the Section 2 narrative.

   ii. For toxins (specifically known toxins), smaller curated databases are utilized with identity cutoffs between 30-50% or E-value cutoffs ranging from 1E-04 to 1E-05. An additional analysis was performed using a *Clostridium*-specific toxin database with an e-value cutoff of 1E-04. A *Clostridium*-specific database was selected as there are currently no curated toxin databases specific to *Butyrivibrio* or Lachnospiraceae. *Butyrivibrio* is in the Clostridiales order, so *Clostridium*-specific databases represent the closest taxonomic relative for which a curated database currently exists. The results are presented in the revised Section 2 narrative.

We acknowledge that there are differences between VFDB (PATRIC) and Victors (PATRIC) and the original databases were queried using 80% identity and 70% coverage cutoffs. Results are provided in the narrative. We have also updated the narrative to include the number of genomic islands and a more thorough description of the IsandViewer tool.

2. The notifier states that PathogenFinder deemed that *B. fibrisolvens* ASCUSDY19 is not a pathogen, but elsewhere in the narrative states that *B. fibrisolvens* ASCUSDY19 is not a predicted "human pathogen". The original publication of PathogenFinder does not mention whether PathogenFinder is able to predict animal pathogens. CVM conducted a brief search in the published test results which revealed that an example organism, Clostridium botulinum BKT015925, known to produce neurotoxins and cause animal botulism, was predicted with "no pathogenicity". Based on this information it is unclear at this time if PathogenFinder is suitable to predict animal pathogens. The notifier should explain in its narrative how PathogenFinder can be used to address animal pathogens.

   Response: We acknowledge that PathogenFinder has limited ability to detect non-human pathogens, and have updated the text in the narrative accordingly. The goal is to use this tool to identify potential pathogenicity using a broad range of genome sequences (i.e. not just *B. fibrisolvens*) in order to comprehensively assess pathogenicity and to detect features that may not have been previously identified in species of *B. fibrisolvens.*

Microbial Safety

1. The notifier states on page 51 that "No reports of toxigenicity or pathogenicity associated with B. fibrisolvens were identified in the published literature", but this is contradicted by statements elsewhere in the notice. The notifier should resolve this discrepancy.

   Response: Section 6.8 has been updated to resolve the discrepancy (see Attachment 3). Additional text has been added to address the few published clinical cases.

2. In response to question 13b of the Pariza et al. (2015) decision tree, "For strains to be used in animal feeds: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? [xviii]" the notifier states "no" but does not address the footnote associated with this question, "Experimental evidence of safety is required. Such evidence may include, but is not necessarily limited to, studies in appropriate animal models and the target animal species." The notifier must address this contradiction.

   Response: Experimental evidence of safety in which *B. fibrisolvens* was fed to dairy cows and cattle has been completed and are presented in the dossier (Shivani et al., 2016 and Klieve et al., 2003). No adverse health impacts were observed in these studies.

   Since the submission of AGRN 42, a study specific to incorporation of *B. fibrisolvens* ASCUSDY19 in feed has been completed (Attachment 4). Thirty lactating multiparous cows were fed a consortia of four microorganisms (including *B. fibrisolvens* ASCUSYDY19) for 39 weeks and another thirty cows served as the control. This study corroborated that cows supplemented

with *B. fibrisolvens* ASCUSYDY19 exhibit no undesirable physiological effects. No adverse effects were observed, thus further supporting the safety assessment.

The text in Section 6 has been updated accordingly (see Attachment 3).

Utility

1. The notifier states on page 9 of its GRAS notice that the intended purpose of *B. fibrisolvens* ASCUSDY19 supplementation is to "augment the digestion of feed in the rumen." The two articles described in section 6.5 (Shivani et al., 2016 and Klieve et al., 2003) do not provide evidence that *B. fibrisolvens* supplementation augments rumen fermentation. However, the notifier states on page 37 of the notice that "based on the results of published comparative studies, *B. fibrisolvens* ASCUSDY19 will act only to support normal ruminal function of digestion of feed, …" Thus, the description of the intended conditions of use of the additive is not accurate and the intended technical effect may be acceptable if as described elsewhere, the notifier indicates that the use is to support rumen fermentation. There was also discussion that the terms "support" as used elsewhere in the document and "augment" have different meanings.

   Response: The text (updated Section 1, included as Attachment 1, and updated Section 2, included as Attachment 2) has been updated throughout the dossier to reflect that the intended technical effect is to "support" normal ruminant digestion with the existing rumen microbiome rather than "augment".

2. The notifier describes in section 2.5 that "the technical effect of *B. fibrisolvens* ASCUSDY19 when fed to dairy cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety. However, the notifier incorporates a section in the notice (section 2.5.1) that describes how modifying the microbiome could influence rumen fermentation processes and provides examples of ways that *B. fibrisolvens* might alter end-products of digestion and subsequently these end-products have altered composition of animal products or animal productivity. The notifier needs to address how supplementing *B. fibrisolvens* ASCUSDY19 would not have a "bearing on safety" if the intended purpose is to augment rumen fermentation and alter the composition of animal products or animal productivity, which could be addressed by removal of this section.

   Response: The intended purpose is to support digestion. Section 2.5.1 has been removed from the dossier (see Attachment 2).

3. The notifier should recognize that it is contradictory to argue that safety does not relate to utility, but then to include a great deal of discussion outlining expected benefits associated with feeding the viable microorganism, such as increased digestion, animal productivity, and altered composition. Some of these do relate to safety. The notifier asked about how to address the argument that safety is not related to utility. CVM responded that, as addressed in other notices, the notifier should provide an argument with supporting information that if the microbe had no effect in the rumen, the other rumen microflora would be expected to metabolize consumed feed.

   Response: The discussion related to possible benefits of feeding has been modified and Section 6 has been updated accordingly (see Attachment 3).

4. Several articles in the scientific literature indicate that *B. fibrisolvens* produces extracellular polysaccharides and that these substances enhance *B. fibrisolvens* ability to bind to fiber particles and to associate with microorganisms that breakdown cellulose and hemicellulose, noting the nutritional interdependence among rumen bacteria is common. These data also indicates that pure cultures of *B. fibrisolvens* readily degrade xylans, and also ferments other hemicelluloses considerably less well. Some articles indicate that *B.fibrisolvens* does not ferment cellulose unless it is co-incubated with other microorganisms. The inconsistency between the need for *B. fibrisolvens* to utilize extracellular polysaccharides to attach to fiber particles to create a microenvironment for nutritional interdependence and supplementation of a live microorganism in an encapsulated form needs to be addressed.

   Response: The purpose of *B.fibrisolvens* ASCUSDY19 encapsulation is a delivery method to ensure that the microorganism survives when it is exposed to the air and other environmental stressors before it reaches the rumen. *B. fibrisolvens* is an anaerobic bacterium that cannot survive when exposed to atmospheric oxygen (Loesche 1969). Loesche (1969) reported that 0.7% oxygen would inhibit the growth of *B. fibrisolvens* and when exposed to air (21% oxygen), *B. fibrisolvens* would not survive more than 100 minutes. Therefore, the encapsulation is used to create a temporary barrier between live *B. fibrisolvens*, oxygen, and other potentially antimicrobial compounds in the environment (i.e. TMR). Upon the exposure to moisture (i.e, in the rumen), the encapsulation would dissolve and allow *B. fibrisolvens* to become active in the rumen. Thus, it is expected that the fed *B. fibrisolvens* ASCUSDY19 will be present in an unencapsulated form in the rumen and the encapsulation would not interfere with the activity of the microorganism

5. Other articles in the scientific literature focused on *B. fibrisolvens* ability to degrade proteins and noted that *B. fibrisolvens* is one of the most important protein degrading species isolated from the rumen and one of the major end-products of this degradation is ammonia. The notifier acknowledges on page 44 that *B. fibrisolvens* is proteolytic but does not address the fact that the major end-product is ammonia. The notifier should address this issue in terms of potential impacts on safety.

   Response: Nitrogen metabolism in the rumen is important. However, proteolytic activity does not always lead to the production of ammonia (Bach, Calsamiglia, and Stern 2005). As noted by Bach, Calsamiglia, and Stern (2005) regarding the fate of peptides and amino acids (AA) produced via rumen proteolytic activity:

   > *"Peptides and AA resulting from the extracellular rumen proteolytic activity are transported inside microbial cells. Peptides can be further degraded by peptidases into AA, and the latter can be incorporated into microbial protein or further deaminated to VFA, CO2, and ammonia (Tamminga, 1979). The fate of absorbed peptides and AA once inside the microbial cell will depend on the availability of energy [carbohydrates (CHO)]. If energy is available, AA will be transaminated or used directly for microbial protein synthesis. However, if energy is limiting, AA will be deaminated, and their carbon skeleton will be fermented into VFA. Some ruminal bacteria lack mechanisms of AA transport from the cytoplasm to the extracellular environment, and AA absorbed in excess must be excreted from the cytoplasm as ammonia (Tamminga, 1979)."*

   Specifically, Sales, Lucas, and Blanchart (2000) found that the presence of ammonia and AA stimulates the growth and proteolytic activity of *B. fibrisolvens*. When casein is used as the sole protein source, the biomass, doubling time, and the proteolytic activity of *B. fibrisolvens*

increases with increasing concentrations of ammonia and AA. The authors reported a linear relationship between the ammonia utilized and the amount of ammonia added in the presence of *B. fibrisolvens*. When AA was supplemented, *B. fibrisolvens* produced very little ammonia (~ 0.1 mM) regardless of the AA concentration. Consistent with the review by Bach, Calsamiglia, and Stern 2005, the study concluded that "the deaminating activity of *B. fibrisolvens* is weak, and it is able to use amino acids in their native form or after transamination." Furthermore, when cows are fed with a standard diet (at dry matter level: 17% CP, 32% NDF), approximately 5 - 22 mM of ammonia is detected in the rumen (Gustafsson and Palmquist, 1993), which is 50 - 220 times higher than the production capability of a pure culture of *B. fibrisolvens* in the presence of AA. Cumulatively, literature suggests:

1) Ammonia is not the major end product of *B. fibrisolvens* proteolytic activity

2) If *B. fibrisolvens* produces ammonia due to the presence of a large amount of AA, the amount of ammonia produced is a fraction compared to the amount of ammonia present in the rumen and does not pose a safety concern.

The text in Section 6 has been updated accordingly (see Attachment 3).

Chemistry, Manufacturing, and Controls (CMC)
Manufacturing Process and Controls

1. In Section 2.2.2 of the submission, the notifier states that *B. fibrisolvens* ASCUSDY19 is produced through a (b) (4) fermentation process. (b) (4)

   Response: (b) (4)

2. Appendix 010, Section 4 – (b) (4)

   (b) (4)

   (b) (4)

   (b) (4)

(b) (4)

3. In the Appendix 010 Section 3, the notifier describes that the (b) (4)
(b) (4)

Response: (b) (4)
(b) (4)

Starting Materials

1. Appendix 010, Table 10, regulatory status citations for the following starting materials are incorrect:

Ingredient	Citation in Table 1	Correct Citation
Monopotassium phosphate	21 CFR 172.892	21 CFR 160.110
Manganese sulfate monohydrate	21 CFR. 5461	21 CFR 582.5461
Corn steep powder	21 CFR 582.1778 and 582.5778	Not cited in the CFR or AAFCO *Official Publication*

Response: Appendix 10 has been amended (see Attachment 5) to update the incorrect references.

According to USDA (see Attachment 6), corn steep is also known as condensed fermented corn extractive. Under this name AAFCO recognizes it and lists it under 48.24, including IFN-4-02-890. AAFCO Definition 48.24: Condensed Fermented Corn Extractives is obtained by the partial removal of water from the liquid resulting from steeping corn in a water and sulphur dioxide solution which is allowed to ferment by the action of naturally occurring lactic acid producing microorganisms as practiced in the wet milling of com. (Proposed 1959, Amended 1960, Adopted 1961)

Appendix 10 Table 1 has been amended with these corrected references (see Attachment 5).

Monopotassium phosphate is addressed below.

2. Monopotassium phosphate is currently approved to be used in frozen eggs for human consumption (21 CFR 160.110). It is not approved/permitted to be used as a food additive in

animal diets. To fully justify the safe use of monopotassium phosphate, the notifier needs to provide a safety assessment based on the intended use and the amount of the monopotassium phosphate used in the seed medium and fermentation medium of commercial production.

Response: (b) (4)
(b) (4)

(b) (4)

3. The specification unit for cadmium, lead, and arsenic in ascorbic acid (Appendix 09K) are listed as g/g, the notifier needs to clarify whether the unit is g/g, mg/kg, µg/g or ppm

Response: The units were reported on the specification sheet from (b) (4) in error. Attached (see Attachment 8) is a letter from (b) (4) clarifying the proper heavy metals units for Ascorbic Acid, which is ppm. Attached also (Attachment 9) is a recent CofA from (b) (4) showing correct units for the heavy metals.

Specifications of the Notified Substance

1. The notifier states that three batches of *B. fibrisolvens* ASCUSDY19 cell concentrate were analyzed for botulinum toxins. The notifier needs to clarify why botulinum toxins are tested. In addition, the footnote of the Table 2.19 indicates that the testing was conducted in the samples collected at the end of fermentation, not in the cell concentrate. The notifier needs to justify why the botulinum toxins are tested at the end of fermentation. It is recommended that botulinum toxins are analyzed at the manufacturing step where the highest concentration of botulinum toxins are expected.

Response: Botulinum toxins are tested out of an abundance of caution, considering the fermentation batch is anaerobic, not because *B. fibrisolvens* ASCUSDY19 is expected to be producing botulinum toxins, rather the bacteria that do produce botulinum toxins are also anaerobic. By testing for the toxins we can rule out the contamination of the batch by botulinum toxin producing bacteria.

We believe that the end of fermentation is the place where, if present, the toxins would be the most concentrated. This is because botulinum toxins are water soluble and once the cells are centrifuged (at the cell concentrate stage) toxin would be removed with the centrifuge waste.

2. The notifier states that the batches tested to establish specifications are representative of the commercial materials and the same batches were also used in the stability study. The notifier should provide necessary information to justify how the tested batches can represent the expected quality of the commercial products.

(b) (4)

Lot	Broth Volume (L)	End of Fermentation Titer (CFU/g)	End of Fermentation Contamination test by microscopy	End of Fermentation or Cell Concentrate Botulinum toxin test	Cell Concentrate Contamination test by microscopy	Preservation Mixture Titer (CFU/g)	Preservation Mixture Contamination test by microscopy
Target / Specification	N/A	> 1.00E+09	Absence	Negative / 2g	Absence	> 1.00E+10	Absence
1801.2033	100	(b) (4)	PASS	PASS	PASS	(b) (4)	PASS
1801.2035	100		PASS	PASS	PASS		PASS
1801.2037	100		PASS	PASS	PASS		PASS
NM0628211	1200		PASS	PASS	PASS		PASS
1801.3005	3000		PASS	PASS	PASS		PASS

(b) (4)

Lot	Broth Volume (L)	Milled Freeze-Dried Titer (CFU/g)	Milled Freeze-Dried Moisture %	Fat Encapsulated Titer (CFU/g)	Fat Encapsulated Listeria Test	Fat Encapsulated Salmonella Test	Fat Encapsulated Coliorms Test	Fat Encapsulated E coli Test
Target / Specification	N/A	> 1.00E+09	< 3.00%	> 2.00E+07	Negative / 25g	Negative / 25g	< 10 CFU/g	< 10 CFU/g
1801.2033	100	(b) (4)			PASS	PASS	PASS	PASS
1801.2035	100				PASS	PASS	PASS	PASS
1801.2037	100				PASS	PASS	PASS	PASS
NM0628211	1200				PASS	PASS	PASS	PASS
1801.3005	3000				PASS	PASS	PASS	PASS

As commercialization of this organism is still forthcoming, no data can be presented in full size runs. However, all efforts and processes will be employed to comply with current Good Manufacturing Practices, including Hazard Analysis and Risk-based Preventive Controls to comply with the specifications as set forth in ARGN 42. Batches that do not meet quality specifications will not be considered for release.

3. The same batch IDs are used for *B. fibrisolvens* ASCUSDY19 cell concentrate, freeze dried powder and final fat encapsulated product as listed in the table below

Batch #	Manufacturing date		
	Cell concentrate	Freeze dried powder	Fat encapsulated
1801.2033	unknown	Oct. 26, 2020	Nov. 24, 2020
1801.2035	unknown	Oct. 26, 2020	Nov. 23, 2020
1801.2037	unknown	Nov. 03, 2020	Nov. 24, 2020

The notifier needs to clarify the relationship among these products bearing the same batch ID, e.g. whether fermentation batch 1801.2033 was processed to produce only freeze dried and fat encapsulated batch 1801.2033. The notifier needs to clarify whether batches 1801.2033, 1801.2035, and 1801.2037 were three independent fermentation batches.

Response: Following are the dates of harvest (to make the cell concentrate). Each lot number represents a unique fermentation, which led to a unique freeze dried powder, and finally a unique fat encapsulated product.

1801.2033: October 3, 2020
1801.2035: October 9, 2020
1801.2037: October 16, 2020

4. The notifier needs to describe the fermentation size, conditions, and post fermentation processes including harvesting, preservation, freeze drying, and fat encapsulating of each batch of presented *B. fibrisolvens* ASCUSDY19 cell concentrate, freeze dried powder and final fat encapsulated product. Considering the size of a commercial fermenter could be thousands of gallons, the notifier needs to explain how the process used to produce the presented batches is representative to the commercial manufacturing process, so the provided analytical results can be used to support the specifications (anticipated viable cell count, microbial contaminants, and heavy metal contents) and stability of the commercial products of *B. fibrisolvens* ASCUSDY19.

Response: Following are the batch sizes and parameters for the three pilot-scale runs used to create the batches used in the AGRN 42 dossier. Details on how the runs were done is found in the Master Production Records (see Attachment 10)

(b) (4)

(b) (4)

Stability

1. The submitted data collected at 40°C, 50°C and 60°C are not adequate to demonstrate/estimate the stability of Fat Encapsulated *B. fibrisolvens* ASCUSDY19 at 2-10°C. The use of Arrhenius equation to predict the stability or viability of microorganisms at different temperatures has been investigated by different authors for different microorganisms but with different conclusions. Several factors could impact the accuracy of the shelf life estimated from Arrhenius equation, including the manufacturing process, intrinsic resistance of the microorganism strain, the protective agents used in the formulation, potential changes in the microorganism's physical state at accelerated temperature, and lipid oxidation. Therefore, accelerated storage testing was found to be a simple technique but with only limited degree of correctness and predictability for long-term storage at 2-10°C. CVM stated to support the claimed shelf life for the Fat Encapsulated *B. fibrisolvens* ASCUSDY19 at 2-10°C, the notifier should provide real time stability data under the recommended storage conditions using representative pilot or commercial batches.

   Response: Stability testing on the same pilot-scale batches used for the ARGN 42 dossier has been completed through 9 months for recommended storage conditions of ambient refrigerated storage (2-10°C) and for room temperature (25°C) which is an accelerated temperature for the declared ambient.

   Attachment 11 shows that little change in viable count among all three lots has been observed over 9 months at ambient (refrigerated) temperatures, giving good indication that the tentative 12 months assigned in the AGRN 42 dossier will be met. A final decision on the stability will be made at the end of the stability testing period. Additionally, accelerated data at 25°C (see Attachment 12) also show less than 1 log reduction of all three lots at 9 months and all three lots

remain above specification (2.0 E+07 CFU/g), giving further evidence that supports the tentative 12 months expiration date set in the dossier.

Analytical Methods

1. The notifier refers to FDA-BAM method for the determination of the botulinum toxins. The referenced FDA-BAM method includes mouse bioassay, amplified ELISA assay, an approach using digoxigenin-labeled IgGs and DIG-ELISA, and PCR method. The notifier needs to clarify which testing approach is used and what type of toxins are tested.

   Response: The testing approach used for botulinum toxin testing is the mouse bioassay, which does not differentiate between toxin types. Official documentation from the accredited testing laboratory is appended to this document as Attachment 13

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AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 1

Amended Dossier Section 1.3

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19

Amended Section 1.3

1.3 Intended Conditions of Use

B. fibrisolvens ASCUSDY19 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. The intended purpose of supplementation of the microorganism is to support the digestion of feed in the rumen. The microbial strain will be delivered in the fat encapsulated form to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing fat encapsulated *B. fibrisolvens* ASCUSDY19 may be incorporated into the diet of dairy cattle include as part of the total mixed ration (TMR), as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. It is anticipated that *B. fibrisolvens* ASCUSDY19 will be incorporated into feed at a recommended level of $1x10^8$ CFU/cow/day.

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GRAS Notice Amendment

ATTACHMENT 2

Amended Dossier Sections 2.1.4 through 2.1.9 and 2.5

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19

Amended Sections 2.1.4 through 2.1.9 and 2.5

2.1.4 Identification of the Microorganism

2.1.4.1 16S rRNA Gene Sequencing

The 16S rRNA gene was amplified from the strain using 27F and 543R primers and paired end sequenced [2x300 base pairs (bp)] using an Illumina Miseq (Schumann 1991; Muyzer, de Waal, and Uitterlinden 1993). The resulting sequence was quality trimmed and compared to National Center for Biotechnology Information (NCBI) databases using the Basic Local Alignment Search Tool (BLAST) to establish the identity of the strain. Details of the analysis including the BLAST output are provided in Appendix 003A and 003B. Strains of *B. fibrisolvens* and unnamed rumen bacterium provided 16S rRNA sequence matches that fall within the minimum 98.7% sequence identity threshold typically used to define a species (Yarza et al. 2014). The best match was to *B. fibrisolvens* InBov1 at 99.7% sequence identity. Results can be found in Table 2.4.

While 16S rRNA alignment of the partial gene returned matches to *B. fibrisolvens* strains above the minimum 98.7% sequence identity threshold, the *B. fibrisolvens* type strain (ATCC 19171) returned alignment of 95.2% to the partial sequence. To confirm that *B. fibrisolvens* ASCUSDY19 should be identified as a strain of *B. fibrisolvens,* a copy of the 1,551 bp full length 16S rRNA sequences was extracted from the *B. fibrisolvens* ASCUSDY19 whole genome sequence and compared to the NCBI database by BLAST. Results confirmed that *B. fibrisolvens* ASCUSDY19 is a strain of *B. fibrisolvens* as alignment of the full length 16S rRNA gene resulted in 99.6% identity and 95% coverage alignment to the *B. fibrisolvens* type strain (ATCC 19171).

Table 2.4: Partial 16S rRNA alignment to *B. fibrisolvens* ASCUSDY19 16S rRNA by BLAST

Genus species (Genbank accession #)	Identity (%)	Coverage (%)
B. fibrisolvens InBov1 (JN642599)	99.7%	100%
Rumen Bacterium NK3B81 (GU324363)	99.7%	99%
Rumen Bacterium NK4A61 (GU324372)	99.3%	99%
Rumen Bacterium NK4A114 (GU324377)	98.9%	99%
B. fibrisolvens WV1 (AF396927)	98.3%	99%

2.1.4.2 *Whole Genome Sequence Assembly and Annotation*

Genomic DNA was isolated from a pure culture of *B. fibrisolvens* ASCUSDY19 and sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA). The resulting libraries were paired-end sequenced (1x300bp) on an Illumina Miseq and in parallel, long-read libraries were prepared from the same extracted DNA using SQK-RAD004 kit (Oxford NanoporeTechnologies, Oxford) following the protocol outlined by Jain *et al.* (2018) and 1D sequenced on the MinION (R9.4 flowcell; Oxford Nanopore, Oxford) (Jain et al. 2018). The genome was assembled through hybrid methods utilizing both short and long reads. Read quality and genome coverage was evaluated using FASTQC for Illumina data and NanoStat for the Oxford Nanopore reads. The *B. fibrisolvens* ASCUSDY19 genome was closed with no gaps and consisted of 2 chromosomes, a main chromosome (b) (4) The presence of a chromid is consistent with previous observations of the species (Rodríguez Hernáez et al. 2018). (b) (4) . The full details of the assembly are provided in Appendix 003C.

Protein coding genes were predicted through GLIMMER2 and through an iterative process of annotating putative genes using the FIGfams database (Delcher 1999; Meyer, Overbeek, and Rodriguez 2009). To identify protein coding open reading frames of potential genes, contigs were first filtered of all potential tRNA coding genes (T. M. Lowe and Eddy 1997) and rRNA genes (Aziz et al. 2008).

The *B. fibrisolvens* ASCUSDY19 genome contains 3,867 coding sequences which were subsequently built into a metabolic reconstruction describing 235 functional subsystems (DeJongh et al. 2007; Becker and Palsson 2005). These subsystems include larger metabolic groups describing metabolism, virulence, plasmids, disease, defense metabolic products, stress response and dormancy.

The assembled genome has been deposited at NCBI under accession number CP065800 for the main chromosome and CP065801 for the chromid.

Table 2.5: Assembly Statistics for *B. fibrisolvens* ASCUSDY19

# of Contigs	2
# of Contigs ≥ 5,000 bp	2
Longest Contig (bp)	(b) (4)
Assembly Length	(b) (4)
N50	(b) (4)
N75	(b) (4)
GC%	(b) (4)

2.1.4.3 *Whole Genome Sequence Comparison*

To determine relatedness of *B. fibrisolvens* ASCUSDY19 to other closely related species at a higher resolution, whole genomes were compared using ANI. Candidate genomes for genome-genome comparison to *B. fibrisolvens* ASCUSDY19 were selected by full length 16S rRNA similarity and downloaded from the NCBI database. MUMmer was used to generate the alignments for ANI on the basis that this software is adept at aligning highly similar sequences and is more stringent than most

other aligners such as BLAST (Kurtz et al. 2004). Results for the MUMmer alignment can be found in Table 2.6.

The only ANI matches to *B. fibrisolvens* ASCUSDY19 above the 95% ANI cutoff to be considered the same species were two strains of *B. fibrisolvens* (Richter and Rosselló-Móra 2009).

