Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Road
Woodbridge. VA 22192-575.5
703 5907337 (Fax 703 580 8637)
Smedley@cfr-services.com

Since 1973
consultants to the regulated industry

February 4, 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: Filing of Animal GRAS Notice
DFM *Succinivibrio dextrinosolvens ASCUSBF53* -
for Beef 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 an Animal General Recognized as Safe N otice for the use of *Succinivibrio dextrinosolvens ASCUSBF53* as a direct fed microorganism for use in Beef Cattle. The submission is compliant with 21 CFR 570.210-255. The GRAS conclusion is based on scientific procedures.

Should you have any questions on the filing, please contact me directly.

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: 2021.02.04 09:50:10-05'00'

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

RECEIVED DATE
FEB 18, 2021

Cc: Mallory Embree, Native Microbials, Inc.

ATTACHMENTS:

Letter of Smedley Authorization to Represent
GRAS Notice *Succinivibrio dextrinosolvens ASCUSBF53* (Narrative Hard Copy, Full Submission on DVD)

February 2, 2021

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

Subject: Authorization for Representation—Kristi O. Smedley Ph.D. for correspondence (written and verbal), agreements, meeting requests, and submission(s) for Native Microbials, Inc.

Dear Dr. Edwards:

We are authorizing Kristi Smedley to act on our behalf for submission and representation of our GRAS notice for *Succinivibrio dextrinosolvens* ASCUSBF53 for use as a Direct Fed Microbial in Beef Cattle.

Her contact information:

Kristi Smedley, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
703-590-7337
Fax 703-580-8637
Smedley@cfr-services.com

Please contact the undersigned with any questions.

Sincerely,

(b)(6)

Mallory Embree, Ph.D
Chief Scientific Officer
Native Microbials, Inc.
10255 Science Center Dr, Suite C2
San Diego, CA 92121

GRAS Notice for *Succinivibrio dextrinosolvens* ASCUSBF53 for Use as a Direct Fed Microbial in Beef Cattle

February 02, 2021

Prepared for: Division of Animal Feeds, (HFV-220)
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Submitted by: Native Microbials, Inc.
10255 Science Center Dr., Suite C2
San Diego, California 92121

GRAS Notice for *Succinivibrio dextrinosolvens* ASCUSBF53 for Use as a Direct Fed Microbial in Beef Cattle

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LIST OF ABBREVIATIONS

AAFCO	Association of American Feed Control Officials
ADF	Acid Detergent Fiber
ANI	Average Nucleotide Identity
AOAC	Association of Official Analytical Chemists
BAM	Bacteriological Analytical Manual
BLAST	Basic Local Alignment Search Tool
CFR	Code of Federal Regulations
CFU	Colony Forming Units
cGMP	current Good Manufacturing Practices
CLSI	Clinical and Laboratory Standards Institute
CV	Coefficient of Variation
CVM	Center for Veterinary Medicine
DFM	Direct Fed Microbial
DM	Dry matter
DNA	DeoxyriboNucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FSMA	Food Safety Modernization Act
GC	Guanine-Cytosine
GRAS	Generally Recognized As Safe
HACCP	Hazards Analysis Critical Control Points
ITS	Internal Transcribed Spacer
MIC	Minimum Inhibitory Concentrations
NCBI	National Center for Biotechnology Information
ND	Not Detected
NDF	Neutral Detergent Fiber
NRRL	Agricultural Research Service Culture Collection
OP	Official Publication
QPS	Qualified Presumption of Safety
RNA	RiboNucleic acid
SD	Standard Deviation
SPC	Spiral Plate Count
TMR	Total Mixed Ration
USC	United States Code
USP	United States Pharmacopoeia
YPD	Yeast extract Peptone Dextrose

NOMENCLATURE

The notified substance is *Succinivibrio dextrinosolvens* ASCUSBF53 and is deposited in NRRL as B-67550. The microbial strain may be encapsulated with hydrogenated glycerides for use in direct fed microbial products for beef cattle which is referred to as 'fat encapsulated *S. dextrinosolvens* ASCUSBF53 cell concentrate.

The microbial strain *S. dextrinosolvens* ASCUSBF53 is often referred to in some appended reports as 'Beef-53', which is the internal research name for *Succinivibrio dextrinosolvens* ASCUSBF53.

GRAS Notice for *Succinivibrio dextrinosolvens* ASCUSBF53 for Use as a Direct Fed Microbial in Beef Cattle

PART 1 – SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §570 Subpart E consisting of §570.203 to 280, Native Microbials, Inc. hereby informs the U.S. Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for *Succinivibrio dextrinosolvens* ASCUSBF53.

1.1 Name and Address of Organization

Native Microbials, Inc.
10255 Science Center Dr., Suite C2
San Diego, CA 92121

1.2 Name of the Notified Substance

The notified substance is *Succinivibrio dextrinosolvens* ASCUSBF53 (microbial strain). It is manufactured as a freeze-dried milled product which is further standardized and stabilized by encapsulation in fat for use in direct fed microbial products for cattle. The standardized product is referred to as 'fat encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53' or '*Succinivibrio dextrinosolvens* ASCUSBF53 encapsulated'. In addition, a number of the appended reports refer to *Succinivibrio dextrinosolvens* ASCUSBF53 or the fat encapsulated product under the internal research name, Beef-53.

1.3 Intended Conditions of Use

S. dextrinosolvens ASCUSBF53 is intended for use as a supplemental source of viable microorganisms in the feed of beef cattle. The intended purpose of supplementation of the microorganism is to augment the digestion of feed in the rumen. The microbial strain will be delivered in the fat encapsulated form to beef cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing fat encapsulated *S. dextrinosolvens* ASCUSBF53 may be incorporated into the diet of 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 *S. dextrinosolvens* ASCUSBF53 will be incorporated into feed at a recommended level of $1x10^8$ CFU/head/day.

1.4 Statutory Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §570.30(a) and (b), *S. dextrinosolvens* ASCUSBF53 manufactured by Native Microbials, has been concluded to have GRAS status for use as a direct fed microbial in beef cattle, as described in Part 1.3, on the basis of scientific procedures.

1.5 Premarket Exception Status

Native Microbials hereby informs the U.S. FDA of the view that *S. dextrinosolvens* ASCUSBF53 is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on Native Microbials conclusion that the notified substance is GRAS under the conditions of intended use as described in Part 1.3 above.

1.6 Availability of Information

The data and information that serve as the basis for this GRAS notification will be made available to the U.S. FDA for review and copying upon request during customary business hours at the offices of:

Native Microbials, Inc.
10255 Science Center Dr., Suite C2
San Diego, CA 92121

In addition, upon request, Native Microbials will supply the U.S. FDA with a complete copy of the data and information either in an electronic format that is accessible for the Agency's evaluation or on paper.

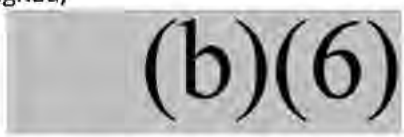
1.7 Freedom of Information Act, 5 U.S.C. 552

In Native Microbials view, all data and information presented in Parts 2 through 7 of this notice do not contain any trade secrets, commercial or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempt from the Freedom of Information Act, 5 U.S.C. Section 552 with the exception of Appendices 10, 11 and 15, which are considered to contain proprietary commercial information which is confidential.

1.8 Certification

As required in 21 CFR 570.250(c)(2), Native Microbials, Inc. hereby certifies that to the best of their knowledge, all data and information presented in this notice constitutes a complete, representative and balanced submission, which includes all unfavorable as well as favorable information known to Native Microbials and pertinent to the evaluation of the safety and GRAS status of *Succinivibrio dextrinosolvens* ASCUSBF53.