Table 2.6: Average Nucleotide Identity (ANI) of Related Species to *B. fibrisolvens* ASCUSDY19 by MUMmer

Genus species (assembly)	ANI (%)	Coverage (%)
B. fibrisolvens INBov1 (GCA_003175155)	97.6	72.1
B. fibrisolvens YRB2005 (GCA_000423985)	96.8	77.3
B. fibrisolvens DSM3071 (GCA_900129945)	89.2	34.8
Butyrivibrio proteoclasticus B316n (GCA_000145035)	86.4	3.69
Butyrivibrio proteoclasticus P6B7 (GCA_000622085)	85.5	2.8
Butyrivibrio hungatei NK4A153 (GCA_000424465)	84.8	2.6
Butyrivibrio hungatei MB2003 (GCA_001858005)	84.4	3.4

2.1.4.4 Summary and Conclusions

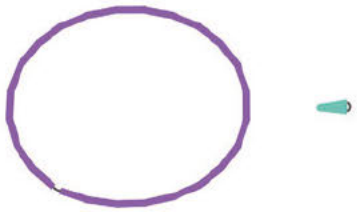
16S rRNA and whole genome analysis confirm that *B. fibrisolvens* ASCUSDY19 represents a member of the species *B. fibrisolvens.*

2.1.5 Plasmid Analysis

To confirm the presence/absence of plasmids, the assembly graph for the *B. fibrisolvens* ASCUSDY19 assembly was analyzed by Bandage (Wick et al. 2015). The assembly graph analysis confirmed that the *B. fibrisolvens* ASCUSDY19 was contained in 2 circular chromosomes with no unincorporated fragments, verifying the completeness of the assembly. Image of the assembly graph can be found in Figure 2.4.

As noted in Part 2.1.4.2, the presence of a smaller, circular second replicon (chromid) is consistent with other assemblies of the species. The annotated features on the putative chromid are associated with general housekeeping and metabolic functions, which is consistent with gene composition of chromids (Harrison et al. 2010). No genes encoding virulence factors, toxins, antimicrobial resistance, or transposable elements were found on the chromid.

Figure 2.4: ***B. fibrisolvens*** **ASCUSDY19 Assembly Graph as Generated by Bandage**



2.1.6 *In-vitro* and *In-silico* Analysis of Antibiotic Susceptibility

Phenotypic testing was conducted on *B. fibrisolvens* ASCUSDY19 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials of relevance to human and veterinary medicine. The full study report is provided in Appendix 004 and results can be found in Table 2.7. The results were evaluated against the resistant breakpoints set by the European Food Safety Authority (EFSA) for "other gram positive bacteria", the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram positive anaerobes" and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). The MIC values reported for *B. fibrisolvens* ASCUSDY19 were equal, or lower than, the cut-off values and break-points established by EFSA, EUCAST and/or CLSI for chloramphenicol, and ampicillin. The isolate would be considered susceptible to Vancomycin and Clindamycin according to EFSA and EUCAST breakpoints but considered intermediately sensitive to Clindamycin per CLSI MIC values were also considered to be in the intermediate range established by CLSI for tetracycline. MIC values reported for *B. fibrisolvens* ASCUSDY19 were higher than the cutoff values and break-points established by EFSA for tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin.

It should be noted that susceptibility to aminoglycosides (gentamicin, kanamycin, streptomycin) and macrolides (erythromycin) decrease significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al. 2016). As such, classifications set forth by EFSA are for general gram-positive organisms and should be carefully applied to *Butyrivibrio fibrisolvens* due to its anaerobic nature. CLSI and EUCAST refrain from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. Tetracycline resistance was indicated by values above the EFSA breakpoint and in the intermediate range by CLSI breakpoint. Tetracycline resistance is not uncommon among ruminal derived organisms. Among 68 livestock derived *Clostridium* strains analyzed by Dutta et al. (1983) 17/68 (25%) strains displaying MIC values above the EFSA microbiological cut-off value. More recent studies have shown that tetracycline resistance is widespread amongst diverse taxa in the rumen (Dutta, Devriese, and Van Assche 1983). Sabino et al. (2019) found that 69% of the ruminal isolates they screened contained tetracycline resistance genes, which were not only expressed, but also reflected in a resistant phenotype (Y. N. V. Sabino et al. 2019).

Table 2.7:	*B. fibrisolvens* ASCUSDY19 Antimicrobial Susceptibility in Relation to EFSA, EUCAST, and CLSI breakpoints				
Antimicrobial	**Tested Range (ug/mL)**	**MIC (ug/mL) of *B. fibrisolvens* ASCUSDY19**	**2018 EFSA Microbiology Cut-off Values (ug/mL) for Other Gram +**	**EUCAST Resistant Breakpoints (ug/mL) Gram + Anaerobes**	**CLSI Resistant Breakpoints (ug/mL) Anaerobes***
Ampicillin	0.5-128	< 0.5	1	8	≥ 2 (R) ≥ 1 (I)
Chloramphenicol	0.5-64	4	4	8	≥ 32 (R) ≥ 16 (I)
Clindamycin	0.03-32	4	4	4	≥ 8 (R) ≥ 4 (I)
Erythromycin	0.5-16	4	1	Not available	Not available
Gentamicin	0.5-32	8	4	Not available	Not available
Kanamycin	0.5-64	> 64	16	Not available	Not available
Streptomycin	0.5-64	16	8	Not available	Not available
Tetracycline	0.0625-64	8	4	Not available	≥ 16 (R) ≥ 8 (I)
Vancomycin	0.125-32	0.25	1	2	Not available

*R = Resistant Breakpoint; I = Intermediate Sensitivity / Susceptible, Increased Exposure. A microorganism is categorized as "I" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.

To evaluate the presence of antimicrobial resistance genes in the *B. fibrisolvens* ASCUSDY19 genome, amino acid sequences from coding regions identified in Part 2.1.4.3 were aligned to the PATRIC database. Included in the PATRIC database is the Comprehensive Antibiotics Resistance Database (CARD) and NCBI's National Database of Antibiotic Resistant Organisms (NDARO) for assessing antimicrobial resistance. In addition to the protein sequences from the databases, PATRIC has compiled protein hits to CARD and NDARO from 331,756 bacterial genomes and included those as redundant gene entries as a means to understand the global distribution of antimicrobial resistance proteins across diverse taxa isolated from a wide range of environments and hosts. Antimicrobial resistance was further explored using the ResFinder web server (Zankari et al. 2012) and BLASTp alignment to the NCBI AMR database as used by AMRFinder (Note: this database differs from NARDO used by PATRIC) (Feldgarden et al. 2019). Between these databases there are a total of 30,748 protein sequences, characteristics of each database can be found in Table 2.8.

Table 2.8: Characteristics of Databases Used to Assess Antimicrobial Resistance				
Database Name	**Number of Entries**	**Number of *Butyrivibrio* Entries**	***B. fibrisolvens* Entries**	**Contains Redundant Entries**
CARD (PATRIC)	17,559 (2,227 non redundant proteins	0	0	Yes
NDARO (PATRIC)	5,138 (4,004 non redundant proteins)	0	0	Yes
ResFinder	3,105	0	0	No
AMRFinder Plus	6,946	0	0	No

To ensure no hits were missed due to codon bias or sequencing error, protein alignments were considered a hit if they have greater than 80% identity over more than 70% query coverage. While there are no widely accepted cutoffs for detecting protein homology at the whole genome level, 80% identity and 70% query coverage is a less stringent cutoff than cutoffs established by many tools examining virulence factor and antimicrobial gene protein homologies at the whole genome level. PATRIC and IslandViewer4, for example, use a minimum of 80% identity and 80% coverage as cutoffs (Mao et al. 2015; Bertelli et al. 2017). Similar approaches have been adopted in published studies investigating virulence factors and antimicrobial resistance (J. Liang et al. 2020; Hu et al. 2013; Abril et al. 2020; Deng et al. 2021; Rojas-Estevez et al. 2020; Y. Pan et al. 2020). Hu et al. (2013), for example, found that 80% identity cutoffs maximized the precision of the identification of antimicrobial resistance genes with 99.1% precision. Lower cutoffs resulted in loss of precision of the alignments. This approach has been proven to return precise results that minimize under and over estimation of the number of virulence, toxin production and antimicrobial resistance genes when detecting protein homology at the whole genome level. Lending further support to our selection of an 80% identity/70% query coverage cutoff is EFSA's use of an identical cutoff for whole genome sequence analysis of microorganisms to be used in the food chain as of 2021. Results can be found in Tables 2.9 to 2.11.

Genetic analysis of *B. fibrisolvens* ASCUSDY19 identified one possible resistance gene (see Tables 2.9 to 2.11).

- The antimicrobial gene in question is a 100% match to the tetracycline resistance gene, tetW, in both the ResFinder and NCBI AMR databases and a 99% match to the same gene in the Card and NDARO databases. TetW confers resistance to tetracycline through ribosomal protection (Aminov, Garrigues-Jeanjean, and Mackie 2001). The tet(W) gene is a ubiquitous gene in the bacterial population of ruminants, humans, and other farm animals (Pal et al. 2016; Joyce et al. 2019; Y. Sabino et al. 2019).

Table 2.9: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by PATRIC								
Source	**Source Organism**	**Gene**	**Product**	**Function**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
CARD/ NDARO	*Bifidobacterium longum*	tetW	Tetracycline resistance, ribosomal protection	MULTISPECIES: tetracycline resistance ribosomal protection protein Tet(W)	100	100	99	0.0

Table 2.10: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by ResFinder				
Gene	**Identity**	**Query Coverage**	**Function**	**Accession number**
tetW	99.9	100% (1920/1920)	Tetracycline Resistance	AJ427422

Table 2.11: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by NCBI AMR BLASTp				
Gene	**e-value**	**Percent Identity**	**Query Coverage**	**Subject Coverage**
tet(W)	0	99.8	100	100

2.1.6.1 Section Summary

In vitro testing demonstrated that *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin. Resistance to aminoglycosides and macrolides such as gentamicin, kanamycin, streptomycin, and erythromycin is reflective of *B. fibrisolvens* ASCUSDY19 being anaerobic rather than any specific resistance mechanism or genotype. *In silico* analyses revealed the presence of tetW, a gene implicated in tetracycline resistance. This finding is consistent with the tetracycline resistant phenotype observed in the MIC testing. *B. fibrisolvens* ASCUSDY19 is susceptible to chloramphenicol, vancomycin, and ampicillin and therefore could easily be controlled with readily available antibiotics.

2.1.7 Antimicrobial Production

Butyrivibrio fibrisolvens ASCUSDY19 supernatant obtained post fermentation was tested for inhibitory activity against reference strains known to be susceptible to a range of antibiotics. No zones of inhibition were observed indicating that the strain is not an antimicrobial producer. Further details of the study are provided in Appendix 005.

2.1.8 Toxigenicity and Pathogenicity

To assess the presence of virulent and pathogenic genes, amino acid sequences from coding regions identified in Part 2.1.4.3 were aligned to several databases. All applicable, publicly available databases were used to identify potential pathogenic genes. The characteristics of these databases are described in Table 2.12. The PATRIC database has compiled relevant genes from external databases including Victors, Virulence Factors Database (VFDB), and the PATRIC_VF database. These genes represent 331,756 bacterial genomes. Redundant gene entries (e.g. the same toxin showing up in multiple microbial

species) are included as a means to understand the global distribution of pathogenicity and virulence associated proteins across diverse taxa isolated from a wide range of environments and hosts. To ensure no toxins or virulence genes were missed, amino acid sequences from *B. fibrisolvens* ASCUSDY19 were aligned to the Victors and VFDB databases downloaded independently from PATRIC due to some entries from these databases being absent in PATRIC. As detailed in section 2.6.1. 80% identity and 70% coverage cutoff was applied to alignments of these databases by *B. fibrisolvens* ASCUSDY19. The contents of the databases are summarized in Table 2.12.

A more conservative alignment approach was taken with the alignment of *B. fibrisolvens* ASCUSDY19 to a subset of protein toxins from the VFDB and DBETH databases. Published studies have established less strict cutoffs of 30-50% identity or e-value cutoffs ranging from 1E-04 to 1E-05, when aligning to known protein toxins (Wei et al. 2015; Surachat et al. 2017; Negi et al. 2017; X. Liang et al. 2019). Therefore, an e-value threshold of 1E-04 was used for the alignment to the toxin databases. It is worth noting that this more conservative approach can result in false positives due to many toxin proteins containing multiple domains with only one of the domains being responsible for the detrimental effects of the toxin (Negi et al. 2017; Xie and Fair 2021). As such, smaller databases containing organism specific toxins should be used and results from low identity alignments should be thoroughly vetted to ensure that the corresponding protein hits are not false positives. As there are no known toxins derived from organisms in the genus *Butyrivibrio* or the family *lachnospiraceae* to which *B. fibrisolvens* ASCUSDY19 belongs to a custom database was used that contained all protein toxin entries in the VFDB and DBETH databases from the order Clostridiales for our alignment.

PathogenFInder and IslandViewer web servers (Cosentino et al. 2013; Bertelli et al. 2017) as well as BLASTp alignment to the Pathogen-Host Interaction Database (Phi-BASE) (Urban et al. 2015) were also utilized to assess the pathogenicity and virulence of *B. fibrisolvens* ASCUSDY19. The total number of sequences in the PATRIC and Phi-BASE databases is 134,396 and includes no sequences from *Butyrivibrio.* IslandViewer contains 4,065 pathogenicity islands including 4 from *Butyrivibrio* species. The analysis in PathogenFinder is database independent and uses a model trained with protein sequences from 886 whole genome sequences.

IslandViewer4 is a software that uses multiple diverse methods to predict genomic islands. These methods include IslandPick (Langille, Hsiao, and Brinkman 2008), SIGI-HMM (Waack et al. 2006), IslandPath (Hsiao et al. 2003), and Islander (Hudson, Lau, and Williams 2014). After identification of genomic islands, the sequences in each island are subject to a search against a curated database of virulence factors, antimicrobial resistance genes, and pathogen associated genes. The database searched includes sequences from VFDB (Chen et al. 2005), PATRIC (Wattam et al. 2013), Victors (Sayers et al. 2019), CARD (Jia et al. 2017), and a database of pathogen associated genes from Ho Sui et al. (Ho Sui et al. 2009). IslandViewer4 then annotates the features in each genomic island using 1e-10 evalue, >90% sequence similarity, and >80% coverage for homologues by BLAST. Any genomic island containing a virulence factor, antimicrobial resistance gene, and/or pathogen associated gene is considered a pathogenicity island.

The PathogenFinder model predicts human pathogenicity based on matches to proteins found differentially in human pathogenic and non-pathogenic bacteria regardless of their annotated function. Therefore, a single hit to a protein found in human pathogenic species does not necessarily suggest the query organism is virulent or pathogenic, but a collection of hits to proteins uniquely found in pathogens

could be enough for PathogenFinder to deem the organism a human pathogen, even if the proteins are not traditionally implicated in virulence or pathogenicity. The program allows the organism to be evaluated more holistically and enables the evaluation of proteins that are potentially involved in virulence and pathogenicity beyond well annotated virulence factors such as toxins.

Table 2.12: Characteristics of Databases Used to Assess Virulence and Pathogenicity

Database Name	Number of Entries	Number of *Butyrivibrio* Entries	*B. fibrisolvens* Entries	Contains Redundant Protein ID entries
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	0	0	Yes
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	1	1	Yes
VFDB	28,982 (3,580 curated entries)	0	No	No
Victors	5,304	0	No	No
PATRIC_VF	38,791(1,570 non-redundant proteins)	0	0	Yes
Phi-Base	6,780	0	0	No
IslandViewer4	4,065 pathogenicity islands	4	0	No
PathogenFInder	N/A	N/A	N/A	N/A

No genes involved in pathogenicity or virulence were identified in the VFDB, PATRIC_VF, or Phi-Base databases. Additionally, 13 genomic islands were discovered by IslandViewer none of which were deemed pathogenicity islands due to the lack of any virulence, pathogenicity, or antimicrobial resistance genes within the genomic island. None of the genomic islands were excluded by the notifier in its analysis for pathogenicity islands. . A site specific recombinase was identified as a potential virulence factor by both Victors and PathogenFinder. While the annotation, protein sequence, and source organism slightly differs between the two databases, the protein in question in the *B. fibrisolvens* ASCUSDY19 genome is the same. The recombinase is homologous to a recombinase found in pathogenic *Streptococcus pneumoniae.* Phage derived site-directed recombinases have been known to excise and insert pathogenic elements in *Streptococcus* species (Carroll et al. 1995). However, excision and insertion of genetic material by the recombinase requires other phage encoded proteins which are not present in the *B. fibrisolvens* ASCUSDY19 genome. Homologues of the recombinase were found to be one of 337 genes necessary to cause lung infections by *S. pneumoniae* in mice, though there was no indication that the recombinase itself was sufficient to cause pathogenicity (Hava and Camilli 2002). There is some evidence that recombinases might play a role in regulation of surface protein production in Streptococci as part of the evolution from commesal to pathogen (Holden et al. 2009). However, there is no evidence linking the recombinase encoded by the *B. fibrisolvens* ASCUSDY19 genome to this type of activity. A global search of the organisms in the PATRIC database was conducted to assess the global distribution of

similar site-directed recombinases. The search returned 134,507 unique protein hits between diverse taxa including pathogenic and non-pathogenic species. Alignment of the recombinase protein identified in the *B. fibrisolvens* ASCUSDY19 genome yielded hits in pathogenic Streptococci and in non pathogenic commensals alike, suggesting that the recombinase does not solely cause pathogenicity or virulence. Results for these analyses can be found in Tables 2.13 to 2.17.

Lower cutoff threshold alignments to curated clostridial toxin databases from VFDB and DBETH yielded 2 and 10 hits respectively. One protein match was identified by both databases, making for a total of 11 unique protein hits between the two databases. Each putative protein toxin identified by the database search was then subjected to a BLASTp search to the NCBI database as means to compare annotations and assess the distribution of the protein globally. Full results can be found in tables 2.18 and 2.20. The 11 unique protein alignments can be summarized as follows:

- All 11 potential toxins identified more closely matched proteins from non-pathogenic *B. fibrisolvens* than to the sequences from the toxin database (table 2.20). Of the 11 proteins only 1 has an annotated function by NCBI that matches its annotated function in the toxin database. This protein is a putative RNA methyltransferase. The other 10 proteins matched more closely to proteins with annotated functions that are not related to toxicity. Additionally, 1 protein has an annotated function by NCBI as hemolysin family protein (HlyC/CorC transporter family protein) and warrants further examination.

- The single protein which had match functional annotations in both the VFDB toxin database and NCBI encodes for a RNA methyltransferase (TlyA). The protein from *B. fibrisolvens* ASCUSDY19 matches a RNA methyltransferase in pathogenic *Clostridium* at 63.6%. Methylation of rRNA is a ubiquitous bacterial cellular process and in some cases differing patterns of methylation between clades can be used for phylogenetic reconstruction (Khaitovich and Mankin 2000; Green and Noller 1996; Liu and Douthwaite 2002). In some cases strain specific patterns of rRNA methylation has been demonstrated to impart antimicrobial resistance and enhance virulence and pathogenicity (Doi and Arakawa 2007; Sałamaszyńska-Guz *et al*. 2020; Rahman *et al*. 2015; Monshupanee 2013; Lata, Paul, and Chattopadhyay 2014). The rRNA methylase homolog in question more closely matches rRNA methylases from non-pathogenic *B. fibrisolvens* than any feature from pathogenic species.

- The hemolysin family protein (HlyC/CorC family transporter) in question shares 31.9% identity with a modulator of ion transport in pathogenic *C. botulinum.* HlyC/CorC domain (pfam PF03471) proteins play an essential role in magnesium and cobalt transport as well as potentially playing a role in modulating the transport of other ions (Harris, Odzer, and Breaker 2019; Huang et al. 2021)The domain is widely distributed across proteins of differing function throughout the phylum *Firmicutes*. The pfam database has 2,145 entries for HlyC/CorC domain proteins in 999 species in the phylum *Firmicutes.* While the protein identified in the *B. fibrisolvens* contains this domain there is no evidence to suggest it engages in hemolytic activity. In fact, importantly, the protein is 99.6% identical with a 100% coverage to proteins in non pathogenic *B. fibrisolvens.*

Table 2.13: Significant Alignments Between Virulence Databases and *B. fibrisolvens* ASCUSDY19						
Organism	**Protein Hits to Victors**	**Protein Hits to VFDB**	**Protein Hits to PATRIC_VF**	**Protein Hits to Phi-Base**	**Pathogenicity Island Hits in IslandViewer**	**Hits to Proteins from Pathogens in PathogenFinder**
B. fibrisolvens ASCUSDY19	1	0	0	0	0	1

Table 2.14: *B. fibrisolvens* ASCUSDY19 Hits to Pathogenic Genes in Victors								
Source	**Source Organism**	**Gene**	**Product**	**Function**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
Victors	*Streptococcus pneumoniae TIGR4*	SP_1040	Site-specific recombinase	Phage Integration	12	100	88	2e-27

Table 2.15: PathogenFinder Results *B. fibrisolvens* ASCUSDY19			
Gene Matches	**Proteins from Pathogens Matched**	**Proteins from Non-Pathogens Matched**	**Predicted as Human Pathogen?**
9	1	8	No

Table 2.16: *B. fibrisolvens* ASCUSDY19 Hits to Pathogenic Genes in PathogenFinder			
Gene	**Genbank Accession Number**	**Source Organism**	**Percent Identity**
Lactobacillales site-specific recombinase	CAW99778	*Streptococcus equi subsp. zooepidemicus H70*	90.1

Table 2.17: Top BLASTp Hits to Site-specific Recombinase found in *B. fibrisolvens* ASCUSDY19 (excluding hits to organisms without standing nomenclature)					
Organism	**Protein Name**	**Genbank Accession Number**	**Percent Identity**	**Query Coverage**	**Known Pathogen?**
Pseudobutyrivibrio xylanivorans	recombinase family protein	WP_072915090	100	100	no
Enterococcus cecorum	Hypothetical protein	KLO65182	99	100	Rare opportunistic pathogen
Peptoanaerobacter stomatis	hypothetical protein	EHL18418	92	100	yes, periodontal disease
Streptococcus pneumoniae	site-specific DNA recombinase	CVU12401	90	100	yes
Coprococcus comes	recombinase family protein	WP_147357729	89	100	no
Eubacterium rectale	recombinase family protein	WP_138305609	89	100	no

Table 2.18: *B. fibrisolvens* ASCUSDY19 Significant Protein Alignments to VFDB Clostridial Toxins						
ASCUSDY19 protein ID	**VFDB ID**	**VFDB Toxin**	**% identity**	**Query Coverage**	**Alignment Length**	**Subject length**
peg.3168	VFG012175	putative RNA methyltransferase	63.64	81	242	270
peg.1367	VFG002280	hyaluronidase (nagK)	51.43	18	35	1163
peg.215	VFG012147	probable enterotoxin (entD)	44.19	16	43	635
peg.461	VFG012150	NlpC/P60 family protein (entB)	35.59	44	59	553
peg.1330	VFG012146	hypothetical protein (entC)	33.33	12	51	744
peg.2976	VFG012149	probable enterotoxin (entB)	32.79	46	61	549
peg.1512	VFG019289	modulator of ion transport	31.94	90	432	441
peg.698	VFG012143	putative enterotoxin (entA)	29.49	31	78	947
peg.769	VFG002288	toxin B (toxB)	28.28	67	244	2366
peg.793	VFG012154	alpha-clostripain (cloSI)	28.28	24	244	522

Table 2.19: *B. fibrisolvens* ASCUSDY19 Significant Protein Alignments to DBETH Clostridial Toxins						
ASCUSDY19 protein ID	**DBETH ID**	**DBETH Toxin**	**% identity**	**Query Coverage**	**Alignment Length**	**Subject length**
peg.2766	Q897D0	Zn-dependent peptidase, insulinase family	45.95	99	962	973
peg.769	C9YJ35	toxin B	30.74	65	244	2366

Table 2.20: Best BLAST Matches to Potential Toxin Sequences in the *B. fibrisolvens* ASCUSDY19 Genome				
ASCUSDY19 protein ID	**Organisms providing best match by BLAST**	**BLAST annotation**	**% identity**	**Query Coverage**
peg.3168	*Butyrivibrio, B. fibrisolvens*	TlyA family RNA methyltransferase	100	100
peg.1367	*B. fibrisolvens* INBov1	carbohydrate-binding protein	92.8	100
peg.215	*B. fibrisolvens* INBov1	SH3 domain-containing protein	98.5	100
peg.461	*B. fibrisolvens*	SH3 domain-containing protein	100	100
peg.1330	*B. fibrisolvens*	Cell wall associated hydrolase, NlpC family	99.77	100
peg.2976	*B. fibrisolvens*	SH3 domain-containing protein	98.8	100
peg.1512	*B. fibrisolvens*	hemolysin family protein, HlyC/CorC transporter family protein	99.6	100
peg.698	*B. fibrisolvens*	SH3 domain-containing protein	98.8	100
peg.769	*B. fibrisolvens* INBov1	hypothetical protein	99.8	100
peg.793	*B. fibrisolvens*	peptidase C11	99.1	100
peg.2766	*B. fibrisolvens*	insulinase family protein	99.8	100

2.1.8.1 Section Summary

No genes directly involved in pathogenesis or toxin production were identified.

All publicly available pathogen and virulence-related databases were queried to determine the pathogenic potential of *B. fibrisolvens* ASCUSDY19. In total, these databases encompass 138,461 known pathogen-related genes spanning all microbial taxonomies. Comprehensive alignment of the *B. fibrisolvens* ASCUSDY19 genome to these databases yielded 1 hit above the 80% identity, 70% query coverage threshold. The single hit was to a site-specific recombinase that does not confer pathogenicity alone, and is found in pathogenic and non-pathogenic species alike. The analysis also included a search of 4,065 pathogenicity islands, 4 of which originated from *Butyrivibrio* species by the IsandViewer web interface. A less stringent alignment using a 1E-4 e-value cutoff of *B. fibrisolvens* ASCUSDY19 to known clostridial toxins yielded 11 unique protein matches to *B. fibrisolvens* ASCUSDY19. All of the potential toxins identifedwere more closely matched proteins from non-pathogenic *B. fibrisolvens* than to toxins from pathogenic species. Additionally, database independent analysis using the PathogenFinder web interface was conducted. IslandViewer did not identify any pathogenicity islands. The same site-specific recombinase identified in the database alignment was also identified by PathogenFinder. Ultimately, PathogenFinder deemed that *B. fibrisolvens* ASCUSDY19 is not likely to be a human pathogen.

2.1.9 Summary of Organism Safety Based on Genomics

B. fibrisolvens ASCUSDY19 was identified as a strain of *B. fibrisolvens* by 16S rRNA and whole genome analysis. *In vitro* antimicrobial susceptibility testing revealed *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin. The strain is susceptible to chloramphenicol, vancomycin, and ampicillin. Consistent with the *in vitro* antimicrobial resistance data, *in silico* analyses revealed one antimicrobial resistance gene in the genome that plays a role in tetracycline resistance. Phenotypic testing confirmed that no antimicrobials were produced by *B. fibrisolvens* ASCUSDY19 during fermentation. Comparison of the *B. fibrisolvens* ASCUSDY19 genome to several databases containing known pathogenic-related genes revealed one protein hit. However, the identified recombinase does confer pathogenicity alone. Homologues of the recombinase are found in pathogens as well as non-pathogens indicating that the feature is not solely responsible for pathogenicity or virulence. A less stringent alignment to known clostridial toxin sequences revealed 11 unique protein matches. However, the potential toxins identified more closely matched proteins from non-pathogenic *B. fibrisolvens* than to toxins from pathogenic species. Based on these analyses, *B. fibrisolvens* ASCUSDY19 is safe for use as a direct fed microbial.

[no changes were made to sections 2.2 through 2.4]

Amended Section 2.5

2.5 Effect of the Notified Substance

This portion of the notice addresses the requirements specified in 21 CFR 570.230(d):

> (d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.

The GRAS Final Rule (81 FR 54960) provides interpretation of this regulation specific to animal feed ingredients in response to comment 144: "We agree that data and information bearing on the physical or other technical effect the notified substance is intended to produce are only necessary when they bear on safety." A product like phytase would require data, however, the intended purpose of supplementation of *B. fibrisolvens* ASCUSDY19 is to support normal rumen digestion. As described below, Native Microbials has determined that the technical effect of *B. fibrisolvens* ASCUSDY19 when fed to dairy cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety. Thus, data and information demonstrating the intended effect of *B. fibrisolvens* ASCUSDY19 in the feed of dairy cattle are not required as part of this GRAS notice.

The use of this organism is to facilitate the digestion of degraded fibrous plant material and ferments polysaccharides (Hespell, Wolf, and Bothast 1987). *B. fibrisolvens* has been found in rumen and silage globally (Kameshwar *et al*., 2019; Avila and Carvaho, 2019; Thi Hoang *et al*., 2020; Seshadri *et al*., 2018) and has been assessed as a probiotic for monogastric animals (Vanbelle *et al*., 1990; Prosekov *et al*., 2015). The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour *et al*., 2015), and is further described below in context of technical effect and animal safety (Part 6.4 of this notice).

B. fibrisolvens is able to degrade fibrous plant material and ferment polysaccharides (Hespell, Wolf, and Bothast 1987). Supplementation of dietary fibrolytic enzymes could improve DMI and milk production has also been reported (Rode et al., 1999). As a commensal microorganism, feeding *B. fibrisolvens* would have no impacts on animal health. Should *B. fibrisolvens* not degrade fibrous plant material and ferment polysaccharides, there would be no safety impact, as the other rumen microorganism will continue fermentation, and the feed was formulated to assure nutrient requirements were met without consideration of the potential for increased digestion of feed.

2.5.1 ****This Section Has Been Removed***

2.5.2 Rumen Microbiome

The most recent authoritative text on the nutrition of major ruminants (NRC, 2016), states that the rumen is a "complex dynamic anaerobic ecosystem." The dynamics of the microbial community arises from variability introduced by feed source, the environment, and physiological state impacts the microbiome (Xue et al. 2018). Experts (NRC, 2016) note that diurnal shifts of a full pH unit are not uncommon, and this can significantly impact the microbial population. The rumen microbial population is well adapted to these standard diurnal shifts in the rumen environment and continue to serve the function of digestion of feed despite these changes (NRC, 2016). This ability to rapidly adapt is due in

part to the rumen microbiome's ability to utilize specialized enzymes and enzyme complexes to convert feed components to end products of digestion and microbial cells (NRC, 2016). It is this specific understanding that Native Microbials uses in their identification of existing, commensal microorganisms in the rumen of high producing ruminants. Particularly, understanding of their unique enzymatic properties and physiology support the selection and use of them as DFMs.

Several studies have linked the rumen microbiome profile to animal performance and digestibility (Lima et al. 2015; Jami et al. 2013; Kumar et al. 2015). The rumen microbiome is highly variable depending on several factors including age, breed, diet composition, time after feeding, season, stage of lactation, location, and farm management practices (Pitta et al. 2016; Furman et al. 2020; Henderson et al. 2015). Additionally, there are groups of microorganisms that are unique to particular breeds of cow (i.e., Jersey or Holstein), regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Diet, in particular, has been shown to be the main driver of microbiome composition (Ghaffari et al. 2014). To better study the microbiome in context of this variability, many studies have focused on identifying and characterizing the core rumen microbiome (Petri et al. 2013; Xue et al. 2018; Henderson et al. 2015; Furman et al. 2020; Kumar et al. 2015; Jami et al. 2013; Lima et al. 2015; Fouts et al. 2012). The concept of core microbiome, a common assemblage of microorganisms that exists in or is associated with a specific habitat, was first introduced and applied to differentiate human microbiomes associated with healthy and diseased conditions (Turnbaugh et al. 2009; Turnbaugh and Gordon 2009; Turnbaugh et al. 2007). Since then, core microbiomes have been identified in a broad spectrum of environments including agroecosystems, monogastric animals, and ruminants (Shade and Handelsman 2012; Yeoh et al. 2017; Toju et al. 2018; B. A. Lowe et al. 2012; Dougal et al. 2013).

There is a core microbiome that appears in the majority of dairy cows that provides the basal level of fermentation required for animal survival. Although the results are variable at times and defining a "normal healthy'' rumen is challenging, there are several phyla that tend to appear across all ruminants. Henderson *et al*. (2015) reported 32 different species of ruminants globally shared a core assembly of rumen bacteria. Xue *et al*. (2018) demonstrates that individual animals within a large cohort of dairy cattle with similar genetics, diet, environment, and management can have significant differences in their rumen microbiome species. The core microbiome identified included microorganisms from over 391 genera covering 26 phyla. The microorganisms unique to individual animals (termed "pan microbiome") along with the core microbiome dictated the variability in rumen fermentation and production. Consistent with other studies (Jami et al. 2013; Jami and Mizrahi 2012; Lima et al. 2015; Deusch et al. 2017; Huws et al. 2018; Xue et al. 2018), members of Bacteroidetes, Firmicutes, Proteobacteria, and Fibrobacteres were among the topmost abundant bacteria identified regardless of animal origin and diet.

As more rumen microbiomes were studied, it became clear that diet was the major determinant of observed microbiome differences (Johnson and Johnson 1995; Brulc et al. 2009; Carberry et al. 2014; Deusch et al. 2017; Alejandro Belanche et al. 2019; Kumar et al. 2015; Mizrahi and Jami 2018). This indicates the direct impact of diet on rumen microbial populations. Hence, modifying either diet or microbiome could influence the rumen fermentation process (Moraïs and Mizrahi 2019; Furman et al. 2020; A. Belanche et al. 2012). *B. fibrisolvens* has been fed to ruminants as well as monograstrics. In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens*

favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported. Furthermore, *B. fibrisolvens* has been utilized as a probiotic in mice, being analyzed for its CLA production (Fukuda et al. 2006) and potential for tumor reduction (Ohkawara et al. 2007) Both studies reported that *B. fibrisolvens* had positive impacts on the health of the mice in the studies and reported no adverse health effects of administration. A strain has also been tested as an aspect of a dietary study in rats to increase intestinal production of short-chain volatile fatty acids (Nielsen et al. 2016). Similarly, this study also did not report any adverse health impacts of *B. fibrisolvens.* Although this species is not commercially available and has not seen widespread application in feed, academic and scientific research has shown that there are no adverse effects when *B. fibrisolvens* is fed to animals, thus it is unlikely that this organism is dramatically altering rumen fermentation processes. The intent of feeding DFMs, particularly *B. fibrisolvens* ASCUSDY19, is to improve the nutrient availability from feed. Feeding *B. fibrisolvens* ASCUSDY19 to dairy cattle supplements the existing populations of *B. fibrisolvens* ASCUSDY19 in the rumen, and ultimately provides additional nutrient availability to the animal. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. This notice includes a more detailed discussion of the core microbiome and microbiome safety in Part 6.4 of this GRAS notice.

2.5.2 Impact of Failure of the Notified Substance

If this product fails, that is, the product fails to support feed digestibility in the rumen, there would not be a safety concern with respect to the animal's health or nutrition. The notified substance supports the digestion of carbohydrates by acting upon the existing feed within the rumen. The diet offered to the animal would be formulated to meet the existing nutritional needs of the animal (NRC, 2001). Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients.

Several published experiments have directly investigated the impacts of DFMs by comparing groups of animals receiving a "dead" microbial against a variety of treatment conditions. Cunha, *et al*. (2019) compared heifers fed a basal diet against heifers fed the same basal diet containing a live yeast or inactive yeast supplement (2 different doses) in a 5x5 Latin square experimental design with 15-day periods. Live and dead yeasts were administered to the appropriate animals after each feeding through infusion directly into the rumen. No differences in digestibility were observed between the control, live yeast, or either of the inactive yeast doses. No differences were observed in feed intake nor animal behavior. Hence the inactive yeast did not alter the overall digestion of the feed, nor impact the health of the animals. Feeding inactive yeast did not decrease rumen function.

Muscato, *et al*. (2002) evaluated the feeding of fresh and inactivated rumen fluid to calves in a series of four experiments. The animals were dosed daily with 8 mL of either fresh or inactivated rumen fluid obtained from a cannulated Holstein cow from 0-6 weeks of age. In the first experiment, calves were either fed a typical basal ration or the same basal ration supplemented with fresh rumen fluid. In the second experiment, calves were fed the basal ration with either the cell pellet of fresh rumen fluid,

supernatant of fresh rumen fluid, or no addition. In the third experiment, calves were fed a basal ration, or a basal ration supplemented with autoclaved rumen fluid. Autoclaving rumen fluid ensures microbial death, thus inactivating the biological component. The fourth experiment had a similar set-up to the third experiment, but rumen fluid was only fed for 5 days rather than 6 weeks. In the studies that evaluated autoclaved rumen fluid, the number of days of scouring were significantly decreased compared to the control. Similarly, the calves receiving autoclaved rumen fluid experienced higher gains in the first two weeks, but by the end of the experimental period there was no impact on growth. There were no differences in the outcomes of calves receiving fresh rumen fluid as compared to calves receiving autoclaved rumen fluid. This study suggests that the feeding of inactivated microorganisms does not decrease rumen function or create a safety concern when fed to animals.

The contribution of members of *Butyrivibrio*, specifically, to the fermentation characteristics of the rumen has been evaluated in the published literature. In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported.

Philippeau, *et al.* (2017) fed multiple DFM treatments to investigate the effects of DFM on rumen fermentation characteristics and digestibility. Animals were assigned one of four treatment groups: control (CON), *Propionibacterium* P63 (P63), *Propionibacterium* P63 and *Lactobacillus plantarum* 115 (P63+Lp), or *Propionibacterium* P63 and *Lactobacillus rhamnosus* 32 (P63+Lr). Each strain was administered at 10^{10} cfu/d. No change in ruminal VFA concentration was observed, and only P63 was found to impact the concentration of some milk fatty acids. pH increased on average 0.18 units in all DFM groups as compared to the control. Although the study did not demonstrate the positive response in performance as was expected, there was no negative change in the assessed parameters that may suggest a decrease in health. Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight, Linn, and Jung 2007; Abu-Tarboush, Al-Saiady, and Keir El-Din 1996; Higginbotham and Bath, 1992; McGilliard and Stallings 1998). In Weiss *et al*. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette *et al*. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette *et al*. (2007), *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman et al. 2011; Hagg et al. 2010; Zebeli et al. 2012; Kung and Hession 1995).

A *Lactobacillus*-based probiotic fed alone and in combination with *S. cerevisiae* showed no change in milk production or efficiency in early-lactation dairy cows (Boga and Gorgulu 2007). In a meta-analysis conducted at INRA, 33 probiotic bacteria studies with or without yeast were evaluated for their impact on the production and health of dairy and beef cattle (Lettat et al. 2012). Variable performance and rument impacts were observed, however the study indicated no negative health consequences were reported. In the studies summarized above, even though the direct fed microbials did not achieve the performance response expected, there was no indication of a safety concern.

In these examples, failure of DFM supplementation or the DFM itself did not cause any harm to the fermentation characteristics of the rumen or animal well-being. In the case of *B. fibrisolvens* ASCUSDY19, if the DFM failed to provide improved digestibility, rumen fermentation of treated cows would be identical to rumen fermentation of untreated cows. Since no alterations are made to the standard feeding regime when using this product, the value of the feed that would be digested and utilized for the nutrients required to sustain life is identical between the control and treated group. Animals would be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001). Any non-performing *B. fibrisolvens* ASCUSDY19 or deceased *B. fibrisolvens* ASCUSDY19 would pass through the GI tract with the normal flow of digesta, providing nutrients for absorption by the animal (NRC, 2016).

In this respect, based on the results of published comparative studies, *B. fibrisolvens* ASCUSDY19 will act only to support normal ruminal function of digestion of animal feed. Like other DFMs, while *B. fibrisolvens* ASCUSDY19 may aid the digestion of feed, the effect is not required for the general well-being and normal performance of dairy cattle. Thus, the absence of the anticipated effect of *B. fibrisolvens* ASCUSDY19 on feed digestion by dairy cattle would not have an impact on safety. Native Microbials product labeling does not suggest a change in normal feeding regime, and its use would be specific for gaining additional nutritional value from a typical balanced ration. Animals would continue to be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001).

2.5.3 Summary

In summary it is Native Microbials' understanding that the regulatory hurdle provided in §570.230(d), is not applicable to the conclusion of the generally recognized as safe substance *B. fibrisolvens* ASCUSDY19, that is "failure" of the intended use will not raise a safety concern, as the intended use is to provide increased nutritive value from nutritionally adequate feeds. As such, failure would result in typical nutrient availability of the diets, as they have been formulated to meet the nutritional requirements of the animal. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. Therefore, there is no regulatory requirement to provide specific utility data to support the intended use.

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 3

Amended Dossier Section 6

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19

Amended Section 6

PART 6 – NARRATIVE

The conclusion that *B. fibrisolvens* ASCUSDY19, as described herein, is GRAS under the conditions of intended use as a direct fed microbial in feed for dairy cattle is based on scientific procedures using product-specific characterization data on the microbial strain together with a body of published information on the prevalence and potential pathogenicity and toxigenicity of the *Butyrivibrio* species.

As mentioned in Part 1.3, fat encapsulated *B. fibrisolvens* ASCUSDY19 will be provided to dairy cattle either alone or in combination with other direct fed microbials. The strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and is intended as a source of commensal microorganisms. In this respect, *B. fibrisolvens* ASCUSDY19 will contribute to the native microbial population in the rumen and the functionality of the direct fed microbial strain is considered in Part 6.1.

The safety of *B. fibrisolvens* ASCUSDY19 for use as a direct fed microbial for dairy cattle is evaluated using several different pieces of data regarding strain characterization and the evaluation of its pathogenic and toxigenic potential. In order to understand the pathogenic and toxigenic potential, the microbial strain must be fully characterized and the body of knowledge pertaining to safety based on its taxonomic unit considered. Full details of the characterization of *B. fibrisolvens* ASCUSDY19 are detailed in Part 2. The microbial has been unambiguously characterized as *B. fibrisolvens* (see Part 2.1.4). Furthermore, whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2.1.8). Whole genome sequence analysis together with phenotypic testing indicate that *B. fibrisolvens* ASCUSDY19 is susceptible to antimicrobials and should not increase the risk of transfer of resistance to other microorganisms (see Part 2.1.5 and 2.1.6). Testing also confirms *B. fibrisolvens* ASCUSDY19 does not produce antimicrobial substances (see Part 2.1.7 and Appendix 005).

In addition to the characterization data, a body of information is available in the public domain pertaining to (a) the identity of *B. fibrisolvens* (see Part 6.2); (b) the history of exposure of the species by animals and humans (see Parts 6.4 and 6.5); and (c) the potential for toxigenicity and pathogenicity (see Part 6.6). These data represent another important component of the safety evaluation of *B. fibrisolvens* ASCUSDY19 spray dried powder and are summarized below.

6.1 Functionality

The microbial population of the rumen plays an important role in the utilization of feed by dairy cattle. Manipulation of rumen microbiota by dietary supplementation with sources of viable microorganisms is common practice in the dairy cattle industry in the U.S. in order to facilitate fermentation and contribute to the general digestive health of the animal (Yoon and Stern 1995; Chaucheyras-Durand and

Durand 2010; Abd El-Tawab et al. 2016). The contribution of bacteria to the fermentation characteristics of the rumen have been extensively evaluated in the published literature, with important functions reported to be stabilization of the rumen pH, increase in volatile fatty acid production, reduction in ammonia concentrations, improved microbial protein synthesis and fiber digestibility (*e.g.*, (McAllister et al. 2011; Nocek et al. 2002; Henning et al. 2010; Krehbiel et al., 2003; Qiao et al. 2010; Weinberg et al. 2007; Jeyanathan et al. 2019; Yoon and Stern 1995). As mentioned in Part 2, *B. fibrisolvens* ASCUSDY19 was isolated from the rumen content of a healthy mid-lactation Holstein and is expected to contribute in the same way as other bacteria to digestion and metabolism in the ruminal environment.

In particular, *B. fibrisolvens* was shown to degrade fibrous plant material and ferment polysaccharides (Hespell, Wolf, and Bothast 1987). It utilizes various carbon sources including simple carbohydrates (e.g., glucose and fructose), reducing sugars derived from plant materials such as xylose and cellobiose, glucosides derived from plant materials such as salicin and esculin, and starch (see Part 2.1). Similar phenotypes are reported in the published literature for other *B. fibrisolvens* strains (M. Cotta and Forster 2006; Hespell, Wolf, and Bothast 1987; Marounek and Petr 1995; M. A. Cotta 1992, 1988; VAN Gylswyk et al. 1996; Emerson and Weimer 2017). Additionally, many strains of the species degrade protein and pectin (M. A. Cotta and Hespell 1986; Sales, Lucas, and Blanchart 2000; M. Cotta and Forster 2006; Marounek and Duskova 1999; Gradel and Dehority 1972). Co-culture experiments have demonstrated that *B. fibrisolvens* is capable of degrading a variety of feedstuffs including barley, sorghum, wheat, lucerne, and cotton stalks (Ben-Ghedalia, Miron, and Solomon 1993; J. Miron and Ben-Ghedalia 1992; J. Miron 1991; J. Miron and Ben-Ghedalia 1993; Joshua Miron and Ben-Ghedalia 1993). Thus, the microorganism has the potential to support digestion by aiding fermentation of forages and partially degraded digesta in the rumen.

Similar to other *B. fibrisolvens* strains, *B. fibrisolvens* ASCUSDY19 has been shown to utilize a range of monosaccharides including glucose, fructose, and xylose to produce relatively high levels of butyrate with lower amounts of acetate, formate, and lactate (Emerson and Weimer 2017; Hespell, Wolf, and Bothast 1987). While butyrate is generally favored, there is some intraspecies heterogeneity and differing growth conditions may result in higher acetate or lactate production (Shane, Gouws, and Kistner 1969; Diez-Gonzalez et al. 1999; Hespell, Wolf, and Bothast 1987; Paillard et al. 2007). Fermentation of pectin by *B. fibrosolvens* generally yields higher proportions of acetate to butyrate/lactate (Marounek and Duskova 1999).

B. fibrisolvens is known to possess proteolytic activity (M. A. Cotta and Hespell 1986; Sales, Lucas, and Blanchart 2000). It is estimated that 30-50% of all ruminal isolates possess proteolytic capability (Fulghum and Moore 1963; Prins, van Rheenen, and van't Klooster 1983). Microbial protein degradation is an important ruminal process needed to break down proteins into smaller peptides and free amino acids that support the rumen microbiota and supply free amino acids to the host (Tamminga 1979; Bach, Calsamiglia, and Stern 2005). As a byproduct of this protein degradation, *B. fibrisolvens* may produce minimal amounts of ammonia under certain conditions (Sales, Lucas, and Blanchart, 2000). To provide

context, when cows are fed with a standard diet (at dry matter level: 17% CP, 32% NDF), approximately 5 - 22 mM of ammonia is detected in the rumen (Gustafsson and Palmquist, 1993), amounts ranging from 50 to 220 times higher than amounts that a pure culture of *B. fibrisolvens* could produce in the presence of amino acids. Hence, the ammonia produced by B. fribriosolvens would have a negligible impact on the overall ammonia concentration in the rumen. Meanwhile the liberated amino acids can support ruminal microbial growth and subsequent VFA production and protein synthesis (Bach, Calsamiglia, and Stern 2005; Argyle and Baldwin 1989; Regueira et al. 2020). Microbial protein synthesis in the rumen accounts for an estimated 50-80% of all absorbable protein supplied to the small intestine of dairy cows (Storm and Ørskov 1983; Clark, Klusmeyer, and Cameron 1992)

Taken together, these examples of the potential functionality of *B. fibrisolvens* in the rumen support the proposed role of *B. fibrisolvens* ASCUSDY19 as a source of viable microorganisms in the diet to support the existing rumen microbiome in the production of VFAs and general colonic health of the animals. While *B. fibrisolvens* ASCUSDY19 may contribute to the native population of *Butyrivibrio* species in the gut of the animal, the technical function has no bearing on the safety when used as a direct fed microbial in feed for dairy cattle. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. On this basis, no further demonstration of the technical effect (utility) of *B. fibrisolvens* ASCUSDY19 was required for the safety evaluation (see Part 2.5).

6.2 Identity

The genus *Butyrivibrio* consists of motile, anaerobic curved rods that gram stain negative, but maintain gram positive structure. Members of the genus ferment glucose or maltose with butyrate as the major fermentation product (Anne Willems and Collins 2015). Heterogeneity exists amongst species in regards to fermentation of additional carbon sources, and fermentation products may differ due to species-specific metabolism, with some members of the genus favoring the production of lactate, acetate, or formate (M. Cotta and Forster 2006; Anne Willems and Collins 2015). 16S phylogeny has placed the genus in the *Clostridium* XIVa cluster. The genus is polyphyletic, with three distinct lineages and 12 identified rRNA subtypes (A. Willems, Amat-Marco, and Collins 1996; Anne Willems and Collins 2015; Forster et al. 1996).

B. fibrisolvens was the only species proposed at the time of the genus description, and while diversity was noted amongst isolates, many were identified as *B. fibrisolvens* based solely on their morphology and phenotype (Bryant and Small 1956). Due to initial phenotype based taxonomic classification, strains of *B. fibrisolvens* are more diverse genetically that what is typically seen between strains of a species, with G+C mol% between 39%-49.2% and 16S rRNA sequence similarity as low as 88% between strains (Mannarelli 1988; Mannarelli et al. 1991; Forster et al. 1996). As previously noted, the genus *Butyrivibrio* is polyphyletic, with species in the genus spread across three phylogenetically distinct clusters. The species *B. fibrisolvens* itself is split between two of the three clusters. *Butyrivibrio* group 1 consists of the *B. fibrisolvens* type strain, strains from *B. hungatei,* and other species from the genus *Clostridium.* Group 2, also known as the *Pseudobutyrivibrio* group, consists of species of *B. fibrisolvens* and species from the genus *Pseudobutyrivibrio*. Group 3 consists of *B. crossotus* and similar species (Anne Willems and Collins 2015).

6.3 Literature Search

A comprehensive literature search was conducted in order to identify all publicly available information pertaining to the safety of *B. fibrisolvens* for the intended use as a source of viable cells for dairy cows. Results can be found in Appendix 17.

6.4 Natural Occurrence

6.4.1 Prevalence in Animals

B. fibrisolvens is ubiquitous in nature and has been isolated from rumen content of cattle, deer, sheep, goats, bison, camels, and giraffes, as well as fecal samples from horses, rabbits, dogs, cats, and humans (Asanuma, Kawato, and Hino 2001; Balamurugan et al. 2009; Vasta et al. 2010; Moore and Holdeman 1974; Cheng et al. 1969; Brown and Moore 1960; Bryant and Small 1956; Sundset et al. 2009; Forster et al. 1996; Henderson et al. 2015)

A total of 9 different strains of *B. fibrisolvens* have been isolated, sequenced, and analyzed in the JGI genome portal to date (b) (4)), and 11 strains in the NCBI GenBank database (b) (4)). The Global Rumen Census found that the *Butyrivibrio* genus had a mean relative abundance of 3.4% in the rumen (Henderson et al. 2015), while several other studies put the relative abundance of *Butyrivibrio fibrisolvens* near 1% (Li et al. 2012; Petri et al. 2013). Species in the *Butyrivibrio* genus were found in 100% of samples across 742 samples taken from 32 animal species in 35 countries (Henderson et al. 2015). Thus, *Butyrivibrio* and *B. fibrisolvens* are highly prevalent as commensal organisms of the rumen microbial ecosystem.

6.4.2 Microbiome Safety

The rumen microbiome is crucial for the digestion of feed and supplies necessary nutrients to ruminants (Faichney 1996; Huws et al. 2018). The rumen hosts a diverse group of microorganisms that work closely to degrade plant materials. The fermentation process converts nearly all dietary carbohydrates to volatile fatty acids (VFA), predominantly butyrate, acetate, and propionate. It has been widely recognized that the rumen VFAs are crucial for digestive system development and animal carbon and nitrogen needs (Storm and Ørskov 1983; Broudiscou and Jouany 1995; Weigand, Young, and McGilliard, 1974; Górka et al. 2018; Leng, Steel, and Luick 1967; Young 1977; Huws et al. 2018; Bach, Calsamiglia, and Stern 2005; Edwards et al. 2008; Wallace, Onodera, and Cotta 1997). Direct infusion of VFAs into the rumen can also improve animal performances. For example, direct infusion of butyrate into the rumen increased milk fat production without changing milk yield (Huhtanen, Miettinen, and Ylinen 1993) and direct infusion of propionate into the rumen increased milk protein production (Rook and Balch 1961).

The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour et al. 2015). Specific species within the genera *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Bacillus, Propionibacterium, Megasphaera and Prevotella* have been fed to animals (Nocek et al. 2002; Yoon and Stern 1995; Ghorbani et al. 2002; Stein et al. 2006; Yang and Beauchemin et al. 2004; Nagaraja et al. 1997; Chiquette, Allison, and Rasmussen 2008; Mohammed et al. 2012; Weiss, Wyatt, and McKelvey 2008; Aikman et al. 2011). There are several studies, for example, that describe the fermentation patterns and feed digestibility of ruminants fed a standard diet supplemented with a DFM compared to ruminants only on a standard diet. Feeding of *Lactobacillus plantarum* via silage in (Mohammed et al. 2012) showed no changes in production, but no deleterious effects on the animal.

Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight, Linn, and Jung 2007; Abu-Tarboush, Al-Saiady, and Keir El-Din 1996; Higginbotham and Bath, 1992; McGilliard and Stallings 1998). In Weiss, et al. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette, et al. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette et al. 2007, *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman et al. 2011; Hagg et al. 2010; Zebeli et al. 2012; Kung and Hession 1995).

Bacteria catabolism also plays an important role in animal nutrient cycling. Hoogenraad et al. (1970) studied how model organisms of gram-negative bacterium (*Escherichia coli*) and gram-positive bacterium (*Bacillus subtilis*) were utilized in adult sheep digestive tract. The study found that the (b) (4) whole cells of either bacteria were quickly digested by the rumen microbiome and cell carbons were incorporated into VFAs. A large amount of the bacterial carbon (70%) was captured by the host animal. Bacterial whole cells and cell components such as cell wall and content were also readily digested and metabolized in abomasum. Despite the common belief that gram-positive cells are more difficult to metabolize due to the presence of peptidoglycan, 73-86% of *B. subtilis* cell and cell component carbon was captured by the animal through lower gut digestion. In contrast, a smaller portion (66-78%) of *E. coli* carbon was captured by the host animal. Notably, although *B. subtilis* cells contain a greater amount of glucose than *E. coli*, a much greater amount of *E. coli* carbon was incorporated into the lower gut glucose pool. The findings suggest that bacteria turnover in ruminant digestive tract is an important process and supplying building blocks to support the host metabolism.

The rumen microbiome is dynamic. Moraïs and Mizrahi (2019) summarized that multiple microbial community states exist within the rumen depending on the rumen metabolic needs. The flow of metabolites and energy were passed on from one functional group to the next rather than from one group to another. Thus, microbial interactions could drive larger changes in overall fermentation patterns and identifying the optimal microbial interactions could improve digestibility (Weimer 2015). Published studies showed that diet contributes to the greatest rumen microbiome shifts observed (Kumar et al. 2015; Deusch et al. 2017; Mizrahi and Jami 2018; Alejandro Belanche et al. 2019; Johnson and Johnson 1995; Brulc et al. 2009; Carberry et al. 2014). Under the same diet, the addition of DFMs does not change the rumen microbiome significantly but can improve rumen digestibility. Westergaard (2015) fed a *Bacillus pumilus* DFM to 21 dairy cows and compared the composition of their rumen microbiomes to 22 control animals. The study reported an insignificant increase in Firmicutes from 14.1% to 15.8% and an insignificant decrease of Bacteroidetes from 64.1% to 62.3% in rumen fluid of animals that received the DFM. Its companion study reported that the animals receiving the DFM were more efficient at feed conversion (ECM:DMI) than the control animals, although not significantly ($p = 0.06$) (Luan et al. 2015). Le et al. (2017) conducted a study comparing the growth performance of 4 week-old dairy calves with and without DFM *Bacillus amyloliquefaciens* in feed. *B. amyloliquefaciens* was administered daily for 9 weeks to 12 calves and another 12 calves were used as controls. The study found that dairy calves administered *B. amyloliquefaciens* gained 20% more weight and suffered less diarrhea than the control

group. Notably, its companion study observed that *B. amyloliquefaciens* supplementation did not change the dairy calf rumen microbiomes significantly, despite confirmation of colonization of the DFM strain in rumen (Schofield et al. 2018). In another study, Fomenky et al. (2018) compared the rumen digesta microbiome of pre- (33 days old) and post-weaned calves (96 days old) fed with control diet alone and control diet supplemented with *S. cerevisiae* (SCB) or *L. acidophilus* (LA) (8 per treatment). The study found that supplementing DFMs did not significantly change the overall rumen microbial community structure, where the p-values for alpha diversity indices ranged from 0.051 to 0.992 and the p-value for beta diversity (PERMANOVA) was 0.512. The study also predicted that pathways involved in lipid and protein metabolism and cellular processes were more abundant in pre-weaned rumen administered DFMs. Once weaned, no predicted pathways in rumen digesta were significantly different between control and LA fed animals. Riboflavin metabolism was the only significantly more abundant pathway in SCB fed animal rumen digesta than control. These studies demonstrated that DFMs could promote better microbial interactions and improve the overall rumen feed digestibility without significantly changing microbial community structures.

The rumen bacterial population composition was investigated using internal animal survey experiments as well as external, peer-reviewed experiments (Appendix 18). Typical ranges of the native bacteria phyla as well as the abundance of the native population of *B. fibrisolvens* were identified, demonstrating that *B. fibrisolvens* is a ubiquitous constituent of the dairy cow microbiome.

The use of *B. fibrisolvens* to facilitate the digestion of fibrous plant material and polysaccharides (Hespell, Wolf, and Bothast 1987) of animal feed within the rumen utilizes enzymes related to amylase, xylanase, and beta-glucanase. Studies conducted on *B. fibrisolvens* have revealed the presence and induction of a collection of xylanases and hemicellulolytic isoenzymes in response to xylan (Sechovcová et al. 2019; Emerson and Weimer 2017; Lin and Thomson 1991; Hespell, Wolf, and Bothast 1987). The species has a demonstrated ability to hydrolyze starch through the expression of extracellular and cell-associated alpha amylase (M. A. Cotta 1992; Rumbak et al. 1991; M. A. Cotta 1988; Ramsay et al. 2006). Furthermore, *B. fibrisolvens* produces beta-glucanase (Pierre van Rensburg, van Zyl, and Pretorius 1994), and when taken together these fibrolytic enzymes are major factors in the digestion of plant material (Rode, Yang, and Beauchemin, 1999; Beauchemin et al., 2003). *B. fibrisolvens* is frequently found in rumen content globally, across many species of ruminants (Bryant and Small 1956; Lee and Moore 1959; Brown and Moore 1960; Cheng et al. 1969; Forster et al. 1996; Sundset et al. 2008; Vasta et al. 2010; Henderson et al. 2015; Anne Willems and Collins 2015) and is also commonly found in monogastric animals (Moore and Holdeman 1974; Asanuma, Kawato, and Hino 2001; Balamurugan et al. 2009; Mi et al. 2018). *B. fibrisolvens* is a common commensal rumen microorganism that has been used previously in non-commercial, research settings as a DFM (see Part 6.5).

Native Microbials conducted a series of experiments in order to obtain a representative sampling of the rumen microbial community in dairy cows under farm-like conditions in the U.S. The full study report is provided in Appendix 018. In two general survey experiments, animals were cannulated and sampling conducted across the different regions of the rumen over a number of days. In all of the experiments, the typical abundance of *B. fibrisolvens* specifically, in the rumen of dairy cows was found to vary from approximately 0.0001% to 1% of the bacterial population. General observations indicated that all animals were in good health. Taken together, these studies provide corroborative experimental evidence that *B. fibrisolvens* is naturally abundant in the rumen of dairy cattle and not associated with any health concerns.

Hence the use of *B. fibrisolvens* as a source of live microorganisms, will have a beneficial effect on the available nutrition from a typical dairy ration. However, with understanding of the typical microbiome shifts as related to influencers such as dietary composition, physiological changes and environmental impacts, the notified substance will not make marked or detrimental changes on the rumen microbiome.

6.4.3 Section Summary

B. fibrisolvens occurs in a wide range of animals, including essentially all ruminants, as a commensal organism in the gastrointestinal tract. Dietary supplementation of *B. fibrisolvens* will not negatively impact the function of the rumen or the well-being of the animal.

6.5 History of Use in Manufacture of Food and Feed Ingredients

In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported. Furthermore, *B. fibrisolvens* has been utilized as a probiotic in mice, being analyzed for its CLA production (Fukuda et al. 2006) and potential for tumor reduction (Ohkawara et al. 2007). Both studies reported that *B. fibrisolvens* had positive impacts on the health of the mice in the studies and reported no adverse health effects of administration. A strain has also been tested as an aspect of a dietary study in rats to increase intestinal production of short-chain volatile fatty acids (Nielsen et al. 2016). Similarly, this study also did not report any adverse health impacts of *B. fibrisolvens.* Although this species is not commercially available and has not seen widespread application in feed, academic and scientific research has shown that there are no adverse effects when *B. fibrisolvens* is fed to animals.

Several other applications of this microorganism have been researched. Due to the high level of production of extracellular polysaccharides similar to xanthan gum, a particular strain *B. fibrisolvens* has been proposed for use as an industrial source of this biopolymer (Wachenheim and Patterson 1992). Some research regarding applications of the genome of *B. fibrisolvens* has been completed. Specifically, genes coding for xylan-degrading enzymes (Sewell et al. 1989; Utt et al. 1991), cinnamoyl ester hydrolase (Dalrymple and Swadling 1997), glucanase (Pierre van Rensburg, van Zyl, and Pretorius 1994; P. van Rensburg, van Zyl, and Pretorius 1997, 1996), glutamine synthase (Goodman and Woods 1993), and cellodextrinase (Berger et al. 1990) from *B. fibrisolvens* have been used in transformation of other bacteria. While these studies focus on a range of different enzymes and transform several species of bacteria, the core intent of all of these studies is to improve the digestive functionality of the transformed bacteria with enzymes from *B. fibrisolvens*.

6.6 Toxigenicity and Pathogenicity

Butyrivibrio species are largely considered to be non-pathogenic commensals and have not commonly been identified as opportunistic pathogens. The American Type Culture Collection (ATCC) lists *B. fibrisolvens* as BSL-1, indicating that it is a low-risk microorganism that poses little to no threat of infection in healthy humans and animals. DSMZ also classifies *B. fibrisolvens* as BSL-1.

Butyrivbrio have been cited in a small number of opportunistic infections since the 1970s. The first suspected infection in animals or humans by *Butyrivbrio* was reported in a farmer who suffered an eye injury from barbed wire in a cattle enclosure. Infection of the eye followed the injury, and *B. fibrisolvens* was suspected as the causative agent (Wahl 1974). *Butyrivbrio* like organisms have been isolated from both liver abscesses and gastrointestinal infections (Chow, Ota, and Guze 1976; Thadepalli et al. 1978; George et al. 1981). In all the cited cases of suspected *Butyrivbrio* infection, identification of the causative organism was based on morphology, metabolism, and antimicrobial susceptibility profiles and no infections have been confirmed using unambiguous molecular methods.

As noted in Part 2.1.5, the *B. fibrisolvens* ASCUSDY19 genome assembly contains a chromid. The presence of plasmids, mega-plasmids, and chromids are common in *Butyrivbrio* genomes and the presence of more than two extrachromosomal replicons have been observed in some cases (Palevich et al. 2017; Yeoman et al. 2011; Teather 1982; Rodríguez Hernáez et al. 2018; Palevich et al. 2019). Plasmids from *B. fibrisolvens* are not known to carry pathogenic genes, though a small collection of plasmids from the species have been characterized with the hope of developing vector systems to transform ruminal microbes (Anne Willems and Collins 2015; Hefford et al. 1997; Beard et al. 1995). The high rate of megaplasmids and chromids within the genus is believed to help lend a competitive advantage over other ruminal organisms by enhancing growth rate and cellular efficiency through copy number increase of key metabolic genes (Palevich et al. 2019; Morrison 1996), rather than bestow pathogenic ability. This hypothesis is at least in part supported by the gene composition observed in the only sequenced *B. fibrisolvens* chromid, which largely consists of genes which encode for carbohydrate degradation enzymes many of which are also encoded by the main chromosome (Rodríguez Hernáez et al. 2018).

6.6.1 Summary

Overall, the available information indicates that *B. fibrisolvens* is a prevalent organism in the gastrointestinal microbiome of animals, including humans. Few instances of infection have been attributed to the genus *Butyrivbrio* or the species *B. fibrisolvens* and no infections have been documented since the wide acceptance and implementation of molecular techniques that allow for unambiguous microbial identification. As indicated in Part 2.1.8, interrogation of the whole genome sequence of *B. fibrisolvens* ASCUSDY19 did not reveal the presence of any protein toxins and the single virulence factor identified is not solely responsible for pathogenicity or virulence.

6.7 Studies in Target Animals

The determination that *B. fibrisolvens* ASCUSDY19 is GRAS under the intended conditions is based on product-specific characterization data together with the body of information in the published literature. The organism is a commensal rumen organism.

In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported. Although this species is not commercially available and has not seen widespread application in feed, academic and scientific research has shown that there are no adverse effects when *B. fibrisolvens* is fed to ruminants.

6.8 Summary and Critical Evaluation of Target Animal Safety

B. fibrisolvens is a common commensal bacteria in the gut of humans and animals. *Butyrivbrio* have been cited in a small number of opportunistic infections, however, all studies were prior to 1981 and relied on morphology and phenotypic characterization for microbial species identification. No cases of pathogenicity have been recorded since 1980 nor has this species been detected in any clinical cases using more sophisticated and accurate methods of species identification. Native Microbials has conducted an assessment of *B. fibrisolvens* ASCUSDY19 and confirmed the absence of any genes encoding for toxin production or other virulence factors known to be associated with pathogenicity (see Part 2.1.8). Furthermore, the susceptibility of *B. fibrisolvens* ASCUSDY19 strains to antibiotics of veterinary and pharmaceutical relevance, and the absence of antimicrobial production has been demonstrated (see Parts 2.1.6 and 2.1.7, and Appendices 004 and 005). Collectively, these data indicate that *B. fibrisolvens* ASCUSDY19 (the notified substance) should not be associated with any safety concerns for dairy cattle under the intended conditions of use as a direct fed microbial.

6.9 Summary and Critical Evaluation of Human Food Safety

As mentioned in Part 3.2, no transfer of viable *B. fibrisolvens* ASCUSDY19 from the rumen to milk or other edible species is anticipated under the conditions of intended use as a direct fed microbial in the feed of dairy cattle. Furthermore, the strain has been unambiguously characterized as *B. fibrisolvens* and whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2.1.8). The absence of pathogenicity or toxigenicity is supported by the ubiquitous nature of *B. fibrisolvens* and its natural occurrence in the rumen and gastrointestinal tract of animals. Taken together, these data indicate that *B. fibrisolvens* ASCUSDY19 should not be associated with any human food safety concerns under the intended conditions of use as a direct fed microbial in the feed of dairy cattle.

In this safety assessment we identified, discussed and placed into context data and information that are, or may appear to be inconsistent with the GRAS status (21 CFR 570.250(c)(1)). Based on the preponderance of evidence, Native Microbials' conclusion of safety is scientifically justified.

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GRAS Notice Amendment

ATTACHMENT 4

DairyExperts Trial Pre-publication

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 5

UPDATED – Dossier Appendix 10

Detailed Manufacturing Description

UPDATED-Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19

Confidential Manufacturing Information

The raw materials used in the manufacture of *B. fibrisolvens* ASCUSDY19 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009U.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *B. fibrisolvens* ASCUSDY19

Material	Function	Regulatory Status	Grade
Ammonium Hydroxide	Seed Medium and Fermentation Medium	21 CFR 184.1139	FCC
L-Cysteine Hydrochloride	Seed Medium and Fermentation Medium	21 CFR 582.5271	USP
Sodium Hydroxide	Seed Medium and Fermentation Medium	21 CFR 582.1763	FCC
Iron (Ferrous) Sulfate Heptahydrate	Seed Medium and Fermentation Medium	AAFCO 57.83; 21 CFR 582.5315	USP
Magnesium Sulfate Heptahydrate	Seed Medium and Fermentation Medium	AAFCO 57.88; 21 CFR 582.5443; IFN 6-02-758	USP
Monopotassium Phosphate	Seed Medium and Fermentation Medium	21 CFR 160.110; see Attached Regulatory Review	FCC
Sodium Acetate, Anhydrous	Seed Medium and Fermentation Medium	21 CFR 582.1721	USP
Sodium Chloride	Seed Medium and Fermentation Medium	AAFCO 57.31	USP
Hydrogenated Glycerides	Fat Encapsulation	AAFCO 33.19	Feed grade

Table continued on the next page.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *B. fibrisolvens* ASCUSDY19 (cont'd)

Material	Function	Regulatory Status	Grade
Polyoxyethylene polyoxypropylene block copolymer	Seed Medium and Fermentation Medium	21 CFR 176.210; FDA-ETA Letter, 2003	Specific product specified. Allowed for Food/feed production
Ascorbic Acid, Vitamin C	DSP and Freeze Drying Processing Aid	IFN 7-00-433; 21 CFR 582.5013	USP or FCC
Manganese Sulfate, Monohydrate	Seed Medium and Fermentation Medium	AAFCO 57.96; 21 CFR 582.5461	USP
Sodium Sulfate	Fat Encapsulation	AAFCO 57.109	FCC, Moisture: ≤ 1% by LOD, Purity: ≥ 98%
Ammonium Chloride	Seed Medium and Fermentation Medium	AAFCO 57.265	USP
Dextrose Monohydrate	Seed Medium and Fermentation Medium	21 CFR 168.111; 21 CFR 184.1857	FCC
Condensed Fermented Corn Extractives	Seed Medium and Fermentation Medium	AAFCO 48.24; IFN-4-02-890	Feed Grade
Mannitol	Fermentation Medium and Freeze Drying	21 CFR 582.5470	USP
Sucrose	Freeze Drying	21 CFR 184.1854	NF
Amberex 1003 AG Yeast Extract	Seed Medium and Fermentation Medium	AAFCO 96.11	Specific food grade product specified.
Hydrochloric Acid	Seed Medium and Fermentation Medium	21 CFR 582.1057	FCC
Phosphoric Acid	Seed Medium and Fermentation Medium	AAFCO 57.19; IFN 6-03-707	FCC

Abbreviations: AAFCO – Association of American Feed Control Officials; IFN – International Feed Identification Number; FCC – Food Chemicals Codex; USP – United States Pharmacopoeia; NF – National Formulary

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19

1 Overview

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Table 2. (b) (4)

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Appendix A. Process Diagram of the Production of Fat Encapsulated *B. fibrisolvens* ASCUSDY19

(b) (4)

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ATTACHMENT 6

USDA Monograph on Corn Steep Liquor

Corn Steep Liquor

Crop Production

Identification of Petitioned Substance

Chemical Name:
Corn Steep Liquor

Other Names:
(Corn steepwater, light steepwater, heavy steepwater, condensed fermented corn extractives

Trade Names:

CAS Number:
66071-94-1

Other Codes:
European Inventory of Existing Commercial Chemical Substances (EINECS) No. 266-113-4

Characterization of Petitioned Substance

Composition of the Substance:

Steeping is a procedure used during wet corn milling. The major objectives for corn steeping are to induce chemical and physical changes in the kernel by leaching the soluble components from the corn. Cleaned shelled corn is soaked for 30-48 hours at 120 - 130° F in a dilute sulfur dioxide solution. The steeped liquid is then separated from the non-soluble corn solids, which are further separated into germ, bran, starch, and gluten protein. The steeped liquor is concentrated by evaporation into Condensed Corn Fermented Extractives or Corn Steep Liquor (CSL). Corn steep liquor is a mixture of soluble protein, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals.

Wet corn milling is used to produce numerous corn based products that are subsequently used as biofuel, ingredients in food, and for livestock feed. These products include starch, high fructose corn syrup, oil, ethanol, bran, gluten feed, and meal. Corn steep liquor is one of the byproducts of corn wet milling directed to the production of animal feed. It is also used as a nutrient for microorganisms in the production of enzymes, antibiotics, and other fermentation products.

Properties of the Substance:

Product Chemistry	
Physical State	Liquid
Melting Point	Not applicable, corn steep liquor is a liquid
Boiling Point	100 – 104 degrees Centigrade
Density	1.2 to 1.4 g/cm^3
Vapor Pressure	17.5 mm, 20 degrees Centigrade
Flammability/Flame Extension	not flammable
Explodability	not explosive
Solubility	Soluble in water

Oxidizer	not an oxidizer

Specific Uses of the Substance:

CSL is a mixture of soluble proteins, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals. It is used as a nutrient for microorganisms in the production of enzymes, antibiotics, and other fermentation products. It is sometimes combined with other ingredients in corn gluten feed and widely used in complete feeds for dairy and beef cattle, poultry, swine, and pet foods. It may also be sold separately as a liquid protein source for beef or dairy rations.

Approved Legal Uses of the Substance:

The Association of American Feed Control Officials, Inc. (AAFCO) has listed corn step liquor as a livestock feed ingredient.

The following is quoted directly from the AAFCO homepage.

"The purpose of the corporation shall be to establish and maintain an Association through which officials of any state, dominion, federal or other governmental agency and employees thereof charged with a responsibility in enforcing the laws regulating the production, labeling, distribution, or sale of animal feeds or livestock remedies may unite to explore the problems encountered in administering such laws, to develop just and equitable standards, definitions and policies to be followed in enforcing such laws, to promote uniformity in such laws, regulations and enforcement policies, and to cooperate with members of the industry producing such products in order to promote the effectiveness and usefulness of such products."

Action of the Substance:

Corn steep liquor is a byproduct of wet corn milling. Its components are soluble proteins, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals. It is sometimes combined with other ingredients in corn gluten feed and widely used in complete feeds for dairy and beef cattle, poultry, swine, and pet foods. Some corn steep liquor is used in the production of acetic acid, food acids, and fermentation processes. Some corn steep liquor is used in the pharmaceutical industry in the production of intravenous solutions and drugs, most notably antibiotics (penicillin).

Status

U.S. Environmental Protection Agency

Corn steep liquor is one of 2800 High Production Volume (HPV) chemicals identified on the US Environmental Protection Agency's (USEPA) 1990 Toxic Substances Control Act (TSCA) Inventory Update Rule (IUR). HPV chemicals are those that are manufactured or imported in quantities greater than 1 million pounds per year.

The following information is quoted directly from the USEPA homepage for New Chemicals.

"Under the Toxic Substances Control Act, section 8(b) provides EPA authority to "compile, keep current, and publish a list of each chemical substance that is manufactured or processed in the United States." TSCA section 3(2)(A) states that "the term 'chemical substance' means any organic or inorganic substance of a

particular molecular identity, including - (i) any combination of such substances occurring in whole or in part as a result of a chemical reaction or occurring in nature, and (ii) any element or uncombined radical." TSCA does not include chemical substances subject to other US statutes such as foods and food additives, pesticides, drugs, cosmetics, tobacco, nuclear material, or munitions."

U.S. Food and Drug Administration

Corn steep liquor is not listed as Generally Recognized as Safe by the FDA (FDA, 2004), but is listed as a component of a color additive allowed in chicken feed.

The following is directly quoted from 21 CFR Sec. 73.275.

"§ 73.275 Dried algae meal.
(a) *Identity.* The color additive dried algae meal is a dried mixture of algae cells (genus *Spongiococcum,* separated from its culture broth), molasses, cornsteep liquor, and a maximum of 0.3 percent ethoxyquin. The algae cells are produced by suitable fermentation, under controlled conditions, from a pure culture of the genus *Spongiococcum.*
(b) *Uses and restrictions.* The color additive dried algae meal may be safely used in chicken feed in accordance with the following prescribed conditions: (1) The color additive is used to enhance the yellow color of chicken skin and eggs. (2) The quantity of the color additive incorporated in the feed is such that the finished feed: (i) Is supplemented sufficiently with xanthophyll and associated carotenoids so as to accomplish the intended effect described in paragraph (b)(1) of this section; and (ii) Meets the tolerance limitation for ethoxyquin in animal feed prescribed in § 573.380 of this chapter."

Association of American Feed Control Officials, Inc.

The Association of American Feed Control Officials, Inc has listed corn steep liquor as a livestock feed ingredient.

International:

The European Union permits the use of stillage and stillage extracts as fertilizers and soil conditioners in organic crop production, however, corn steep liquor is not mentioned specifically (European Union, 2008). Stillage is defined as the mash from the fermentation of grains after the removal of alcohol by distillation (Association of American Feed Control Officials, 2005). Maize bran and gluten from wet corn milling are permitted as feed materials used in livestock production (European Union, 2008). European manufacturers refer to corn wet milling as maize processing. The processes are the same, which includes the use of sulfur dioxide.

The Codex Alimentarius permits the use of stillage and stillage extracts as fertilizers and soil conditioners in organic crop production, however, corn steep liquor is not mentioned specifically (Codex Alimentarius, 2008).

Corn steep liquor is included on the chemical inventory of the Domestic Substances List by the Canadian government.

Evaluation Questions for Substances to be used in Organic Crop or Livestock Production

Evaluation Question #1: Is the petitioned substance formulated or manufactured by a chemical process? (From 7 U.S.C. § 6502 (21).)

Corn steep liquor is produced by steeping corn grain in water for up to 48 hours. The soluble components in the corn are removed because a natural lactic fermentation is taking place during steeping. Sulfur dioxide is added at rates of 0.1 to 0.2 percent and is used to cleave disulfide linkages, resulting in the degradation of the corn protein that encapsulates the starch granules. The starch is then released from the encapsulating material. The steep water containing the corn solubles are concentrated with evaporators to

form corn steep liquor. Corn steep liquor is a mixture of soluble protein, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals. The nitrogen fraction is high in free amino acids and small peptides. In four samples of corn steep water, Hull et al., (1996) found a number of small poly-peptides present. Concentrations of poly-peptides generally increased during steeping. In the same study, Hull et al., (1996) found the amino acids glutamine, leucine, proline, and asparagine at the highest concentrations. Lower concentrations of lysine, cysteine, and methionine were reported. Concentrations of amino acids generally increased during steeping. The composition of amino acids in the four corn steep liquor samples compared characteristically similar to corn albumin, globulin, glutelin, and zein proteins (Wilson, 1987). Hull et al., (1996) found various non-protein nitrogenous compounds in corn steep water. Enzymatic activities provided no evidence for proteases during steeping, however, the length of steeping time (up to 30 hours), coupled with the higher temperature (50 to 55 degrees Centigrade) and the presence of micro-organisms could contribute to the enhancement of proteolytic activity during steeping (Hull et al., 1996). Corn steep liquor is very high in phosphorus, potassium, and sulfur (Kalscheur, et al., 2008).

Therefore, the chemical composition of corn steep liquor will probably vary and is reflective of the processing strategy used by a particular manufacturer, depending on which corn component they are interested in isolating. Factors affecting the composition of CSL are corn hybrid, steeping time, temperature, and the presence of micro-organisms.

Evaluation Question #2: Is the petitioned substance formulated or manufactured by a process that chemically changes the substance extracted from naturally occurring plant, animal, or mineral sources? (From 7 U.S.C. § 6502 (21).)