Signed,



Mallory Embree, PhD, Chief Scientific Officer

Date

PART 2 – IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

2.1.1 Taxonomic Classification

The current taxonomic classification of the microbial strain, *S. dextrinosolvens* ASCUSBF53, is provided in Table 2.1. *S. dextrinosolvens* is a prominent member of the rumen of both bovine and sheep and is enriched in animals on high grain diets where it acts to degrade starch and produce volatile fatty acids (VFAs) (Bryant and Small 1956; Wozny et al. 1977a; Hespell 1992; Hippe et al. 1999). Higher abundance of *S. dextrinosolvens* has been associated with more efficient dairy and beef cattle (Elolimy et al. 2018; Hailemariam, Zhao, and Wang 2020; Hernandez-Sanabria et al. 2012). Reduction of *S. dextrinosolvens* has been associated with transportation stress in beef cattle and subsequent weight loss confirming the species as a critical member of a healthy ruminal microbiome (Deng et al. 2017). Additionally, supplementation of ginkgo to *in vitro* rumen models have demonstrated the ability to reduce methane emissions, which also was correlated to the increase in abundance of *S. dextrinosolvens* (Oh et al. 2017a; Oh et al. 2017b).

Table 2.1: Taxonomic Classification of *S. dextrinosolvens*

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Aeromonadales
Family	*Succinivibrionaceae*
Genus	*Succinivibrio*
Species	*dextrinosolvens*

2.1.2 Source of the Microorganism

S. dextrinosolvens ASCUSBF53 was identified and isolated to axenicity from the rumen content of a healthy steer by Native Microbials. The isolate was deposited in the NRRL, Agricultural Research Service Culture Collection, and referenced as B-67550.

2.1.3 Description of the Microorganism

S. dextrinosolvens ASCUSBF53 is an anaerobic, non-spore forming bacterium composed of helically twisted, curved rods with 1 to 3 cells per grouping (Figure 2.1). Cells are motile and stain gram-negative (Figure 2.2). Our observations of *S. dextrinosolvens* ASCUSBF53 are consistent with the original description of the species by (Bryant and Small 1956).

Figure 2.1: ***S. dextrinosolvens*** **ASCUSBF53 Methylene Blue Stain after 48 hours of incubation (1000x magnification)**

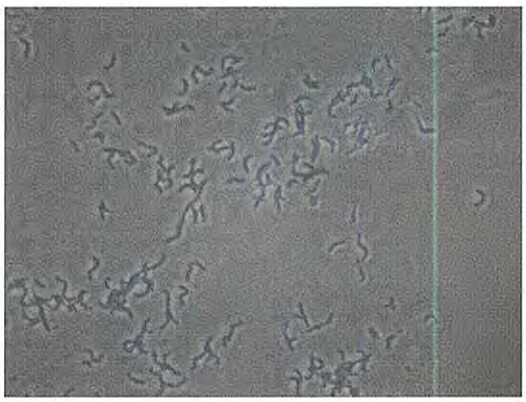
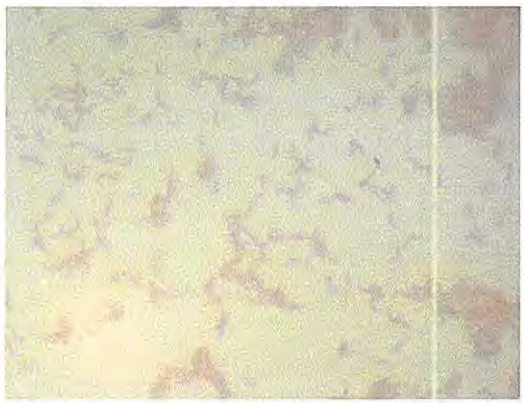


Figure 2.2: ***S. dextrinosolvens*** **ASCUSBF53 Gram Stain after 48 hours of incubation (1000x magnification)**



In vitro assays demonstrate that *S. dextrinosolvens* ASCUSBF53 grows on a variety of monosaccharides, disaccharides, and sugar alcohols including arabinose, ribose, xylose, galactose, glucose, fructose, lactose, maltose, melibiose, sorbitol and arabitol. Additionally, the strain ferments starch. Carbon source utilization results are summarized in Table 2.2. Fermentation of starch, disaccharides, and monosaccharides are consistent with observations of the species, though there is some interspecies variability (Bryant 2015; Bryant and Small 1956). The results align with the proposed function of *S. dextrinosolvens* in the rumen as a key degrader of starch and dextrins in ruminants on high grain diets (Bryant 2015; Cotta 1988).

Table 2.2: Growth of *S. dextrinosolvens* ASCUSBF53 on Different Carbon Sources			
Carbon Source	**Growth**	**Carbon Source**	**Growth**
No Carbon Control	No Growth	Inositol	No Growth
Glycerol	No Growth	D-Mannitol	No Growth
Erythritol	No Growth	D-Sorbitol	Growth
D-Arabinose	No Growth	Methyl-aD-Mannopyranoside	No Growth
L-Arabinose	Growth	Methyl-aD-Glucopyranoside	No Growth
D-Ribose	Growth	N-AcetylGlucosamine	No Growth
D-Xylose	Growth	Amygdalin	No Growth
L-Xylose	No Growth	Arbutin	No Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	No Growth
Methyl-BD-xylopyranoside	No Growth	Salicin	No Growth
D-Galactose	Growth	D-Cellobiose	No Growth
D-Glucose	Growth	D-Maltose	Growth
D-Fructose	Growth	D-Lactose	Growth
D-Mannose	No Growth	D-Melibiose	Growth
L-Sorbose	No Growth	D-Saccharose	Growth
L-Rhamnose	No Growth	D-Trehalose	No Growth
Dulcitol	No Growth	Inulin	No Growth
D-Melezitose	No Growth	D-Tagatose	No Growth
D-Raffinose	No Growth	D-Fucose	No Growth
Starch	Growth	L-Fucose	No Growth

Glycogen	No Growth	D-Arabitol	Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	No Growth	Potassium Gluconate	No Growth
D-Turanose	No Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth		

Metabolite production of *S. dextrinosolvens* ASCUSBF53 was measured at 40 hours elapsed fermentation time using an Agilent 1260 series HPLC with refractive index (RI) detector. The results are summarized in Table 2.3 and Appendix 002. Major fermentation products include succinate, lactate, and acetate.

Table 2.3: Metabolite Production of *S. dextrinosolvens* ASCUSBF53 on Complex Media with Sorbitol

Metabolite	Production (g/L)
Pyruvic acid	0.00
Succinic acid	1.44
Lactic acid	1.08
Glycerol	0.07
Acetic acid	0.73
Propionic acid	0.00
Butyric acid	0.00
Ethanol	0.00
1-Butanol	0.00

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. The results indicated that *S. dextrinosolvens* ASCUSBF53 was most closely related to *Succinivibrio dextrinosolvens* CA76 (99.8%), followed by *Succinivibrio dextrinosolvens* CG79 (99.8%), and *Succinivibrio dextrinosolvens* Z6 (99.2%). The closest match not from the *Succinivibrio* genus is *Anaerobiospirillum thomasii* DSM 11806 (92%).

2.1.4.2 *Whole Genome Sequence Assembly and Annotation*

Genomic DNA was isolated from a pure culture of *S. dextrinosolvens* ASCUSBF53 and sequencing libraries were prepared using the (b) (4). The resulting libraries (b) (4) (b) (4) and in parallel, long-read libraries were prepared from the same extracted DNA using (b) (4) following the protocol outlined by Jain *et al.* (2018) and 1D sequenced on the (b) (4) (b) (4) 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 (b) (4) (b) (4) (b) (4) Assembly statistics can be found in Table 2.4. The full details of the assembly are provided in Appendix 003C.