Corn steep liquor is derived from corn which is a naturally occurring plant. Clean corn is steeped in warm water containing small amounts of sulfur dioxide. Soaking softens the kernels and the dilute sulfurous acid formed when the sulfur dioxide reacts with water prevents excessive bacterial growth and loosens the gluten bonds within the corn and releases the starch. The steep water absorbs the soluble components and is later evaporated and concentrated to a solid content of about 50%. As mentioned in the response to Question 1, the chemical composition of corn steep liquor will probably vary and is reflective of the processing strategy used by a particular manufacturer, depending on which corn component they are interested in isolating. This is affected by steeping time, temperature reached during the lactic acid fermentation, and the microbial environment of the fermentation (Hull et al., 1996). These factors will also likely affect the quality of the fermentation end-products.

Evaluation Question #3: Is the petitioned substance created by naturally occurring biological processes? (From 7 U.S.C. § 6502 (21).)

Corn steep liquor is not created by a naturally occurring biological process. It is created as a result of a process designed to separate corn into its four basic components, starch, germ, fiber, and protein in an aqueous medium. It is a complicated process of chemical and biochemical reactions that, despite the long history of the wet-milling industry, are still not fully understood. A summary of the process is provided in evaluation question #1.

Evaluation Question #4: Is there environmental contamination during the petitioned substance's manufacture, use, misuse, or disposal? (From 7 U.S.C. § 6518 (m) (3).)

Manufacture

Corn steep liquor, itself, should not cause any environmental contamination, because the material is approximately 50% water and the soluble proteins, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals would be readily metabolized and utilized by micro-organisms. The sulfur dioxide added to the fermented material to cleave the disulfide linkages may need to be vented to the atmosphere. However, the wet corn milling process that generates corn steep liquor may have some issues

of concern related to environmental contamination. The wet milling process is designed to separate the corn into its components, starch, germ, protein (gluten) and fiber and convert them into higher value products such as starch, high fructose corn syrup, corn oil, ethanol, bran, gluten feed, and meal. It is the making of the high value products that result in the generation of millions of pounds of waste at wet corn milling plants annually. If the waste is not managed properly it will stress the environment. The USEPA has funded a pilot project to assist small and medium-size manufacturers who want to minimize their generation of waste but who lack the expertise to do so. For more information see: http://www.p2pays.org/ref/02/01481.pdf.

Corn dust produced during the handling and cleaning processes could be a safety hazard, due to the fact that the corn dust is explosive. The organic materials used to extract the corn oil from the germ may be a concern, due to accidental spills and the release of volatile organic compounds. There are no reported incidences on environmental contamination due to the production of corn steep liquor.

Evaluation Question #5: Is the petitioned substance harmful to the environment? (From 7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i).)

Corn steep liquor, itself, should not cause any environmental contamination, because the material is approximately 50% water and the soluble proteins, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals would be readily metabolized and utilized by micro-organisms. Corn steep liquor could be used in crop production to add organic matter and other nutrients to the soil, however, there are probably other materials (animal manures) that are more cost effective. Corn steep liquor is used in the diets of ruminants (Kalscheur et al., 2008).

Evaluation Question #6: Is there potential for the petitioned substance to cause chemical interaction with other substances used in organic crop or livestock production? (From 7 U.S.C. § 6518 (m) (1).)

The water, soluble proteins, amino acids, carbohydrates, organic acids (e.g., lactic acid), vitamins, and minerals in corn steep liquor would be readily metabolized and utilized by microorganisms. Corn steep liquor should not interact chemically with other substances used in organic crop or livestock production.

Evaluation Question #7: Are there adverse biological or chemical interactions in the agro-ecosystem by using the petitioned substance? (From 7 U.S.C. § 6518 (m) (5).)

Corn steep liquor should not cause any adverse biological or chemical interactions in the agro-ecosystem. The release of lactic acid, which comprises 10 to 25% of corn steep liquor, to the environment, may be an issue, if large quantities were released to the environment. However, this would not be expected since the production of corn steep liquor is performed by a controlled process. Any lactic acid released to the environment would be readily metabolized and utilized as an energy source by micro-organisms, therefore, it should have little to no long-term impact on the agro-ecosystem.

Evaluation Question #8: Are there detrimental physiological effects on soil, organisms, crops, or livestock by using the petitioned substance? (From 7 U.S.C. § 6518 (m) (5).)

There is no information available to indicate that using corn steep liquor has detrimental physiological effects on soil, organisms, crops, or livestock. Because it is rich in nutrients, it can be applied to soils as a fertilizer or soil conditioner and it has been successfully fed to livestock for many years (Kalscheur et al., 2008).

Evaluation Question #9: Is there a toxic or other adverse action of the petitioned substance or its breakdown products? (From 7 U.S.C. § 6518 (m) (2).)

Corn steep liquor should not have any toxic or other adverse actions. The components of corn steep liquor are readily metabolized and utilized by micro-organisms as an energy source. Because corn steep liquor is a nutrient source, algal growth is possible, if corn steep liquor reaches bodies of water in concentrated

form. However, the manufacturing of corn steep liquor is a controlled process and given the current uses of corn steep liquor, one would not expect large quantities of corn steep liquor being released to bodies of water.

Hull et al., (1996) analyzed four different corn steep waters for chemical composition. When analyzed for heavy metals, iron was the most prevalent heavy metal present in corn steep water. Chromium and cadmium were not detected in the four samples. Copper and nickel were detected at levels approximately 5 to 10% of that of iron (1.6 mg/L or less). Lead was detected in one sample (36 ug/L).

Evaluation Question #10: Is there undesirable persistence or concentration of the petitioned substance or its breakdown products in the environment? (From 7 U.S.C. § 6518 (m) (2).)

The components of corn steep liquor are readily metabolized and utilized by micro-organisms as energy sources, therefore, corn steep liquor would not persist and concentrate in the natural environment.

Evaluation Question #11: Is there any harmful effect on human health by using the petitioned substance? (From 7 U.S.C. § 6517 (c) (1) (A) (i), 7 U.S.C. § 6517 (c) (2) (A) (i) and), 7 U.S.C. § 6518 (m) (4).)

Corn steep liquor has no harmful effects on human health. The components of corn steep liquor are used as ingredients in foods for human consumption (proteins, amino acids, carbohydrates, vitamins, and minerals). Corn steep liquor has been successfully fed to livestock for many years (Kalscheur et al., 2008) without any adverse effects on human health.

Individuals who handle corn steep liquor should wear gloves, protective clothing, and protective eyeware.

Evaluation Question #12: Is there a wholly natural product that could be substituted for the petitioned substance? (From 7 U.S.C. § 6517 (c) (1) (A) (ii).)

In the case of adding organic matter to soils for crop production, composted and raw manures could be used depending on the crop being grown, time of harvest, and whether the crop will be used for human consumption (Organic Materials Review Institute, 2007). For adding inorganic nutrients to soils, unprocessed mined materials could be used (Organic Materials Review Institute, 2007).

In the case of supplementing livestock feeds with vitamins and minerals, natural vitamin supplements and non-synthetic minerals, respectively, can be used (Organic Materials Review Institute, 2007).

Wet corn milling is defined as corn steeped in water with or without sulfur dioxide to soften the kernel in order to facilitate the separation of the various component parts (Association of American Feed Control Officials, 2005). Therefore, the wet corn milling could be conducted without sulfur dioxide, the lactic acid fermentation and the subsequent separation of the corn components (including natural drying to concentrate the soluble materials in the liquid portion) may be another method of processing the corn. This may be an alternative to adding sulfur dioxide after the lactic acid fermentation and the concentrating of the corn steep liquor with evaporators. However, the quantities and quality of the end-products may be different.

In the case of organic crop production, corn steep liquor would be used in very few, if any, products on the National List of Allowed and Prohibited Substances. As in (7 CFR 206.601), herbicides (soap-based) for use in farm stead maintenance and ornamental crops would be a mixture of either calcium or sodium fatty acids and corn steep liquor should not be used in their manufacture. However, in the case of organic livestock production, trace mineral and vitamin supplements are allowed for enrichment or fortification when FDA approved. If feed ingredient manufacturers use corn steep liquor to produce trace mineral and vitamin supplements, this would be a significant use of corn steep liquor in organic livestock production.

Evaluation Question #13: Are there other already allowed substances that could be substituted for the petitioned substance? (From 7 U.S.C. § 6517 (m) (6).)

As alternatives, organic crop producers could use synthetic substances that are already allowed in organic crop production to amend soils listed in 7 CFR 205.601. They include: 1) elemental sulfur; 2) magnesium sulfate; 3) soluble boron products; 4) sulfates, carbonates, oxides, or silicates of zinc, copper, iron, manganese, molybdenum, selenium, and cobalt; and 5) vitamins B_1, C, and E. Depending on the crop of interest and the micro-nutrient that is in deficiency, some decision would have to be made about which one would be the most appropriate to use.

As alternatives, organic livestock producers could use synthetic substances that are already allowed in organic livestock production to maintain productive and healthy animals listed in 7 CFR 205.603. They include the following feed additives: 1) magnesium sulfate; 2) trace minerals (used for enrichment or fortification when approved by the FDA); and 3) vitamins (used for enrichment or fortification when approved by the FDA). Depending on the livestock species and the micro-nutrient or vitamin that is in deficiency, some decision would have to be made about which one would be the most appropriate to use. In both cases (crop production and livestock production), the conditions for using materials on the National List of Synthetic Substances must be documented in the organic farming system plan.

Evaluation Question #14: Are there alternative practices that would make the use of the petitioned substance unnecessary? (From 7 U.S.C. § 6517 (m) (6).)

As found in 7 CFR 205.205, organic crop producers must implement a crop rotation including but not limited to sod, cover crops, green manure crops, and catch crops that provides for maintaining and improving soil organic matter content and managing deficient or excess plant nutrients. More specifically 7 CFR 205.203 states that organic crop producers: 1) must select and implement tillage and cultivation practices that maintain or improve the physical, chemical, and biological condition of soil and minimize erosion; 2) must manage crop nutrients and soil fertility through rotations, cover crops, and the application of plant and animal materials; and 3) must manage plant and animal materials to maintain or improve soil organic matter content in a manner that does not contribute to contamination of crops, soil, or water by plant nutrients, pathogenic organisms, heavy metals, or residues of prohibited substances. When these practices prove insufficient to prevent deficient or excess nutrients in soils or plants, a substance on the National List of Synthetic Substances allowed for use in organic crop production (7 CFR 205.601) may be applied to maintain adequate nutrients for plant productivity and health (see the information in response to Question13). .

As found in 7 CFR 205.237, organic livestock producers must provide livestock with a total feed ration composed of agricultural products, including pasture and forage, that are organically produced and if applicable, organically handled. Non-synthetic substances and synthetic substances allowed in 7 CFR 205.603 may be used as feed additives and supplements (see the information in response to Question 13).

References

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Wilson, C.M. 1987. Proteins of the kernel. In *Corn: Chemistry and Technology;* Watson, S.A. and P.E. Ramsted, Eds.; Amer. Assoc. of Cereal Chemists; St. Paul, Minnesota; pages 273 – 310.

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 7

Monopotassium Phosphate Animal Use Regulatory Review

Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

Native Microbials

October 2021

Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

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LIST OF TABLES

Safety Evaluation of Monopotassium Phosphate for Use as Mineral Substance for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

1. INTRODUCTION

Native Microbials, Inc. (hereafter referred to as "Native Microbials") develops direct-fed microbial (DFM) productsfor use as supplementary feeds for poultry and cattle in the United States (U.S.). One of the raw materials used to charge the fermenter for the production of the DFM strains is monopotassium phosphate, FCC grade. While dipotassium phosphate is permitted for use as a sequestrant in feed in accordance with good manufacturing or feeding practice under 21 CFR §582.6282[1], monopotassium phosphate is currently not currently acceptable for feeding to animals in the U.S. Considering that all raw materials used in the production of DFM products should be accepted feed substances in the U.S., Native Microbials has conducted a safety evaluation to confirm the suitability of monopotassium phosphate for the intended use as a processing aid in the fermentation of its microbial strains.

2. REGULATORY STATUS

2.1 Regulatory Status in Animal Feed in the U.S.

A number of related phosphate salts are acceptable for use in animal feed in the U.S. and are summarized in Table 2.1.

Table 2.1: Examples of Related Phosphate Salts Accepted for Use in Animal Feed in the U.S.

Mineral Substance	Function in Feed	Regulatory Status
Diammonium phosphate	Mineral product and general purpose food additive	21 CFR §582.1141 and AAFCO ingredient definition 57.16
Dicalcium phosphate	Mineral product and general purpose food additive	21 CFR §582.1217, 21 CFR §582.5217 and AAFCO ingredient definition 57.71
Disodium phosphate	Mineral product and general purpose food additive	21 CFR §582.1778, 21 CFR §582.5778 and AAFCO ingredient definition 57.32
Monoammonium phosphate	Mineral product and general purpose food additive	21 CFR §582.1141 and AAFCO ingredient definition 57.33
Monocalcium phosphate	Mineral product and general purpose food additive	21 CFR §582.1217, 21 CFR §582.5217 and AAFCO ingredient definition 57.98
Monosodium phosphate	Mineral product and general purpose food additive	21 CFR §582.1778, 21 CFR §582.5778 and AAFCO ingredient definition 57.99
Phosphoric acid	Mineral product and general purpose food additive	21 CFR §582.1073 and AAFCO ingredient definition 57.19
Dipotassium phosphate	Sequestrant	21 CFR §582.6282

[1] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=582.6285&SearchTerm=dipotassium%20phosphate

2.2 Regulatory Status in Animal Feed in Canada

Monopotassium phosphate is permitted for use in animal feed as in Canada as a Class 6 – Mineral Product under Schedule IV, Part I of the Feed Regulations (1983). The substance must be labelled with guarantees for minimum percent potassium, minimum percent phosphorus and maximum milligrams fluorine, arsenic and iron per kilogram

2.3 Regulatory Status in Animal Feed in the European Union (EU)

Monopotassium phosphate is a recognized feed material in the EU and listed in the Feed Materials Catalogue laid down under Commission Regulation (EU) No 68/2013 (European Commission, 2013). The substance must be labelled with total phosphorus, potassium and, where greater than 10%, the content of phosphorus insoluble in citric acid.

2.4 Regulatory Status in Human Food in the U.S.

Monopotassium phosphate is generally recognized as safe as a food additive in frozen eggs at levels of less than 0.5% in accordance with 21 CFR §160.110.

3. SAFETY EVALUATION FOR TARGET ANIMALS

3.1 History of Use

As mentioned in Section 2, monopotassium phosphate has a long and established history of use as a mineral substance for use in animal feed in Canada and the EU. The levels of monopotassium phosphate as a source of phosphorus in feed is expected to be higher than the residues arising from carry-over of the fermentation process in DFM products. On this basis, the history of safe use of monopotassium phosphate in Canada and the EU for use in animal feed supports the suitability of the additive for use as a raw material in the fermentation of microbial strains by Native Microbials.

3.2 Natural Occurrence

Potassium is present in most feedstuffs with the highest levels typically reported in protein sources such as soybean meal. Thus, deficiencies in animals, particularly non ruminants are rare (NRC, 2005). Where diets contain high levels of industrial by-products such as brewer's grains or corn gluten, supplementation can be required.

Likewise, phosphates are widely available from the feed, with oilseed meals and other plant-based materials, mineral feeds, and meat and marine animal feeds serving as major sources in the diet of animals. Availability of phosphorus from the diet can vary with the source and is generally taken into account in the formulation of livestock diets (NRC, 2005).

It is reasonable to assume that these background sources will provide potassium and phosphorus as significantly higher levels in the diet of poultry and cattle than will be carried over from the use as a fermentation aid in the production of microbial strains by Native Microbials.

3.3 Metabolic Fate

On ingestion by animals, monopotassium phosphate will dissociate to the respective potassium, hydrogen and phosphate ions. Equivalent behaviour in the gastrointestinal tract is observed on ingestion

of related salts such as mono- and di-sodium phosphate and dipotassium phosphate. Thus, the use of monopotassium phosphate will result in exposure by animals to ions commonly consumed in animal feed. On this basis, the available safety data on sodium, calcium and ammonium phosphate salts as well as dipotassium phosphate may be extrapolated to support the safety of monopotassium phosphate (see Section 3.3 and 3.4).

3.4 Mineral Tolerances

Both potassium and phosphorus are required nutrients for poultry and cattle and are considered by the National Research Council (NRC) to be of medium concern for animal health. The NRC has set maximum tolerable levels for potassium of 1% in the diet of poultry and cattle on a dry matter basis, and for phosphorus of 1% for growing birds, 0.8% for laying hens and 0.7% for cattle on a dry matter basis (NRC, 2005). Any carry-over in the diet of monopotassium phosphate from the production of microbial strains for use as DFM products will contribute to the levels of these minerals in the feed but the overall impact on the daily intakes by animals is expected to be very low.

3.5 Evaluations by Scientific Bodies

3.5.1 JECFA Evaluation

The Joint FAO/WHO Committee on Food Additives (JECFA) has evaluated the safety of phosphoric acid and phosphate salts as a group, including within the scope of the review, mono-, di- and tri-potassium phosphate (JECFA, 1982). In the latest evaluation conducted in 1982, JECFA concluded that:

"*Metabolically, the phosphate salts provide a source of the various cations and phosphate ion. Of the greatest concern is the toxicity arising from calcium, magnesium and phosphate imbalance in the diet. Phosphate salts were not mutagenic in a number of test systems. Teratogenic effects have not been observed in mammalian test systems.*

Numerous animal studies have shown that excessive dietary phosphorus causes an increase of plasma phosphorus and a decrease in serum calcium. The resulting hypocalcaemia stimulates excretion of PTH which in turn increases the rate of bone resorption and decreases calcium excretion. These homeostatic adjustment to high dietary phosphorus may result in bone loss and calcification of soft tissues in animals.

The dose levels of phosphate producing nephrocalcinosis were not consistent among the various rat feeding studies. However, the rat is exquisitely susceptible to calcification and hydronephrosis upon exposure to acids forming calcium chelates or complexes. The lowest dose levels that produce nephrocalcinosis overlap the higher dose levels failing to do so. However, this may be related to other dietary imbalances, such as the level of magnesium in the diet. There is still uncertainty on the optimal Ca:P ratio and whether this ratio is of any dietary significance in man.

The lowest level of phosphate that produced nephrocalcinosis in the rat (1% P in the diet) is used as the basis for the evaluation and, by extrapolation based on the daily food intake of 2800 calories, this gives a dose level of 6600 mg P per day as the best estimate of the lowest level that might conceivably cause nephrocalcinosis in man. The usual calculation for provision of a margin of safety is probably not suitable for food additives which are also nutrients. Ingested phosphates from natural sources should be considered together with that from food additive sources. Since phosphorus (as phosphates) is an

essential nutrient and an unavoidable constituent of food, it is not feasible or appropriate to give a range of values from zero to maximum."

On the basis of the above, the maximum tolerable daily intake for man was estimated to be 70 mg/kg body weight.

3.5.2 SCF Evaluation

The Scientific Committee on Food (SCF) in the European Union (EU) evaluated the group of phosphate salts used as food additives in 1990 and agreed with the JECFA estimate of 70 mg/kg body weight for man, calculated as phosphorus (SCF, 1990).

3.5.3 Summary

Taken together the body of available data indicate that the safety of monopotassium phosphate can be considered from the available data on phosphoric acid and phosphate, which have been previously evaluated by JECFA and the SCF for use as food additives. These evaluations highlighted the role of phosphate salts to provide a metabolic source of cations and the phosphate ion. Safety was primarily based on the absence of any genotoxicity and the requirement to provide nutritionally balanced levels in the diet which do not exceed the maximum that can be tolerated by the body.

4. EXPOSURE ANALYSIS

(b) (4)

5. SUMMARY AND CONCLUSIONS

Monopotassium phosphate has an established history of safe use as a mineral substance for use in animal feed in Canada and in the EU. On ingestion by poultry or cattle, monopotassium phosphate will dissociate into the potassium, hydrogen and phosphate ions. For this reason, and consistent with the evaluations of the additive for use in food by JECFA and the SCF, the safety can be primarily derived from the body of available data on phosphoric acid and phosphate salts. Potassium and phosphate are both essential nutrients for animals and present naturally in the feed as well as being added in the form of supplemental salts. The carry-over of potassium and phosphate from its use as a monopotassium salt in the fermentation of microbial strains for use as DFMs in poultry and cattle feed is shown in the example above to make insignificant contribution to the levels present in the diet from natural and supplemental sources.

Together, it is concluded that there are no safety concerns associated with the use of monopotassium phosphate by Native Microbials as a fermentation aid under the conditions of

intended use.

6. REFERENCES

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AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 8

(b) (4) Ascorbic Acid Letter

(b) (4)

October 21, 2021

RE: Elemental Impurities – *Ascorbic Acid, USP* (Cat# *AS102*)

To Whom It May Concern:

Thank you for your interest in (b) (4) high quality chemicals.

The above material complies with the USP<232>, <233> Elemental Impurities and the ICH Q3D Elemental Impurities Guideline. Per the current supply chain, the following elemental impurities are likely to be present:

Elemental Impurity		Class	Expected Concentration
Cadmium	Cd	1	< 0.01 ppm
Lead	Pb	1	< 2 ppm
Arsenic	As	1	< 3 ppm
Mercury	Hg	1	< 1 ppm

Other elemental impurities considered by USP <232>, <233> and ICH Q3D which are not addressed in the above mentioned table are not likely to be present. These substances are not used in the production process, are not intentionally added or known to be present in the above mentioned material.

This information is subject to change and is intended for risk assessment only. It is responsibility of the end user to evaluate suitability of any chemical for the intended use as well as to assess compound-specific limits of daily intake of metal impurities. For lot-specific information, please refer to the respective Certificate of Analysis.

(b) (4)

Sincerely,

(b) (6)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

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ATTACHMENT 9

(b) (4) Ascorbic Acid Certificate of Analysis

(b) (4)

Printed: 10/21/2021 Page 1 of 1

Catalog : AS102 Ascorbic Acid, USP **Lot :** 2JI0075

Chemical Formula : $C_6H_8O_6$
CAS# : 50-81-7

Formula Weight : 176.13

Test	Limit Min.	Limit Max.	Results
ASSAY	99.0	100.5 %	(b) (4)
SPECIFIC ROTATION $[a]_D$	+20.5 to	+21.5	
RESIDUE ON IGNITION	--	0.1 %	
ELEMENTAL IMPURITIES:			
CADMIUM (Cd)	--AS REPORTED		
LEAD (Pb)	--AS REPORTED		
ARSENIC (As)	--AS REPORTED		
MERCURY (Hg)	--AS REPORTED		
IDENTIFICATION A (FTIR)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
IDENTIFICATION (B)	REDUCES ALKALINE CUPRIC TARTRATE TS		REDUCES ALKALINE CUPRIC TARTRATE TS
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			29-MAR-2022
DATE OF MANUFACTURE			30-MAR-2019
APPEARANCE			WHITE CRYSTALLINE POWDER
RESIDUAL SOLVENTS	--AS REPORTED		
CLASS 2 (SOLVENT) / METHANOL			(b) (4)

All pharmaceutical ingredients are tested using current edition of applicable pharmacopeia at time of release.

Read and understand label and MSDS/SDS before handling any chemical. All (b) (4) chemicals are for manufacturing, processing, repacking or research purposes by experienced personnel only. The customer must ensure to provide its users adequate hazardous material training and appropriate protective equipment before handling our chemicals.

The Elemental Impurities standards implemented by USP and other Pharmaceutical Compendia reflect growing understanding of the toxicology of trace levels of elemental impurities that can remain in drug substances originating from either raw materials or manufacturing processes. Identifying and quantifying impurities can be critical to predicting the best possible patient outcomes. Elemental Impurities has been a requirement of all products meeting USP/NF, EP and BP monographs since January 1, 2018. More information can be found in USP sections <232> Elemental Impurities - Limits and <233> Elemental Impurities - Procedures. Data for drug substances furnished by (b) (4) can be used to ensure that patient daily exposures by oral administration to the selected elements are not exceed in the formulation of pharmaceutical products.