Protein coding genes were predicted through (b) (4) 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 (Lowe and Eddy 1997) and rRNA genes (Aziz et al. 2008).

The *S. dextrinosolvens* ASCUSBF53 genome contains (b) (4) coding sequences which were subsequently built into a metabolic reconstruction describing 210 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 CP068345 for the main chromosome and CP068346 for the chromid.

Table 2.4:	**Assembly Statistics for *S. dextrinosolvens* ASCUSBF53**
# of Contigs	2
# of Contigs ≥ 5,000 bp	2
Longest Contig (bp)	(b) (4)
Assembly Length	
N50	
N75	
GC%	39.1

2.1.4.3 *Whole Genome Sequence Comparison*

To determine relatedness of *S. dextrinosolvens* ASCUSBF53 to other closely related species at a higher resolution, whole genomes were compared using ANI. Candidate genomes for genome-genome comparison to *S. dextrinosolvens* ASCUSBF53 were selected by full length 16S rRNA similarity and downloaded from the NCBI database. (b) (4) 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 (b) (4) alignment can be found in Table 2.5.

The only ANI matches to *S. dextrinosolvens* ASCUSBF53 above the 95% ANI cutoff to be considered the same species were two strains of *S. dextrinosolvens* (DSM 3072 and H5) (Richter and Rosselló-Móra 2009).

Table 2.5: Average Nucleotide Identity (ANI) of Related Species to *S. dextrinosolvens* ASCUSBF53 by (b) (4)

Genus species (assembly)	ANI (%)	Coverage (%)
Succinivibrio dextrinosolvens DSM 3072 (GCA_900167015)	97.9	74.9
Succinivibrio dextrinosolvens H5 (GCA_000702045)	96.8	72.9
Succinivibrio dextrinosolvens ACV-10 (GCA_900116345)	88.3	43.0
Succinivibrio dextrinosolvens 22B (GCA_900114195)	87.9	35.0
Succinivibrio dextrinosolvens Z6 (GCA_011065405)	87.9	31.7
Anaerobiospirillum succiniciproducens DSM 6400 (GCA_000482845)	82.4	0.64
Anaerobiospirillum thomasii NCTC13093 (GCA_900445225)	82.3	0.62
Succinatimonas hippei YIT12066 (GCA_000188195)	82	0.22

2.1.4.4 *Summary and Conclusions*

16S rRNA and whole genome analysis confirm that *S. dextrinosolvens* ASCUSBF53 is a strain of the species *S. dextrinosolvens.*

2.1.5 Plasmid Analysis

To confirm the presence/absence of plasmids, the assembly graph for the *S. dextrinosolvens* ASCUSBF53 was analyzed by (b) (4) (Wick et al. 2015). The assembly graph analysis confirmed that the *S. dextrinosolvens* ASCUSBF53 assembly was contained in 2 circular chromosomes with no unincorporated fragments, verifying the completeness of the assembly. The image of the assembly graph can be found in Figure 2.3.

The smaller chromosome (163,867 bp) was evaluated using three different methods to determine if it should be classified as plasmid or a chromid:

1. PlasmidFinder (Carattoli et al. 2014): The PlasmidFinder database utilizes 469 origin of replication sequences from species in the family *Enterobacteriaceae*.
2. Alignment to proteins in the NCBI plasmid database by BLAST (Brooks, Kaze, and Sistrom 2019): The NCBI plasmid database consists of 1,295,867 plasmid derived proteins from 29,505 plasmids, covering 5,161 species.
3. MOB-suite (Robertson and Nash 2018). The MOB-suite database consists of 23,240 plasmids, of which 10,224 are derived from *Gammaproteobacteria,* with 223 originating in *Aeromonadales.* The analysis with MOB-suite emphasizes origin of replication, relaxase, and mate-pair formation genes with the goal of identifying plasmids and determining their potential for mobility.