Certificate of Analysis Results Certified By:

(b) (6)

(b) (4)

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
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ATTACHMENT 10

Master Production Records

Master Production Record for DY19 Milled Preservation by Vaporization (mPBV)
Version 1

Objective

(b) (4)

Materials

(b) (4)

Method

(b) (4)

(b) (4)

(b) (4)

(b) (4)

2. (b) (4)

(b) (4)

(b) (4)

Reasons for Revision

(b) (4)

MPR Approvals

Name & Title	Signature & Date
Martin Mayhew VP – Process Development and Manufacturing	
(b)(6)	

ASCUSDY19 Master Production Record

OVERVIEW

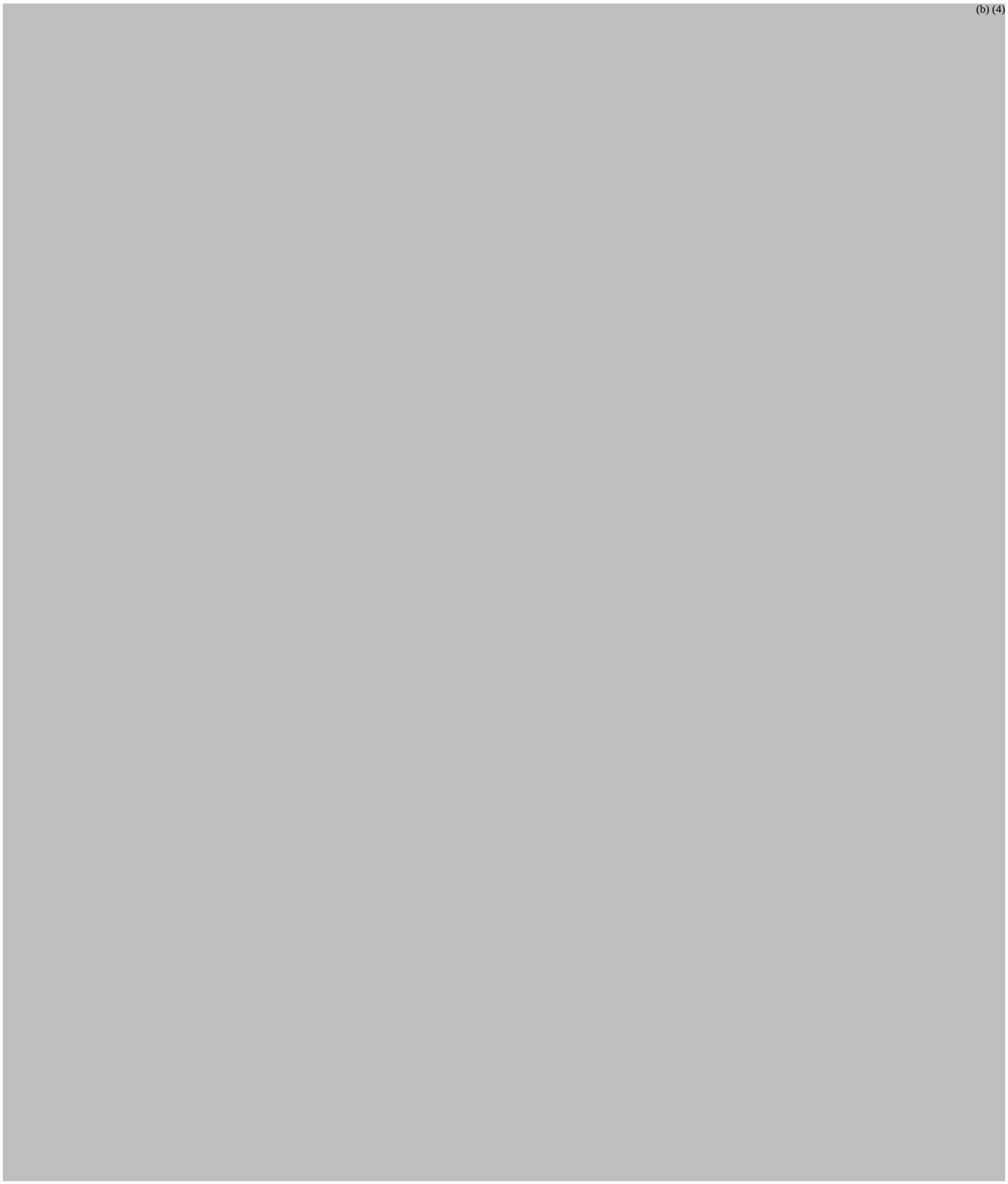
(b) (4)

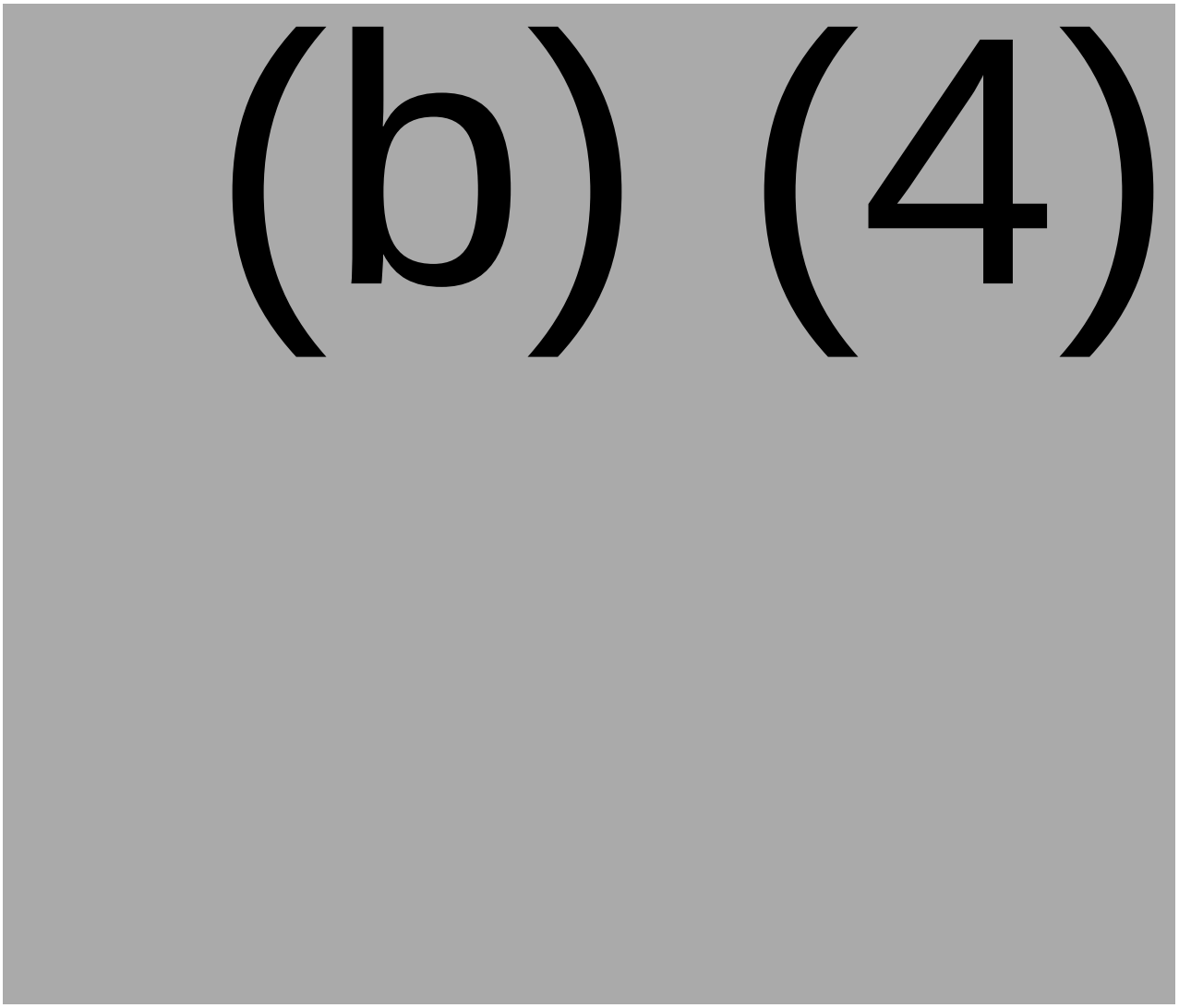
(b) (4)

(b) (4)

Table 3. Dairy-19 Fermentation Media

(b) (6)


(b) (4)


(b) (4)

Manufacturing Process Record: Preparation of DY19 Fat Encapsulate

Objective

(b) (4)

(b) (4)

Media & Reagents

(b) (4)

Method

(b) (4)

(b) (4)

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
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ATTACHMENT 11

Stability Report 5°C (Ambient)

DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 5°C Stability Report

Purpose

The purpose of this report is to analyze the real time data of DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate lots 1801.2033, 1801.2035, and 1801.2037 stored at 5°C to support the prediction of product stability at 2-10°C.

Results

Samples were placed at 5°C and analyzed at (b) (4) for viable cell count according to the approved Stability Protocol for DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate. See Table 1 below for test timepoints. The study is still ongoing, so the 12 month time point has not been reached yet.

Table 1 – Tests and timepoints.

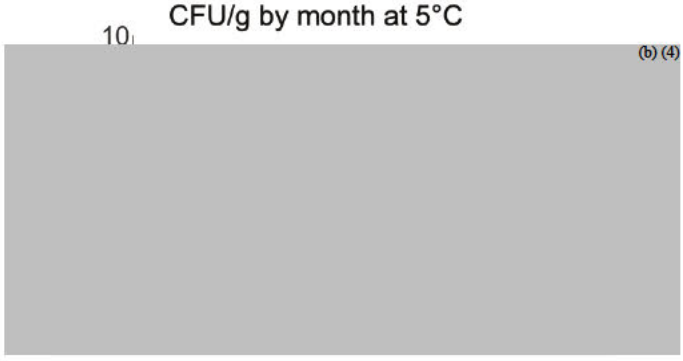
Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY19 Solid Intermediate Microbe Enumeration method	X	(b) (4)					

The CFU/g for each lot are displayed in Table 2 below and graphed in Figure 1.

Table 2 – Test Results

Month	1801.2033 CFU/g	1801.2035 CFU/g	1801.2037 CFU/g
0	6.61E+08	1.31E+09	3.98E+08
1	(b) (4)		
2			
3			
6			
9			

Figure 1 – CFU/g by month



Log_{10} CFU/g measurements are plotted, with the minimum specification (2.0 X 107 CFU/g) represented as zero on the y-axis. Shaded area represents the 95% confidence interval.

Conclusion

Real time stability data collected for 9 months at 5°C demonstrates that all 3 lots of DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate remain above the minimum specification (2.0 X 107 CFU/g) for the duration tested.

Data Availability

All data is retained and available on the company Google Drive:

(b) (4)

Stability Protocol

native

nativemicrobials.com

Stability Protocol Title:	DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 5°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY19 Solid Intermediate Microbe Enumeration method	X	(b) (4)					

Protocol Approvals:

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACDDDAD433BF48... 12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F... 12/1/2020
(b)(6)	(b)(6) 12/1/2020

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 12

Stability Report 25°C (Accelerated)

DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 25°C Stability Report

Purpose

The purpose of this report is to analyze the real time data of DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate lots 1801.2033, 1801.2035, and 1801.2037 stored at 25°C to support the prediction of product stability at 2-10°C.

Results

Samples were placed at 25°C and analyzed at (b) (4) for viable cell count according to the approved Stability Protocol for DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate. See Table 1 below for test timepoints. The study is still ongoing, so the 12 month time point has not been reached yet.

Table 1 – Tests and timepoints.

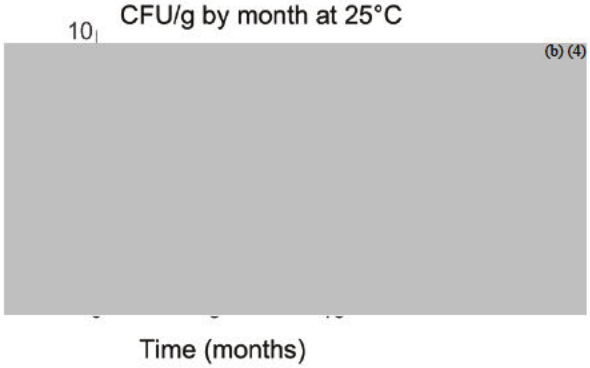
Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY19 Solid Intermediate Microbe Enumeration method	X	(b) (4)					

The CFU/g for each lot are displayed in Table 2 below and graphed in Figure 1.

Table 2 – Test Results

Month	1801.2033 CFU/g	1801.2035 CFU/g	1801.2037 CFU/g
0	6.61E+08	1.31E+09	3.98E+08
1	(b) (4)		
2			
3			
6			
9			

Figure 1 – CFU/g by month



Log_{10} CFU/g measurements are plotted, with the minimum specification (2.0 X 107 CFU/g) represented as zero on the y-axis. Shaded area represents the 95% confidence interval.

Conclusion

Real time stability data collected for 9 months at 25°C demonstrates that all 3 lots of DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate remain above the minimum specification (2.0 X 107 CFU/g) for the duration tested.

Data Availability

All data is retained and available on the company Google Drive:

(b) (4)

Stability Protocol

native

nativemicrobials.com

Stability Protocol Title:	DY19 *Butyrivibrio fibrisolvens* ASCUSDY19 Fat Encapsulate 25°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	
Acceptance Criteria:	

Tests and Timepoints:

Assay	T_0	1 Month	2 Months	3 Months	6 Months	9 Months	12 Months
DY19 Solid Intermediate Microbe Enumeration method	X	(b) (4)					

Protocol Approvals:

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew 12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 12/1/2020
(b)(6)	12/1/2020

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19
GRAS Notice Amendment

ATTACHMENT 13

(b) (4) Botulinum Testing Letter

(b) (4)

April 14, 2021

Native Microbials
10255 Science Center Dr
San Diego, CA 92121

To Whom It May Concern:

(b) (4). is a Tier 1 Select Agent facility regulated by the Centers for Disease Control and Prevention (CDC) and is approved to work with botulinal toxins and neurotoxin-producing strains of *Clostridium botulinum*. The lab is audited by the CDC routinely to ensure compliance to internal procedures and federal regulations.

Sample analysis follows procedures in the Food and Drug Administration (FDA) Bacteriological Analytical Manual https://www.fda.gov/food/laboratory-methods-food/bam-chapter-17-clostridium-botulinum. The lab performs routine botulinal toxin screens on uninoculated client samples via the mouse bioassay. This assays for total biologically active botulinal toxin and does not differentiate by toxin type. Trypsin is added to a portion of the supernatant to activate toxin from nonproteolytic strains, if present. If the assay is negative, the result is reported to the client and no further testing is performed. If the assay is presumptive, additional testing can be performed to confirm the presence of botulinal toxin and the toxin type(s).

Regards,

(b) (4)

(b) (4)

From: Kristi Smedley
To: Animalfood-premarket
Cc: Adams, Carissa; Kevin Korth
Subject: [EXTERNAL] RE: AGRN #42 Amendment Clarification
Date: Tuesday, January 11, 2022 6:58:20 PM
Attachments: image002.png
CFR-FDA Cover letter AGRN 42 amendment Jan 11 2022.pdf
ASCUSDY19 (AGRN 42) Amended Section 2.pdf
Updated Detailed Manufacturing Summary-CONFIDENTIAL AscusDY19.pdf

Megan:

In response to the request for clarification on AGRN 42, we have provided a cover letter and two attachments that support the response.

Please let me know if you have any further questions or have any issues in receiving these documents.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
(b) (6)
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From: Animalfood-premarket [mailto:Animalfood-premarket@fda.hhs.gov]
Sent: Tuesday, December 28, 2021 3:22 PM
To: Kristi Smedley
Cc: Animalfood-premarket; Adams, Carissa
Subject: AGRN #42 Amendment Clarification

Good afternoon,

We are seeking two further points of clarification to the information provided by Native Microbials in its November 4, 2021 amendment.

In the amendment, Native Microbials describes that the technical effect of *B. fibrisolvens* ASCUSDY19 is to support digestion and that Section 2.5.1 "has been removed from the dossier (see Attachment 2)" [in response to Utility question 2]. Further, the notifier states in Attachment 2 that "2.5.1 ****This Section Has Been Removed***". CVM notes that in the GRAS notice, dated December 30, 2020, that the title of Section 2.5.1 was "Rumen

Microbiome". We also note that Section 2.5.2 within Attachment 2 (i.e., Amended Section 2.5) contains the information that was in the "Rumen Microbiome" section of the original notice. It appears that this section was mistakenly left in Attachment 2; the notifier should resubmit Attachment 2 with the section removed as was described in the amendment.

When evaluating the commercial manufacturing process in Attachment 5 of the amendment and the pilot scale production process in Attachment 10 of the amendment, we noted several differences. The notifier should clarify if the notified substance and associated safety assessment is based on the process described for the commercial manufacturing process in attachment 5.

If the notifier is able to provide this clarification within the next two weeks, **no later than January 12, 2022**, we will continue our evaluation of the notice. If the notifier is not able to provide this clarification, it may, as always, request that we cease to evaluate the GRAS notice. Please send any information to animalfood-premarket@fda.hhs.gov.

If you have any questions, please do not hesitate to reach out.

Have a lovely day!

Megan

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Since 1973
Consultant to the Regulated Industry

January 12, 2021

David Edwards, Director
Division of Animal Feeds (HFV-220} Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855

Subject: Amendment Animal GRAS Notice 42
Butyrivibrio fibrisolvens ASCUSDY19 For Dairy Cattle
Notifier: Native Microbials, Inc.
10255 Science Center Dr. Suite C2
San Diego, California 92121

Dear Dr. Edwards:

In response to the Division email of December 28, 2021 requesting clarification regarding our Amendment to AGRN#42 for ASCUSDY19 (November 4, 2021) , the following clarification is provided.

1. In reference to the first point, regarding the duplication of section 2.5.1 in our amended dossier Part 2, the agency is correct in that we intended on eliminating the original section 2.5.1 titled "Rumen Microbiome" but unintentionally left it in creating a duplicate 2.5.2, such that we ended up with two sections 2.5.2. Attached is the corrected amendment to Part 2, with changes only found in section 2.5.

2. In keeping with the regulatory requirements for GRAS conclusions we included a summary of the description of the manufacturing process (21 CFR 570.230 (b)). In the amendment to answer some of the concerns we provided a Master Production Records that had a few tighter tolerances than those found in the summary document. To alleviate any confusion, the summary document has been updated to match the Master Production Records in all ways except one. In that one case we found an error in the provided Master Production Records for (b) (4) We have provided a revised (Updated) "Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19" as an attachment to this amendment which reflects the changes.

We are confident that these clarifications are suitable for the FDA's continued evaluation of ASCUSDY19, AGRN#42 and are happy to respond to any further questions.

Sincerely,

Kristi Smedley

Digitally signed by Kristi Smedley
DN: cn=Kristi Smedley, o=Center for Regulatory Services, Inc., ou, email=smedley@cfr-services.com, c=US
Date: 2022.01.11 17:52:58 -05'00'

Kristi O. Smedley, Ph.D.
Consultant to Native Microbials, Inc.

Cc: Mallory Embree, Native Microbials, Inc.

ATTACHMENTS:
Updated Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19
Amended Section 2.1.4 through 2.1.9 and 2.5

AGRN 42 *Butyrivibrio fibrisolvens* ASCUSDY19

Amended Sections 2.1.4 through 2.1.9 and 2.5

2.1.4 Identification of the Microorganism

2.1.4.1 16S rRNA Gene Sequencing

The 16S rRNA gene was amplified from the strain using 27F and 543R primers and paired end sequenced [2x300 base pairs (bp)] using an Illumina Miseq (Schumann 1991; Muyzer, de Waal, and Uitterlinden 1993). The resulting sequence was quality trimmed and compared to National Center for Biotechnology Information (NCBI) databases using the Basic Local Alignment Search Tool (BLAST) to establish the identity of the strain. Details of the analysis including the BLAST output are provided in Appendix 003A and 003B. Strains of *B. fibrisolvens* and unnamed rumen bacterium provided 16S rRNA sequence matches that fall within the minimum 98.7% sequence identity threshold typically used to define a species (Yarza et al. 2014). The best match was to *B. fibrisolvens* InBov1 at 99.7% sequence identity. Results can be found in Table 2.4.

While 16S rRNA alignment of the partial gene returned matches to *B. fibrisolvens* strains above the minimum 98.7% sequence identity threshold, the *B. fibrisolvens* type strain (ATCC 19171) returned alignment of 95.2% to the partial sequence. To confirm that *B. fibrisolvens* ASCUSDY19 should be identified as a strain of *B. fibrisolvens,* a copy of the 1,551 bp full length 16S rRNA sequences was extracted from the *B. fibrisolvens* ASCUSDY19 whole genome sequence and compared to the NCBI database by BLAST. Results confirmed that *B. fibrisolvens* ASCUSDY19 is a strain of *B. fibrisolvens* as alignment of the full length 16S rRNA gene resulted in 99.6% identity and 95% coverage alignment to the *B. fibrisolvens* type strain (ATCC 19171).

Table 2.4: Partial 16S rRNA alignment to *B. fibrisolvens* ASCUSDY19 16S rRNA by BLAST

Genus species (Genbank accession #)	Identity (%)	Coverage (%)
B. fibrisolvens InBov1 (JN642599)	99.7%	100%
Rumen Bacterium NK3B81 (GU324363)	99.7%	99%
Rumen Bacterium NK4A61 (GU324372)	99.3%	99%
Rumen Bacterium NK4A114 (GU324377)	98.9%	99%
B. fibrisolvens WV1 (AF396927)	98.3%	99%

2.1.4.2 *Whole Genome Sequence Assembly and Annotation*

Genomic DNA was isolated from a pure culture of *B. fibrisolvens* ASCUSDY19 and sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA). The resulting libraries were paired-end sequenced (1x300bp) on an Illumina Miseq and in parallel, long-read libraries were prepared from the same extracted DNA using SQK-RAD004 kit (Oxford NanoporeTechnologies, Oxford) following the protocol outlined by Jain *et al*. (2018) and 1D sequenced on the MinION (R9.4 flowcell; Oxford Nanopore, Oxford) (Jain et al. 2018). The genome was assembled through hybrid methods utilizing both short and long reads. Read quality and genome coverage was evaluated using FASTQC for Illumina data and NanoStat for the Oxford Nanopore reads. The *B. fibrisolvens* ASCUSDY19 genome was closed with no gaps and consisted of 2 chromosomes, a main chromosome (b) (4)) and a chromid (b) (4) The presence of a chromid is consistent with previous observations of the species (Rodríguez Hernáez et al. 2018). (b) (4) Assembly statistics can be found in Table 2.5. The full details of the assembly are provided in Appendix 003C.

Protein coding genes were predicted through GLIMMER2 and through an iterative process of annotating putative genes using the FIGfams database (Delcher 1999; Meyer, Overbeek, and Rodriguez 2009). To identify protein coding open reading frames of potential genes, contigs were first filtered of all potential tRNA coding genes (T. M. Lowe and Eddy 1997) and rRNA genes (Aziz et al. 2008).

The *B. fibrisolvens* ASCUSDY19 genome contains 3,867 coding sequences which were subsequently built into a metabolic reconstruction describing 235 functional subsystems (DeJongh et al. 2007; Becker and Palsson 2005). These subsystems include larger metabolic groups describing metabolism, virulence, plasmids, disease, defense metabolic products, stress response and dormancy.

The assembled genome has been deposited at NCBI under accession number CP065800 for the main chromosome and CP065801 for the chromid.

Table 2.5: Assembly Statistics for *B. fibrisolvens* ASCUSDY19

# of Contigs	2
# of Contigs ≥ 5,000 bp	2
Longest Contig (bp)	(b) (4)
Assembly Length	(b) (4)
N50	(b) (4)
N75	(b) (4)
GC%	(b) (4)

2.1.4.3 *Whole Genome Sequence Comparison*

To determine relatedness of *B. fibrisolvens* ASCUSDY19 to other closely related species at a higher resolution, whole genomes were compared using ANI. Candidate genomes for genome-genome comparison to *B. fibrisolvens* ASCUSDY19 were selected by full length 16S rRNA similarity and downloaded from the NCBI database. MUMmer was used to generate the alignments for ANI on the basis that this software is adept at aligning highly similar sequences and is more stringent than most

other aligners such as BLAST (Kurtz et al. 2004). Results for the MUMmer alignment can be found in Table 2.6.

The only ANI matches to *B. fibrisolvens* ASCUSDY19 above the 95% ANI cutoff to be considered the same species were two strains of *B. fibrisolvens* (Richter and Rosselló-Móra 2009).

Table 2.6: Average Nucleotide Identity (ANI) of Related Species to *B. fibrisolvens* ASCUSDY19 by MUMmer

Genus species (assembly)	ANI (%)	Coverage (%)
B. fibrisolvens INBov1 (GCA_003175155)	97.6	72.1
B. fibrisolvens YRB2005 (GCA_000423985)	96.8	77.3
B. fibrisolvens DSM3071 (GCA_900129945)	89.2	34.8
Butyrivibrio proteoclasticus B316n (GCA_000145035)	86.4	3.69
Butyrivibrio proteoclasticus P6B7 (GCA_000622085)	85.5	2.8
Butyrivibrio hungatei NK4A153 (GCA_000424465)	84.8	2.6
Butyrivibrio hungatei MB2003 (GCA_001858005)	84.4	3.4

2.1.4.4 Summary and Conclusions

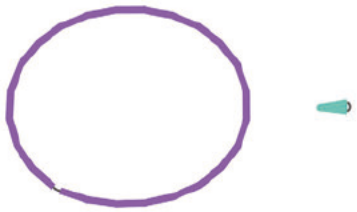
16S rRNA and whole genome analysis confirm that *B. fibrisolvens* ASCUSDY19 represents a member of the species *B. fibrisolvens.*

2.1.5 Plasmid Analysis

To confirm the presence/absence of plasmids, the assembly graph for the *B. fibrisolvens* ASCUSDY19 assembly was analyzed by Bandage (Wick et al. 2015). The assembly graph analysis confirmed that the *B. fibrisolvens* ASCUSDY19 was contained in 2 circular chromosomes with no unincorporated fragments, verifying the completeness of the assembly. Image of the assembly graph can be found in Figure 2.4.

As noted in Part 2.1.4.2, the presence of a smaller, circular second replicon (chromid) is consistent with other assemblies of the species. The annotated features on the putative chromid are associated with general housekeeping and metabolic functions, which is consistent with gene composition of chromids (Harrison et al. 2010). No genes encoding virulence factors, toxins, antimicrobial resistance, or transposable elements were found on the chromid.

Figure 2.4: ***B. fibrisolvens*** **ASCUSDY19 Assembly Graph as Generated by Bandage**



2.1.6 *In-vitro* and *In-silico* Analysis of Antibiotic Susceptibility

Phenotypic testing was conducted on *B. fibrisolvens* ASCUSDY19 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials of relevance to human and veterinary medicine. The full study report is provided in Appendix 004 and results can be found in Table 2.7. The results were evaluated against the resistant breakpoints set by the European Food Safety Authority (EFSA) for "other gram positive bacteria", the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram positive anaerobes" and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). The MIC values reported for *B. fibrisolvens* ASCUSDY19 were equal, or lower than, the cut-off values and break-points established by EFSA, EUCAST and/or CLSI for chloramphenicol, and ampicillin. The isolate would be considered susceptible to Vancomycin and Clindamycin according to EFSA and EUCAST breakpoints but considered intermediately sensitive to Clindamycin per CLSI MIC values were also considered to be in the intermediate range established by CLSI for tetracycline. MIC values reported for *B. fibrisolvens* ASCUSDY19 were higher than the cutoff values and break-points established by EFSA for tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin.

It should be noted that susceptibility to aminoglycosides (gentamicin, kanamycin, streptomycin) and macrolides (erythromycin) decrease significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al. 2016). As such, classifications set forth by EFSA are for general gram-positive organisms and should be carefully applied to *Butyrivibrio fibrisolvens* due to its anaerobic nature. CLSI and EUCAST refrain from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. Tetracycline resistance was indicated by values above the EFSA breakpoint and in the intermediate range by CLSI breakpoint. Tetracycline resistance is not uncommon among ruminal derived organisms. Among 68 livestock derived *Clostridium* strains analyzed by Dutta et al. (1983) 17/68 (25%) strains displaying MIC values above the EFSA microbiological cut-off value. More recent studies have shown that tetracycline resistance is widespread amongst diverse taxa in the rumen (Dutta, Devriese, and Van Assche 1983). Sabino et al. (2019) found that 69% of the ruminal isolates they screened contained tetracycline resistance genes, which were not only expressed, but also reflected in a resistant phenotype (Y. N. V. Sabino et al. 2019).