Characteristics of the databases can be found in Table 2.6. To ensure no hits were missed due to codon bias or sequencing error, protein alignments to the NCBI plasmid database were considered a hit if they have greater than 80% identity over more than 70% query coverage. PlasmidFinder and MOB-suite yielded no hits. Additionally, MOB-suite determined the smaller chromosome of *S. dextrinosolvens* ASCUSBF53 to be non-mobile. Alignment to the NCBI plasmid database yielded one hit to the *S. dextrinosolvens* ASCUSBF53 genome. NCBI plasmid alignment results can be found in Table 2.7.

The hit to the NCBI plasmid database was to a ubiquitous membrane potential regulating protein that aids in regulation of osmotic stress and maintains membrane potential, YqaE/PMP3 (Inada et al. 2005; Kwok et al. 2020; Navarre and Goffeau 2000; Raivio, Leblanc, and Price 2013). Homologues of this protein are ubiquitously found in plants, prokaryotes, yeasts and other eukaryotes (Kwok et al. 2020).

Given the lack of plasmid features on the secondary chromosome of *S. dextrinosolvens* ASCUSBF53 and the predicted immobility of the chromosome by MOB-suite, it should be designated as a chromid rather than a plasmid. Though there is no literature describing chromids in *S. dextrinosolvens* to date, it is estimated that one in ten bacteria carry a chromid (Harrison et al. 2010). With only six representative sequences from the species, only one of which is a closed sequence, the species has likely not been sequenced extensively enough to document chromid carrying members.

Figure 2.3: ***S. dextrinosolvens*** **ASCUSBF53 Assembly Graph as Generated by** (b) (4)

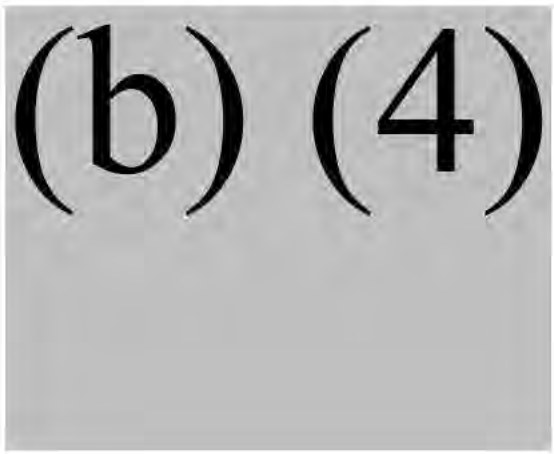


Table 2.6: Characteristics of Databases Used to Identify Plasmids			
Database Name	**Number of Plasmid Entries**	**Features Evaluated**	***S. dextrinosolvens* Entries**
PlasmidFinder	No full Plasmids	Origin of replication	0
NCBI Plasmid DB	29,505	All plasmid proteins	0
MOB-suite	23,240	Origin of replication, relaxases, mate-pair formation genes	0

Table 2.7: *S. dextrinosolvens* ASCUSBF53 Hits to the NCBI Plasmid Database						
Source Organism	**Gene**	**Genbank Accession #**	**Function**	**Query Coverage**	**identity**	**E-Value**
Enterobacterales	MULTISPECIES: YqaE/Pmp3 family protein	WP_057393895	Proteolipid membrane potential modulator	92	81.4	5E-28

2.1.6 *In-vitro* and *In-silico* Analysis of Antibiotic Susceptibility

Phenotypic testing was conducted on *S. dextrinosolvens* ASCUSBF53 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials with relevance to human and veterinary medicine. The full study report is provided in Appendix 004. The results were evaluated against the resistant breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram negative anaerobes," European Food Safety Authority (EFSA) for "gram negative bacteria," and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). Results for *S. dextrinosolvens* ASCUSBF53 can be found in Table 2.8. The MIC values reported for *S. dextrinosolvens* ASCUSBF53 were equal, or lower than, the cut-off values and break-points established by EFSA, EUCAST and/or CLSI for chloramphenicol, tetracycline, and ampicillin. MIC values reported for *S. dextrinosolvens* ASCUSBF53 were higher than the cutoff values and break-points established by EFSA, EUCAST and/or CLSI for gentamicin, kanamycin, clindamycin and streptomycin.