Table 2.7:	*B. fibrisolvens* ASCUSDY19 Antimicrobial Susceptibility in Relation to EFSA, EUCAST, and CLSI breakpoints				
Antimicrobial	**Tested Range (ug/mL)**	**MIC (ug/mL) of *B. fibrisolvens* ASCUSDY19**	**2018 EFSA Microbiology Cut-off Values (ug/mL) for Other Gram +**	**EUCAST Resistant Breakpoints (ug/mL) Gram + Anaerobes**	**CLSI Resistant Breakpoints (ug/mL) Anaerobes***
Ampicillin	0.5-128	< 0.5	1	8	≥ 2 (R) ≥ 1 (I)
Chloramphenicol	0.5-64	4	4	8	≥ 32 (R) ≥ 16 (I)
Clindamycin	0.03-32	4	4	4	≥ 8 (R) ≥ 4 (I)
Erythromycin	0.5-16	4	1	Not available	Not available
Gentamicin	0.5-32	8	4	Not available	Not available
Kanamycin	0.5-64	> 64	16	Not available	Not available
Streptomycin	0.5-64	16	8	Not available	Not available
Tetracycline	0.0625-64	8	4	Not available	≥ 16 (R) ≥ 8 (I)
Vancomycin	0.125-32	0.25	1	2	Not available

*R = Resistant Breakpoint; I = Intermediate Sensitivity / Susceptible, Increased Exposure. A microorganism is categorized as "I" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.

To evaluate the presence of antimicrobial resistance genes in the *B. fibrisolvens* ASCUSDY19 genome, amino acid sequences from coding regions identified in Part 2.1.4.3 were aligned to the PATRIC database. Included in the PATRIC database is the Comprehensive Antibiotics Resistance Database (CARD) and NCBI's National Database of Antibiotic Resistant Organisms (NDARO) for assessing antimicrobial resistance. In addition to the protein sequences from the databases, PATRIC has compiled protein hits to CARD and NDARO from 331,756 bacterial genomes and included those as redundant gene entries as a means to understand the global distribution of antimicrobial resistance proteins across diverse taxa isolated from a wide range of environments and hosts. Antimicrobial resistance was further explored using the ResFinder web server (Zankari et al. 2012) and BLASTp alignment to the NCBI AMR database as used by AMRFinder (Note: this database differs from NARDO used by PATRIC) (Feldgarden et al. 2019). Between these databases there are a total of 30,748 protein sequences, characteristics of each database can be found in Table 2.8.

Table 2.8: Characteristics of Databases Used to Assess Antimicrobial Resistance				
Database Name	**Number of Entries**	**Number of *Butyrivibrio* Entries**	***B. fibrisolvens* Entries**	**Contains Redundant Entries**
CARD (PATRIC)	17,559 (2,227 non redundant proteins	0	0	Yes
NDARO (PATRIC)	5,138 (4,004 non redundant proteins)	0	0	Yes
ResFinder	3,105	0	0	No
AMRFinder Plus	6,946	0	0	No

To ensure no hits were missed due to codon bias or sequencing error, protein alignments were considered a hit if they have greater than 80% identity over more than 70% query coverage. While there are no widely accepted cutoffs for detecting protein homology at the whole genome level, 80% identity and 70% query coverage is a less stringent cutoff than cutoffs established by many tools examining virulence factor and antimicrobial gene protein homologies at the whole genome level. PATRIC and IslandViewer4, for example, use a minimum of 80% identity and 80% coverage as cutoffs (Mao et al. 2015; Bertelli et al. 2017). Similar approaches have been adopted in published studies investigating virulence factors and antimicrobial resistance (J. Liang et al. 2020; Hu et al. 2013; Abril et al. 2020; Deng et al. 2021; Rojas-Estevez et al. 2020; Y. Pan et al. 2020). Hu et al. (2013), for example, found that 80% identity cutoffs maximized the precision of the identification of antimicrobial resistance genes with 99.1% precision. Lower cutoffs resulted in loss of precision of the alignments. This approach has been proven to return precise results that minimize under and over estimation of the number of virulence, toxin production and antimicrobial resistance genes when detecting protein homology at the whole genome level. Lending further support to our selection of an 80% identity/70% query coverage cutoff is EFSA's use of an identical cutoff for whole genome sequence analysis of microorganisms to be used in the food chain as of 2021. Results can be found in Tables 2.9 to 2.11.

Genetic analysis of *B. fibrisolvens* ASCUSDY19 identified one possible resistance gene (see Tables 2.9 to 2.11).

- The antimicrobial gene in question is a 100% match to the tetracycline resistance gene, tetW, in both the ResFinder and NCBI AMR databases and a 99% match to the same gene in the Card and NDARO databases. TetW confers resistance to tetracycline through ribosomal protection (Aminov, Garrigues-Jeanjean, and Mackie 2001). The tet(W) gene is a ubiquitous gene in the bacterial population of ruminants, humans, and other farm animals (Pal et al. 2016; Joyce et al. 2019; Y. Sabino et al. 2019).

Table 2.9: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by PATRIC								
Source	**Source Organism**	**Gene**	**Product**	**Function**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
CARD/ NDARO	*Bifidobacterium longum*	tetW	Tetracycline resistance, ribosomal protection	MULTISPECIES: tetracycline resistance ribosomal protection protein Tet(W)	100	100	99	0.0

Table 2.10: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by ResFinder				
Gene	**Identity**	**Query Coverage**	**Function**	**Accession number**
tetW	99.9	100% (1920/1920)	Tetracycline Resistance	AJ427422

Table 2.11: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by NCBI AMR BLASTp				
Gene	**e-value**	**Percent Identity**	**Query Coverage**	**Subject Coverage**
tet(W)	0	99.8	100	100

2.1.6.1 Section Summary

In vitro testing demonstrated that *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin. Resistance to aminoglycosides and macrolides such as gentamicin, kanamycin, streptomycin, and erythromycin is reflective of *B. fibrisolvens* ASCUSDY19 being anaerobic rather than any specific resistance mechanism or genotype. *In silico* analyses revealed the presence of tetW, a gene implicated in tetracycline resistance. This finding is consistent with the tetracycline resistant phenotype observed in the MIC testing. *B. fibrisolvens* ASCUSDY19 is susceptible to chloramphenicol, vancomycin, and ampicillin and therefore could easily be controlled with readily available antibiotics.

2.1.7 Antimicrobial Production

Butyrivibrio fibrisolvens ASCUSDY19 supernatant obtained post fermentation was tested for inhibitory activity against reference strains known to be susceptible to a range of antibiotics. No zones of inhibition were observed indicating that the strain is not an antimicrobial producer. Further details of the study are provided in Appendix 005.

2.1.8 Toxigenicity and Pathogenicity

To assess the presence of virulent and pathogenic genes, amino acid sequences from coding regions identified in Part 2.1.4.3 were aligned to several databases. All applicable, publicly available databases were used to identify potential pathogenic genes. The characteristics of these databases are described in Table 2.12. The PATRIC database has compiled relevant genes from external databases including Victors, Virulence Factors Database (VFDB), and the PATRIC_VF database. These genes represent 331,756 bacterial genomes. Redundant gene entries (e.g. the same toxin showing up in multiple microbial

species) are included as a means to understand the global distribution of pathogenicity and virulence associated proteins across diverse taxa isolated from a wide range of environments and hosts. To ensure no toxins or virulence genes were missed, amino acid sequences from *B. fibrisolvens* ASCUSDY19 were aligned to the Victors and VFDB databases downloaded independently from PATRIC due to some entries from these databases being absent in PATRIC. As detailed in section 2.6.1. 80% identity and 70% coverage cutoff was applied to alignments of these databases by *B. fibrisolvens* ASCUSDY19. The contents of the databases are summarized in Table 2.12.

A more conservative alignment approach was taken with the alignment of *B. fibrisolvens* ASCUSDY19 to a subset of protein toxins from the VFDB and DBETH databases. Published studies have established less strict cutoffs of 30-50% identity or e-value cutoffs ranging from 1E-04 to 1E-05, when aligning to known protein toxins (Wei et al. 2015; Surachat et al. 2017; Negi et al. 2017; X. Liang et al. 2019). Therefore, an e-value threshold of 1E-04 was used for the alignment to the toxin databases. It is worth noting that this more conservative approach can result in false positives due to many toxin proteins containing multiple domains with only one of the domains being responsible for the detrimental effects of the toxin (Negi et al. 2017; Xie and Fair 2021). As such, smaller databases containing organism specific toxins should be used and results from low identity alignments should be thoroughly vetted to ensure that the corresponding protein hits are not false positives. As there are no known toxins derived from organisms in the genus *Butyrivibrio* or the family *lachnospiraceae* to which *B. fibrisolvens* ASCUSDY19 belongs to a custom database was used that contained all protein toxin entries in the VFDB and DBETH databases from the order Clostridiales for our alignment.

PathogenFInder and IslandViewer web servers (Cosentino et al. 2013; Bertelli et al. 2017) as well as BLASTp alignment to the Pathogen-Host Interaction Database (Phi-BASE) (Urban et al. 2015) were also utilized to assess the pathogenicity and virulence of *B. fibrisolvens* ASCUSDY19. The total number of sequences in the PATRIC and Phi-BASE databases is 134,396 and includes no sequences from *Butyrivibrio.* IslandViewer contains 4,065 pathogenicity islands including 4 from *Butyrivibrio* species. The analysis in PathogenFinder is database independent and uses a model trained with protein sequences from 886 whole genome sequences.

IslandViewer4 is a software that uses multiple diverse methods to predict genomic islands. These methods include IslandPick (Langille, Hsiao, and Brinkman 2008), SIGI-HMM (Waack et al. 2006), IslandPath (Hsiao et al. 2003), and Islander (Hudson, Lau, and Williams 2014). After identification of genomic islands, the sequences in each island are subject to a search against a curated database of virulence factors, antimicrobial resistance genes, and pathogen associated genes. The database searched includes sequences from VFDB (Chen et al. 2005), PATRIC (Wattam et al. 2013), Victors (Sayers et al. 2019), CARD (Jia et al. 2017), and a database of pathogen associated genes from Ho Sui et al. (Ho Sui et al. 2009). IslandViewer4 then annotates the features in each genomic island using 1e-10 evalue, >90% sequence similarity, and >80% coverage for homologues by BLAST. Any genomic island containing a virulence factor, antimicrobial resistance gene, and/or pathogen associated gene is considered a pathogenicity island.

The PathogenFinder model predicts human pathogenicity based on matches to proteins found differentially in human pathogenic and non-pathogenic bacteria regardless of their annotated function. Therefore, a single hit to a protein found in human pathogenic species does not necessarily suggest the query organism is virulent or pathogenic, but a collection of hits to proteins uniquely found in pathogens

could be enough for PathogenFinder to deem the organism a human pathogen, even if the proteins are not traditionally implicated in virulence or pathogenicity. The program allows the organism to be evaluated more holistically and enables the evaluation of proteins that are potentially involved in virulence and pathogenicity beyond well annotated virulence factors such as toxins.

Table 2.12: Characteristics of Databases Used to Assess Virulence and Pathogenicity

Database Name	Number of Entries	Number of *Butyrivibrio* Entries	*B. fibrisolvens* Entries	Contains Redundant Protein ID entries
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	0	0	Yes
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	1	1	Yes
VFDB	28,982 (3,580 curated entries)	0	No	No
Victors	5,304	0	No	No
PATRIC_VF	38,791(1,570 non-redundant proteins)	0	0	Yes
Phi-Base	6,780	0	0	No
IslandViewer4	4,065 pathogenicity islands	4	0	No
PathogenFInder	N/A	N/A	N/A	N/A

No genes involved in pathogenicity or virulence were identified in the VFDB, PATRIC_VF, or Phi-Base databases. Additionally, 13 genomic islands were discovered by IslandViewer none of which were deemed pathogenicity islands due to the lack of any virulence, pathogenicity, or antimicrobial resistance genes within the genomic island. None of the genomic islands were excluded by the notifier in its analysis for pathogenicity islands. . A site specific recombinase was identified as a potential virulence factor by both Victors and PathogenFinder. While the annotation, protein sequence, and source organism slightly differs between the two databases, the protein in question in the *B. fibrisolvens* ASCUSDY19 genome is the same. The recombinase is homologous to a recombinase found in pathogenic *Streptococcus pneumoniae.* Phage derived site-directed recombinases have been known to excise and insert pathogenic elements in *Streptococcus* species (Carroll et al. 1995). However, excision and insertion of genetic material by the recombinase requires other phage encoded proteins which are not present in the *B. fibrisolvens* ASCUSDY19 genome. Homologues of the recombinase were found to be one of 337 genes necessary to cause lung infections by *S. pneumoniae* in mice, though there was no indication that the recombinase itself was sufficient to cause pathogenicity (Hava and Camilli 2002). There is some evidence that recombinases might play a role in regulation of surface protein production in Streptococci as part of the evolution from commesal to pathogen (Holden et al. 2009). However, there is no evidence linking the recombinase encoded by the *B. fibrisolvens* ASCUSDY19 genome to this type of activity. A global search of the organisms in the PATRIC database was conducted to assess the global distribution of

similar site-directed recombinases. The search returned 134,507 unique protein hits between diverse taxa including pathogenic and non-pathogenic species. Alignment of the recombinase protein identified in the *B. fibrisolvens* ASCUSDY19 genome yielded hits in pathogenic Streptococci and in non pathogenic commensals alike, suggesting that the recombinase does not solely cause pathogenicity or virulence. Results for these analyses can be found in Tables 2.13 to 2.17.

Lower cutoff threshold alignments to curated clostridial toxin databases from VFDB and DBETH yielded 2 and 10 hits respectively. One protein match was identified by both databases, making for a total of 11 unique protein hits between the two databases. Each putative protein toxin identified by the database search was then subjected to a BLASTp search to the NCBI database as means to compare annotations and assess the distribution of the protein globally. Full results can be found in tables 2.18 and 2.20. The 11 unique protein alignments can be summarized as follows:

- All 11 potential toxins identified more closely matched proteins from non-pathogenic *B. fibrisolvens* than to the sequences from the toxin database (table 2.20). Of the 11 proteins only 1 has an annotated function by NCBI that matches its annotated function in the toxin database. This protein is a putative RNA methyltransferase. The other 10 proteins matched more closely to proteins with annotated functions that are not related to toxicity. Additionally, 1 protein has an annotated function by NCBI as hemolysin family protein (HlyC/CorC transporter family protein) and warrants further examination.

- The single protein which had match functional annotations in both the VFDB toxin database and NCBI encodes for a RNA methyltransferase (TlyA). The protein from *B. fibrisolvens* ASCUSDY19 matches a RNA methyltransferase in pathogenic *Clostridium* at 63.6%. Methylation of rRNA is a ubiquitous bacterial cellular process and in some cases differing patterns of methylation between clades can be used for phylogenetic reconstruction (Khaitovich and Mankin 2000; Green and Noller 1996; Liu and Douthwaite 2002). In some cases strain specific patterns of rRNA methylation has been demonstrated to impart antimicrobial resistance and enhance virulence and pathogenicity (Doi and Arakawa 2007; Sałamaszyńska-Guz *et al*. 2020; Rahman *et al*. 2015; Monshupanee 2013; Lata, Paul, and Chattopadhyay 2014). The rRNA methylase homolog in question more closely matches rRNA methylases from non-pathogenic *B. fibrisolvens* than any feature from pathogenic species.

- The hemolysin family protein (HlyC/CorC family transporter) in question shares 31.9% identity with a modulator of ion transport in pathogenic *C. botulinum.* HlyC/CorC domain (pfam PF03471) proteins play an essential role in magnesium and cobalt transport as well as potentially playing a role in modulating the transport of other ions (Harris, Odzer, and Breaker 2019; Huang et al. 2021)The domain is widely distributed across proteins of differing function throughout the phylum *Firmicutes*. The pfam database has 2,145 entries for HlyC/CorC domain proteins in 999 species in the phylum *Firmicutes.* While the protein identified in the *B. fibrisolvens* contains this domain there is no evidence to suggest it engages in hemolytic activity. In fact, importantly, the protein is 99.6% identical with a 100% coverage to proteins in non pathogenic *B. fibrisolvens.*

Table 2.13: Significant Alignments Between Virulence Databases and *B. fibrisolvens* ASCUSDY19						
Organism	**Protein Hits to Victors**	**Protein Hits to VFDB**	**Protein Hits to PATRIC_VF**	**Protein Hits to Phi-Base**	**Pathogenicity Island Hits in IslandViewer**	**Hits to Proteins from Pathogens in PathogenFinder**
B. fibrisolvens ASCUSDY19	1	0	0	0	0	1

Table 2.14: *B. fibrisolvens* ASCUSDY19 Hits to Pathogenic Genes in Victors								
Source	**Source Organism**	**Gene**	**Product**	**Function**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
Victors	*Streptococcus pneumoniae TIGR4*	SP_1040	Site-specific recombinase	Phage Integration	12	100	88	2e-27

Table 2.15: PathogenFinder Results *B. fibrisolvens* ASCUSDY19			
Gene Matches	**Proteins from Pathogens Matched**	**Proteins from Non-Pathogens Matched**	**Predicted as Human Pathogen?**
9	1	8	No

Table 2.16: *B. fibrisolvens* ASCUSDY19 Hits to Pathogenic Genes in PathogenFinder			
Gene	**Genbank Accession Number**	**Source Organism**	**Percent Identity**
Lactobacillales site-specific recombinase	CAW99778	*Streptococcus equi subsp. zooepidemicus H70*	90.1

Table 2.17: Top BLASTp Hits to Site-specific Recombinase found in *B. fibrisolvens* ASCUSDY19 (excluding hits to organisms without standing nomenclature)					
Organism	**Protein Name**	**Genbank Accession Number**	**Percent Identity**	**Query Coverage**	**Known Pathogen?**
Pseudobutyrivibrio xylanivorans	recombinase family protein	WP_072915090	100	100	no
Enterococcus cecorum	Hypothetical protein	KLO65182	99	100	Rare opportunistic pathogen
Peptoanaerobacter stomatis	hypothetical protein	EHL18418	92	100	yes, periodontal disease
Streptococcus pneumoniae	site-specific DNA recombinase	CVU12401	90	100	yes
Coprococcus comes	recombinase family protein	WP_147357729	89	100	no
Eubacterium rectale	recombinase family protein	WP_138305609	89	100	no

Table 2.18: *B. fibrisolvens* ASCUSDY19 Significant Protein Alignments to VFDB Clostridial Toxins						
ASCUSDY19 protein ID	**VFDB ID**	**VFDB Toxin**	**% identity**	**Query Coverage**	**Alignment Length**	**Subject length**
peg.3168	VFG012175	putative RNA methyltransferase	63.64	81	242	270
peg.1367	VFG002280	hyaluronidase (nagK)	51.43	18	35	1163
peg.215	VFG012147	probable enterotoxin (entD)	44.19	16	43	635
peg.461	VFG012150	NlpC/P60 family protein (entB)	35.59	44	59	553
peg.1330	VFG012146	hypothetical protein (entC)	33.33	12	51	744
peg.2976	VFG012149	probable enterotoxin (entB)	32.79	46	61	549
peg.1512	VFG019289	modulator of ion transport	31.94	90	432	441
peg.698	VFG012143	putative enterotoxin (entA)	29.49	31	78	947
peg.769	VFG002288	toxin B (toxB)	28.28	67	244	2366
peg.793	VFG012154	alpha-clostripain (cloSI)	28.28	24	244	522

Table 2.19: *B. fibrisolvens* ASCUSDY19 Significant Protein Alignments to DBETH Clostridial Toxins						
ASCUSDY19 protein ID	**DBETH ID**	**DBETH Toxin**	**% identity**	**Query Coverage**	**Alignment Length**	**Subject length**
peg.2766	Q897D0	Zn-dependent peptidase, insulinase family	45.95	99	962	973
peg.769	C9YJ35	toxin B	30.74	65	244	2366

Table 2.20: Best BLAST Matches to Potential Toxin Sequences in the *B. fibrisolvens* ASCUSDY19 Genome				
ASCUSDY19 protein ID	**Organisms providing best match by BLAST**	**BLAST annotation**	**% identity**	**Query Coverage**
peg.3168	*Butyrivibrio, B. fibrisolvens*	TlyA family RNA methyltransferase	100	100
peg.1367	*B. fibrisolvens* INBov1	carbohydrate-binding protein	92.8	100
peg.215	*B. fibrisolvens* INBov1	SH3 domain-containing protein	98.5	100
peg.461	*B. fibrisolvens*	SH3 domain-containing protein	100	100
peg.1330	*B. fibrisolvens*	Cell wall associated hydrolase, NlpC family	99.77	100
peg.2976	*B. fibrisolvens*	SH3 domain-containing protein	98.8	100
peg.1512	*B. fibrisolvens*	hemolysin family protein, HlyC/CorC transporter family protein	99.6	100
peg.698	*B. fibrisolvens*	SH3 domain-containing protein	98.8	100
peg.769	*B. fibrisolvens* INBov1	hypothetical protein	99.8	100
peg.793	*B. fibrisolvens*	peptidase C11	99.1	100
peg.2766	*B. fibrisolvens*	insulinase family protein	99.8	100

2.1.8.1 Section Summary

No genes directly involved in pathogenesis or toxin production were identified.

All publicly available pathogen and virulence-related databases were queried to determine the pathogenic potential of *B. fibrisolvens* ASCUSDY19. In total, these databases encompass 138,461 known pathogen-related genes spanning all microbial taxonomies. Comprehensive alignment of the *B. fibrisolvens* ASCUSDY19 genome to these databases yielded 1 hit above the 80% identity, 70% query coverage threshold. The single hit was to a site-specific recombinase that does not confer pathogenicity alone, and is found in pathogenic and non-pathogenic species alike. The analysis also included a search of 4,065 pathogenicity islands, 4 of which originated from *Butyrivibrio* species by the IsandViewer web interface. A less stringent alignment using a 1E-4 e-value cutoff of *B. fibrisolvens* ASCUSDY19 to known clostridial toxins yielded 11 unique protein matches to *B. fibrisolvens* ASCUSDY19. All of the potential toxins identifedwere more closely matched proteins from non-pathogenic *B. fibrisolvens* than to toxins from pathogenic species. Additionally, database independent analysis using the PathogenFinder web interface was conducted. IslandViewer did not identify any pathogenicity islands. The same site-specific recombinase identified in the database alignment was also identified by PathogenFinder. Ultimately, PathogenFinder deemed that *B. fibrisolvens* ASCUSDY19 is not likely to be a human pathogen.

2.1.9 Summary of Organism Safety Based on Genomics

B. fibrisolvens ASCUSDY19 was identified as a strain of *B. fibrisolvens* by 16S rRNA and whole genome analysis. *In vitro* antimicrobial susceptibility testing revealed *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin. The strain is susceptible to chloramphenicol, vancomycin, and ampicillin. Consistent with the *in vitro* antimicrobial resistance data, *in silico* analyses revealed one antimicrobial resistance gene in the genome that plays a role in tetracycline resistance. Phenotypic testing confirmed that no antimicrobials were produced by *B. fibrisolvens* ASCUSDY19 during fermentation. Comparison of the *B. fibrisolvens* ASCUSDY19 genome to several databases containing known pathogenic-related genes revealed one protein hit. However, the identified recombinase does confer pathogenicity alone. Homologues of the recombinase are found in pathogens as well as non-pathogens indicating that the feature is not solely responsible for pathogenicity or virulence. A less stringent alignment to known clostridial toxin sequences revealed 11 unique protein matches. However, the potential toxins identified more closely matched proteins from non-pathogenic *B. fibrisolvens* than to toxins from pathogenic species. Based on these analyses, *B. fibrisolvens* ASCUSDY19 is safe for use as a direct fed microbial.

[no changes were made to sections 2.2 through 2.4]

Amended Section 2.5

2.5 Effect of the Notified Substance

This portion of the notice addresses the requirements specified in 21 CFR 570.230(d):

> (d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.

The GRAS Final Rule (81 FR 54960) provides interpretation of this regulation specific to animal feed ingredients in response to comment 144: "We agree that data and information bearing on the physical or other technical effect the notified substance is intended to produce are only necessary when they bear on safety." A product like phytase would require data, however, the intended purpose of supplementation of *B. fibrisolvens* ASCUSDY19 is to support normal rumen digestion. As described below, Native Microbials has determined that the technical effect of *B. fibrisolvens* ASCUSDY19 when fed to dairy cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety. Thus, data and information demonstrating the intended effect of *B. fibrisolvens* ASCUSDY19 in the feed of dairy cattle are not required as part of this GRAS notice.

The use of this organism is to facilitate the digestion of degraded fibrous plant material and ferments polysaccharides (Hespell, Wolf, and Bothast 1987). *B. fibrisolvens* has been found in rumen and silage globally (Kameshwar *et al*., 2019; Avila and Carvaho, 2019; Thi Hoang *et al*., 2020; Seshadri *et al*., 2018) and has been assessed as a probiotic for monogastric animals (Vanbelle *et al*., 1990; Prosekov *et al*., 2015). The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour *et al*., 2015), and is further described below in context of technical effect and animal safety (Part 6.4 of this notice).

B. fibrisolvens is able to degrade fibrous plant material and ferment polysaccharides (Hespell, Wolf, and Bothast 1987). Supplementation of dietary fibrolytic enzymes could improve DMI and milk production has also been reported (Rode et al., 1999). As a commensal microorganism, feeding *B. fibrisolvens* would have no impacts on animal health. Should *B. fibrisolvens* not degrade fibrous plant material and ferment polysaccharides, there would be no safety impact, as the other rumen microorganism will continue fermentation, and the feed was formulated to assure nutrient requirements were met without consideration of the potential for increased digestion of feed.

2.5.1 ****This Section Has Been Removed***

2.5.2 Impact of Failure of the Notified Substance

If this product fails, that is, the product fails to enhance feed digestibility in the rumen, there would not be a safety concern with respect to the animal's health or nutrition. The notified substance increases the digestion of carbohydrates by acting upon the existing feed within the rumen. The diet offered to the animal would be formulated to meet the existing nutritional needs of the animal (NRC, 2001). Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients.

Several published experiments have directly investigated the impacts of DFMs by comparing groups of animals receiving a "dead" microbial against a variety of treatment conditions. Cunha, *et al*. (2019) compared heifers fed a basal diet against heifers fed the same basal diet containing a live yeast or inactive yeast supplement (2 different doses) in a 5x5 Latin square experimental design with 15-day periods. Live and dead yeasts were administered to the appropriate animals after each feeding through infusion directly into the rumen. No differences in digestibility were observed between the control, live yeast, or either of the inactive yeast doses. No differences were observed in feed intake nor animal behavior. Hence the inactive yeast did not alter the overall digestion of the feed, nor impact the health of the animals. Feeding inactive yeast did not decrease rumen function.

Muscato, *et al*. (2002) evaluated the feeding of fresh and inactivated rumen fluid to calves in a series of four experiments. The animals were dosed daily with 8 mL of either fresh or inactivated rumen fluid obtained from a cannulated Holstein cow from 0-6 weeks of age. In the first experiment, calves were either fed a typical basal ration or the same basal ration supplemented with fresh rumen fluid. In the second experiment, calves were fed the basal ration with either the cell pellet of fresh rumen fluid, supernatant of fresh rumen fluid, or no addition. In the third experiment, calves were fed a basal ration, or a basal ration supplemented with autoclaved rumen fluid. Autoclaving rumen fluid ensures microbial death, thus inactivating the biological component. The fourth experiment had a similar set-up to the third experiment, but rumen fluid was only fed for 5 days rather than 6 weeks. In the studies that evaluated autoclaved rumen fluid, the number of days of scouring were significantly decreased compared to the control. Similarly, the calves receiving autoclaved rumen fluid experienced higher gains in the first two weeks, but by the end of the experimental period there was no impact on growth. There were no differences in the outcomes of calves receiving fresh rumen fluid as compared to calves receiving autoclaved rumen fluid. This study suggests that the feeding of inactivated microorganisms does not decrease rumen function or create a safety concern when fed to animals.

The contribution of members of *Butyrivibrio*, specifically, to the fermentation characteristics of the rumen has been evaluated in the published literature. In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported.

Philippeau, *et al.* (2017) fed multiple DFM treatments to investigate the effects of DFM on rumen fermentation characteristics and digestibility. Animals were assigned one of four treatment groups: control (CON), *Propionibacterium* P63 (P63), *Propionibacterium* P63 and *Lactobacillus plantarum* 115 (P63+Lp), or *Propionibacterium* P63 and *Lactobacillus rhamnosus* 32 (P63+Lr). Each strain was administered at 10^{10} cfu/d. No change in ruminal VFA concentration was observed, and only P63 was found to impact the concentration of some milk fatty acids. pH increased on average 0.18 units in all DFM groups as compared to the control. Although the study did not demonstrate the positive response in performance as was expected, there was no negative change in the assessed parameters that may suggest a decrease in health. Similar results were observed in studies feeding *Lactobacillus acidophilus*

(Raeth-Knight, Linn, and Jung 2007; Abu-Tarboush, Al-Saiady, and Keir El-Din 1996; Higginbotham and Bath, 1992; McGilliard and Stallings 1998). In Weiss *et al*. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette *et al*. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette *et al*. (2007), *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman et al. 2011; Hagg et al. 2010; Zebeli et al. 2012; Kung and Hession 1995). A *Lactobacillus*-based probiotic fed alone and in combination with *S. cerevisiae* showed no change in milk production or efficiency in early-lactation dairy cows (Boga and Gorgulu 2007). In a meta-analysis conducted at INRA, 33 probiotic bacteria studies with or without yeast were evaluated for their impact on the production and health of dairy and beef cattle (Lettat et al. 2012). Variable performance and rument impacts were observed, however the study indicated no negative health consequences were reported. In the studies summarized above, even though the direct fed microbials did not achieve the performance response expected, there was no indication of a safety concern.

In these examples, failure of DFM supplementation or the DFM itself did not cause any harm to the fermentation characteristics of the rumen or animal well-being. In the case of *B. fibrisolvens* ASCUSDY19, if the DFM failed to provide improved digestibility, rumen fermentation of treated cows would be identical to rumen fermentation of untreated cows. Since no alterations are made to the standard feeding regime when using this product, the value of the feed that would be digested and utilized for the nutrients required to sustain life is identical between the control and treated group. Animals would be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001). Any non-performing *B. fibrisolvens* ASCUSDY19 or deceased *B. fibrisolvens* ASCUSDY19 would pass through the GI tract with the normal flow of digesta, providing nutrients for absorption by the animal (NRC, 2016).

In this respect, based on the results of published comparative studies, *B. fibrisolvens* ASCUSDY19 will act only to support normal ruminal function of digestion of animal feed. Like other DFMs, while *B. fibrisolvens* ASCUSDY19 may aid the digestion of feed, the effect is not required for the general well-being and normal performance of dairy cattle. Thus, the absence of the anticipated effect of *B. fibrisolvens* ASCUSDY19 on feed digestion by dairy cattle would not have an impact on safety. Native Microbials product labeling does not suggest a change in normal feeding regime, and its use would be specific for gaining additional nutritional value from a typical balanced ration. Animals would continue to be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001).

2.5.3 Summary

In summary it is Native Microbials' understanding that the regulatory hurdle provided in §570.230(d), is not applicable to the conclusion of the generally recognized as safe substance *B. fibrisolvens* ASCUSDY19, that is "failure" of the intended use will not raise a safety concern, as the intended use is to provide increased nutritive value from nutritionally adequate feeds. As such, failure would result in typical nutrient availability of the diets, as they have been formulated to meet the nutritional requirements of the animal. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. Therefore, there is no regulatory requirement to provide specific utility data to support the intended use.

UPDATED-Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19

Confidential Manufacturing Information

The raw materials used in the manufacture of *B. fibrisolvens* ASCUSDY19 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009U.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *B. fibrisolvens* ASCUSDY19

Material	Function	Regulatory Status	Grade
Ammonium Hydroxide	Seed Medium and Fermentation Medium	21 CFR 184.1139	FCC
L-Cysteine Hydrochloride	Seed Medium and Fermentation Medium	21 CFR 582.5271	USP
Sodium Hydroxide	Seed Medium and Fermentation Medium	21 CFR 582.1763	FCC
Iron (Ferrous) Sulfate Heptahydrate	Seed Medium and Fermentation Medium	AAFCO 57.83; 21 CFR 582.5315	USP
Magnesium Sulfate Heptahydrate	Seed Medium and Fermentation Medium	AAFCO 57.88; 21 CFR 582.5443; IFN 6-02-758	USP
Monopotassium Phosphate	Seed Medium and Fermentation Medium	21 CFR 160.110; see Attached Regulatory Review	FCC
Sodium Acetate, Anhydrous	Seed Medium and Fermentation Medium	21 CFR 582.1721	USP
Sodium Chloride	Seed Medium and Fermentation Medium	AAFCO 57.31	USP
Hydrogenated Glycerides	Fat Encapsulation	AAFCO 33.19	Feed grade

Table continued on the next page.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *B. fibrisolvens* ASCUSDY19 (cont'd)

Material	Function	Regulatory Status	Grade
Polyoxyethylene polyoxypropylene block copolymer (e.g., KFO-402)	Seed Medium and Fermentation Medium	21 CFR 176.210; FDA-ETA Letter, 2003	Specific product specified. Allowed for Food/feed production
Ascorbic Acid, Vitamin C	DSP and Freeze Drying Processing Aid	IFN 7-00-433; 21 CFR 582.5013	USP or FCC
Manganese Sulfate, Monohydrate	Seed Medium and Fermentation Medium	AAFCO 57.96; 21 CFR 582.5461	USP
Sodium Sulfate	Fat Encapsulation	AAFCO 57.109	FCC, Moisture: ≤ 1% by LOD, Purity: ≥ 98%
Ammonium Chloride	Seed Medium and Fermentation Medium	AAFCO 57.265	USP
Dextrose Monohydrate	Seed Medium and Fermentation Medium	21 CFR 168.111; 21 CFR 184.1857	FCC
Condensed Fermented Corn Extractives (Corn Steep)	Seed Medium and Fermentation Medium	AAFCO 48.24; IFN-4-02-890	Feed Grade
Mannitol	Fermentation Medium and Freeze Drying	21 CFR 582.5470	USP
Sucrose	Freeze Drying	21 CFR 184.1854	NF
Amberex 1003 AG Yeast Extract	Seed Medium and Fermentation Medium	AAFCO 96.11	Specific food grade product specified.
Hydrochloric Acid	Seed Medium and Fermentation Medium	21 CFR 582.1057	FCC
Phosphoric Acid	Seed Medium and Fermentation Medium	AAFCO 57.19; IFN 6-03-707	FCC

Abbreviations: AAFCO – Association of American Feed Control Officials; IFN – International Feed Identification Number; FCC – Food Chemicals Codex; USP – United States Pharmacopoeia; NF – National Formulary

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19

1 Overview

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Table 2. (b) (4)

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Table 3. (b) (4)

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Appendix A. Process Diagram of the Production of Fat Encapsulated *B. fibrisolvens* ASCUSDY19

(b) (4)

From: Kristi Smedley
To: Edwards, David
Cc: "Mallory Embree"; "Kevin Korth"; Schell, Timothy; Conway, Charlotte; Adams, Carissa
Subject: RE: [EXTERNAL] Discussion on April 26, 2022--AGRN 42
Date: Tuesday, May 24, 2022 8:30:22 AM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png
220523_DY19_GRAS_Safety_Summary_FINAL.pdf

I apologize, the attachment.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

Ph. 703-590-7337
Cell (b)(6)
Fax 703-580-8637

From: Kristi Smedley [mailto:smedley@cfr-services.com]
Sent: Monday, May 23, 2022 8:33 PM
To: 'Edwards, David'
Cc: 'Mallory Embree'; 'Kevin Korth'; 'Schell, Timothy'; 'Conway, Charlotte'; 'Adams, Carissa'
Subject: RE: [EXTERNAL] Discussion on April 26, 2022--AGRN 42

Dr. Edwards:

Based on the email received on May 6, 2022 (below); Native Microbials has provided a summary of the safety data that had been previously submitted in support of the Animal GRAS Notice 42. Attached is a conclusive summary hopefully, addressing the need as outlined in the email to address the safety of the direct fed microbial as a cohesive narrative.

Should we have misunderstood your request, we would appreciate further discussion.

Kristi O. Smedley, Ph.D.

Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192

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Fax 703-580-8637

From: Edwards, David [mailto:David.Edwards@fda.hhs.gov]
Sent: Friday, May 06, 2022 11:08 AM
To: Kristi Smedley
Cc: Mallory Embree; Kevin Korth; Schell, Timothy; Conway, Charlotte; Adams, Carissa
Subject: RE: [EXTERNAL] Discussion on April 26, 2022--AGRN 42

Dr. Smedley,

We appreciate the opportunity to further discuss AGRN 42 with you and with Native Microbials. We came away with further understanding of the portions of communication on GRAS Notices in which we can improve, and I hope there was more clarity on the difficulties we have encountered with evaluation of this notice.

One of our challenges with evaluating GRAS Notices is that we are tasked with evaluation of someone else's conclusions. This leads us to rely upon notifiers to pull together a strong narrative that ties together the data and information in a way that fully explains their GRAS conclusion .

Data, information, and scientific studies are different for viable microorganisms than they are for nutrients. These differences mean that showing a reasonable certainty of no harm may be different as well. We grant that target animal safety studies may not be easy to run for viable microorganisms, and the data that come from these studies may be difficult to interpret, especially for those microorganisms that are already commensal to the rumen. Saying that, a change to the populations of microorganisms does impact the rumen, the animal, and potentially the human food resulting from the animal. We do want to ensure a reasonable certainty of no harm for all of these aspects, thus providing for public health.

We have had the opportunity to further discuss a path forward for this notice internally, and we appreciate your suggestion on a further narrative that would discuss target animal safety from a perspective different than a traditional feeding study. We, too, were considering giving this guidance for a path forward. The GRAS Notice process is not iterative, thus our asks for a minor amendment should only be for clarification of information already received. Granting this, we know that there are not many examples to follow for GRAS notices for viable microorganisms. Thus, we would like to exercise some flexibility to be able to get this notice through the evaluation process, so that it can serve as a model for future ones.

We would like to take you up on your offer to provide a narrative that addresses target animal safety through support of the safety conclusion derived from your molecular and microbial safety data and information. A robust narrative should be submitted that explains why these data and information bridge to target animal safety, given the difficulties in running adequate and well controlled target animal safety studies for viable microorganisms in animal feed.

We would then evaluate the narrative to see if that completes the conclusion of reasonable certainty of no harm for the intended use of these microorganisms. We hope this provides the guidance to get this evaluation completed. We are ready for that, and we know you are more than ready, too.

We will be providing the minutes of our recent, very fruitful meeting under separate cover.

Best regards,

David Edwards, PhD
Director, Division of Animal Food Ingredients

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration
Tel: 240-402-6205
david.edwards@fda.hhs.gov

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Wednesday, April 27, 2022 5:33 PM
To: Schell, Timothy <Timothy.Schell@fda.hhs.gov>; Edwards, David <David.Edwards@fda.hhs.gov>; Conway, Charlotte <Charlotte.Conway@fda.hhs.gov>; Adams, Carissa <Carissa.Adams@fda.hhs.gov>
Cc: Mallory Embree <mallory@ascusbiosciences.com>; Kevin Korth <kevin@nativemicrobials.com>
Subject: [EXTERNAL] Discussion on April 26, 2022--AGRN 42

All:

Thank you for the discussion on issues specific to AGRN 42. We would like to offer any needed clarification on the issues raised in the meeting.

There was one point that may have been lost, as I jumped to a new subject. The point is that the Division and the industry are aware that some strains of the listed DFMs in AAFCO definition 36.14, may be toxigenic (certain listed species). Hence, a number of years ago, FDA requested the addition of a statement in the 36.14 header: "These microorganisms must be nontoxigenic. " Industry, who are responsible for marketing safe ingredients, must assure that the DFMs they are

marketing are nontoxigenic. There was no advice offered by AAFCO/CVM as to how to assure the nontoxigenic nature of the microorganism strains. Reputable companies will typically evaluate the genetic sequence of their DFM, and use available databases to assure that the genetic constructs are not toxigenic. They would accept this as due diligence (as I would suspect FDA would agree). I am not aware of animal studies being conducted to assure their compliance with this requirement. Basically, this is satisfactory approach for DFMs in which known toxigenic strains reported in the literature for the listed species; whereas for this GRAS notice microorganism there is no report of toxigenic strains.

Specific to the issue of the appropriateness of the literature search at a species level, in May 2020 (and previously) we discussed with the Division Scientists a novel microbial species. And as we could not rely on a literature review for that species, how was it best to proceed? The CVM advice is captured in the Division minutes "Currently, the firm's identification of the strain is at the family level and for a literature search it would need to address the other organisms that are also in that family. CVM also noted that a traditional TAS study would not be appropriate." This example was consistent with other advice that the phylogenic higher order was appropriate for the literature search, it is a much more conservative approach. Also, these notes also captured the inapplicability of a traditional safety study.

One path forward that we did not discuss was removal any reliance on the Pariza Decision tree as a tool to support our genetic and literature conclusions provided in the GRAS notice, as the agency and Native Microbials disagree on the adequacy of the response to question 13b in the tool. We believe that reasonable certainty in the minds of competent scientists that the substance is not harmful under the conditions of its intended use can be met without reliance on the Pariza decision tree tool.

We are requesting the notes of this meeting, when they are available.

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GRAS Safety Summary and Target Animal Safety for the Direct Fed Microbial *Butyrivibrio fibrisolvens* ASCUSDY19

Historically, safety assessments of Direct Fed Microbials are dependent on the natural exposure to the microorganism and information from the open literature that provides known understanding of the safety of the species. Feeding studies to assess target animal safety are inherently more challenging to interpret for a live, commensal microorganism sourced from the gastrointestinal ecosystem, as the microorganism already exists within the ecosystem at a baseline abundance that can vary based on environmental conditions and natural variability between individual animals. Because of this, the use of typical target animal safety studies is of limited value. This was discussed in numerous meetings with FDA and is documented in the FDA notes of those meetings. Recent technological advancements have improved the ability to accurately *de novo* sequence and assemble the whole genome of strains of interest. The accompanying growth of databases that can identify genomic sequences specific to potential pathogenicity, virulence factors, antimicrobial synthesis, or other hazard identification have assured the identification of the bacterial strain and its safety at a greater depth with far more confidence than in the mid-1980s, when the identification of the microorganism was based on phenotypic measures and the published data was minimal. Together, information derived from deep analysis of the whole genome accompanied with corroborating *in vitro* data can substantiate the safety of specific strains of microorganisms that are known to be common commensals in absence of target animal safety studies.

Specific to GRAS conclusion for *Butyrivibrio fibrisolvens* ASCUSDY19, as detailed below, Native Microbials has provided current scientific rigor specific to:

1. Conduct a thorough literature search that provides the basis of the safety assessment (importantly *B. fibrisolvens* has been robustly studied and reported on by microbiologists studying the rumen microbiome)
2. Identify *B. fibrisolvens* as a common member of the core rumen microbiome of dairy cattle
3. Identify of the strain using genomic methods
4. Thorough evaluation of the closed genome by established and public databases to assess genetic material for potential pathogenicity, virulence factors, or other hazard identification
5. Corroborate safety by published studies in which ruminants were fed *B. fibrisolvens* or in Native Microbials studies in which lactating dairy cows were fed *B. fibrisolvens* ASCUSDY19.

Based on our detailed understanding of the impact of feeding *B. fibrisolvens* ASCUSDY19 in dairy cattle, Native Microbials has met the standard of safety “that there is a reasonable certainty in the minds of competent scientists that the substance is not harmful under the conditions of its intended use.”

Butyrivibio fibrisolvens is a common member of the core rumen microbiome of lactating dairy cows

As discussed in Section 6.1 of the main text of the dossier, commensal rumen microorganisms are essential for maintaining health and nutrition in ruminants. *B. fibrisolvens* is known to be a rumen commensal, and it has been shown to perform a wide array of beneficial biochemical functions. This assessment is supported by the *in vitro* and *in vivo* observations of the species as presented in the cited literature in Section 6.1.

As stated in Section 6.4.1 of the main dossier, *B. fibrisolvens* is found ubiquitously in dairy cattle and other ruminants worldwide. This data has been corroborated by survey studies conducted by Native Microbials as presented in Section 6.4.2 of the main dossier and dossier Appendix 19 (Microbiome Safety for *Butyrivibrio fibrisolvens* ASCUSDY19). Both internal and external datasets were utilized to identify the prevalence and range of abundance of *B. fibrisolvens* in lactating dairy cows. For external datasets, sequencing reads from 4 published studies were downloaded and the 16S rRNA sequences were queried for the *B. fibrisolvens* ASCUSDY19 16S rRNA sequence. *B. fibrisolvens* ASCUSDY19 was detected in all 4 datasets, at percent abundances ranging from 0.001%-3.39%. In internal datasets, *B. fibrisolvens* ASCUSDY19 was detected in nearly all healthy dairy cow rumen microbiome datasets, at abundances ranging from ~0.0001%-1%. This evidence suggests that *B. fibrisolvens* is a common and prevalent member of the rumen microbiome of lactating dairy cows.

Isolation and Ecology

As presented in Section 2.1.1-2.1.3 of the main text of the dossier, *B. fibrisolvens* ASCUSDY19 was isolated from the rumen content of a healthy, mid-lactation Holstein cow rumen obtained via cannula. *B. fibrisolvens* is a prominent anaerobic, non-spore-forming, member of the ruminant gut microbiome. In the rumen the species degrades fibrous plant material and ferments polysaccharides to produce volatile fatty acids, and potentially plays a role in the biohydrogenation of fatty acids. The species is widely understood to be a non-pathogenic commensal organism in published literature. As such, The American Type Culture Collection (ATCC) lists *B. fibrisolvens* as BSL-1, indicating that it is a low-risk microorganism that poses little to no threat of infection in healthy humans and animals. The German Collection of Microorganisms and Cell Cultures (DSMZ) classifies *B. fibrisolvens* as TRBA Risk Group 1, indicating that the organism is unlikely to cause disease. The source of isolation (a healthy cow) together with the species classification by experts in the field (BSL-1) suggests that *B. fibrisolvens* is a low-risk microorganism that is unlikely to cause disease in humans and animals.

DNA Sequencing, Genome Assembly, and Identity

Using methods outlined in Section 2.1.4 of the main text of the dossier, 16S rRNA and whole genome sequencing were employed to unambiguously identify the species. The 16S rRNA sequence from *B. fibrisolvens* ASCUSDY19 most closely matched 16S rRNA sequences from other *B. fibrisolvens* strains. The 16S rRNA alignment between *B. fibrisolvens* ASCUSDY19 and other *B. fibrisolvens* strains were well above the 98.7% sequence identity threshold commonly used to define a species.

Whole genome average nucleotide identity (ANI) was utilized to more thoroughly confirm the identity of *B. fibrisolvens* ASCUSDY19. Matches between *B. fibrisolvens* ASCUSDY19 and other strains of *B. fibrisolvens* provided whole genome alignment values above the 95% sequence identity threshold used to

define a species using ANI. It should be noted that the type strain, DSM3071, did not provide a match above the 95% sequence identity (89%) threshold to *B. fibrisolvens* ASCUSDY19. This is likely due to the incomplete genome assembly of the wild type and the more error-prone technologies used to sequence and construct the DSM3071 assembly, and not due to true biological divergences in taxonomy. The *B. fibrisolvens* ASCUSDY19 genome, which is fully closed with no gaps, aligns closely with more complete, higher quality, and/or more recent assemblies of strains within the species. The assembly providing the best alignment values by ANI to *B. fibrisolvens* ASCUSDY19 has been published and the accuracy of the taxonomic classification has been confirmed by the scientific community. Together, the 16S rRNA and ANI analyses confirm that *B. fibrisolvens* ASCUSDY19 has been identified correctly.

In Silico Safety Assessment

The genome assembly for *B. fibrisolvens* ASCUSDY19 generated in Section 2.1.4 of the main dossier was used to confirm that it was free of any genomic elements that would cause safety concerns. The assembly graph of the complete, un-gapped, genome was inspected for the presence of plasmids as detailed in Section 2.1.5 of the main dossier. The genome is comprised of a main chromosome and a smaller chromid. As discussed in Section 6.6 of the dossier, chromids are common structural elements found in strains of *B. fibrisolvens.* Unlike plasmids, chromids are larger and are comprised largely of housekeeping and metabolic genes responsible for general cellular function. In contrast to plasmids, chromids do not contain, or act to transfer, antimicrobial resistance, virulence or pathogenicity factors. No elements containing features or structures typical of plasmids were observed in the *B. fibrisolvens* ASCUSDY19 genome sequence, suggesting that *B. fibrisolvens* has not acquired any pathogenicity or resistance genes via plasmid transfer from the environment or other microorganisms.

As detailed in Section 2.1.6, *B. fibrisolvens* ASCUSDY19 was aligned to various databases containing antimicrobial resistance genes. A single gene for tetracycline resistance, TetW, was found to be encoded by the genome. Literature review of antimicrobial resistance in Section 2.1.6 revealed that tetracycline resistance, and specifically the presence of TetW, is widely distributed in the rumen.

To assess genome encoded toxins, pathogenicity, and virulence factors, *B. fibrisolvens* ASCUSDY19 was aligned to a collection of databases as detailed in Section 2.1.8. A single feature was identified by the database alignment: a site-specific recombinase. This recombinase was demonstrated to commonly be found in pathogenic and non-pathogenic species alike. Additionally, there appears to be no mechanism in which this element would contribute to virulence or pathogenicity.

Thus, based on a thorough screening of the *B. fibrisolvens* ASCUSDY19 genome using all applicable and relevant databases and the current state of the art, nothing of concern was identified suggesting that *B. fibrisolvens* ASCUSDY19 is safe for humans and animals.

Safety Based on *In Vitro* Experiments

Phenotypic testing was conducted to evaluate antimicrobial resistance and antimicrobial production by *B. fibrisolvens* ASCUSDY19 using methods described in Section 2.1.6 and 2.1.7 in the main text of the dossier.

B. fibrisolvens ASCUSDY19, an anaerobic bacteria, was demonstrated to be resistant to aminoglycosides and macrolides. Resistance to aminoglycosides and macrolides is reflective of *B. fibrisolvens* ASCUSDY19

being anaerobic rather than any specific resistance mechanism or genotype. Consistent with the presence of TetW in the genomic analysis, *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, and susceptible to chloramphenicol, vancomycin, and ampicillin. *B. fibrisolvens* ASCUSDY19 was not found to produce any antimicrobial compounds.

Feeding Trial Summary

As presented in Section 6.7 of the main text of the dossier, *B. fibrisolvens* has been fed to cattle and lactating goats as a DFM in studies published in 2003 and 2016. Findings of these studies are described in Section 6.7. No negative health effects due to the feeding of *B. fibrisolvens* were reported.

In addition, *B. fibrisolvens* ASCUSDY19 was fed to lactating cows for 39 weeks as mentioned in the Microbial Safety section of AGRN 42 *B. fibrisolvens* ASCUSDY19 GRAS Notice Amendment (herein referred to as the Amendment). No adverse health effects due to the feeding of *B. fibrisolvens* ASCUSDY19 were observed. The risk of clinical mastitis was also evaluated and no association of clinical mastitis occurrence and supplementation of *B. fibrisolvens* ASCUSDY19 was found (Attachment 4 of the Amendment).

The manuscript for the above-mentioned *B. fibrisolvens* ASCUSDY19 study is currently undergoing minor revisions in the peer review process for *Journal of Animal Science*. It is important to note that although the same mixing wagon was used to prepare feed for both control and treatment groups, precautions were taken to minimize the potential cross-contamination by loading ~74kg of Bermuda grass hay and mixing for 4 minutes before discharging it to sweep away the previous TMR residues. Microtracers were utilized to confirm minimal cross contamination between TMR batches. Although control and treatment cows were housed in a single pen, the animals could only access assigned feed bins via calan gates. Moreover, as *B. fibrisolvens* ASCUSDY19 is a commensal organism and is naturally present in cow rumens, low level cross contamination through animal interactions would have a negligible impact. This feeding study, although not necessary for GRAS determination, corroborates the safety of feeding *B. fibrisolvens* ASCUSDY19 as no adverse health impacts were observed.

Overall Summary of Safety

B. fibrisolvens ASCUSDY19 belongs to species *B. fibrisolvens* and is a well understood and studied commensal microorganism in the rumen. *B. fibrisolvens* is naturally present in the rumen and considered beneficial. This has been demonstrated by both literature and in a study conducted by Native Microbials. The species has been classified in the lowest risk group (BSL-1/Risk Group 1) by various international agencies. Through comprehensive evaluation of the genome, Native Microbials found no antimicrobial resistance, plasmids, pathogenicity, or virulence factors of concern. *In vitro* assessment of antimicrobial resistance and production demonstrated that *B. fibrisolvens* ASCUSDY19 is susceptible to a wide variety of common antibiotics and does not produce any antimicrobial compounds. Studies that fed *B. fibrisolvens* ASCUSDY19 to ruminants showed that the microorganism is well tolerated by the study animals, and no adverse health effects were observed.

Native Microbials, Inc., therefore, continues to conclude that *B. fibrisolvens* ASCUSDY19 is generally recognized as safe as a direct fed microbial in dairy cattle at the intended rate of inclusion.