It should be noted that susceptibility to aminoglycosides and macrolides 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-negative organisms and should not be applied to *S. dextrinosolvens* due to its anaerobic nature. CLSI and EUCAST refrain from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. Furthermore, since *S. dextrinosolvens* ASCUSBF53 is considered to be a gram-negative bacteria, vancomycin will be ineffective against this organism, as gram-negative bacteria are known to be unresponsive to vancomycin (Antonoplis et al. 2019).

Table 2.8:	*S. dextrinosolvens* ASCUSBF53 Antimicrobial Susceptibility in Relation to EUCAST, and CLSI Breakpoints				
Antibiotic	**Range Tested (ug/mL)**	**MIC (ug/mL) of *S. dextrinosolvens* ASCUSBF53**	**EFSA Interpretation**	**EUCAST Interpretation**	**CLSI Interpretation**
Ampicillin	0.5 – 128	< 0.5	S	S	S
Vancomycin	0.125 – 32	> 32	N/A	N/A	N/A
Gentamicin	0.5 – 32	16	R	-	N/A
Kanamycin	0.5 – 64	16	R	N/A	N/A
Streptomycin	0.5 – 64	32	R	N/A	N/A
Erythromycin	0.5 – 16	16	N/A	-	N/A
Clindamycin	0.03 – 32	> 32	N/A	R	R
Tetracycline	0.0625 – 64	0.25	S	N/A	S
Chloramphenicol	0.5 – 64	< 0.5	N/A	S	S

To evaluate the presence of antimicrobial resistance genes in the *S. dextrinosolvens* ASCUSBF53 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 toCARD 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 Characteristics of each database can be found in Table 2.9.

Table 2.9:	Characteristics of Databases Used to Assess Antimicrobial Resistance			
Database Name	**Number of Entries**	**Number of *Succinivibrio* Entries**	***S. dextrinosolvens* 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. No hits were identified by PATRIC or ResFinder. BLAST alignment to the AMRFinder database revealed one hit in the *S.*

dextrinosolvens ASCUSBF53 genome. The hit corresponds to the tufA gene. This gene is ubiquitous and encodes for a translation elongation factor in bacteria (Pramanik and Schwartz 1984; Filer and Furano 1980, 1981). Point mutations in the tufA gene in some cases have resulted in resistance to the polyketides; kirromycin and pulvomycin (Kraal et al. 1995; Tubulekas, Buckingham, and Hughes 1991; Zeef et al. 1994). Results for the BLAST search to the AMRFinder database can be found in Table 2.10.

Table 2.10: *S. dextrinosolvens* ASCUSBF53 Antimicrobial Resistance by NCBI AMR BLASTp

Gene	e-value	Percent Identity	Query Coverage
tufA	0	82.9	99

2.1.6.1 Section Summary

In vitro testing demonstrated that *S. dextrinosolvens* ASCUSBF53 is resistant to gentamicin, kanamycin, streptomycin, and clindamycin. Resistance to aminoglycosides and macrolides such as is reflective of *S. dextrinosolvens* ASCUSBF53 being anaerobic rather than any specific resistance mechanism or genotype. Furthermore, being unresponsive to vancomycin is a function of *S. dextrinosolvens* ASCUSBF53 being gram-negative, rather than an organism-specific resistance. *In silico* analyses revealed the presence of tufA, a gene that can have point mutations that could lead to resistance to kirromycin and pulvomycin. *S. dextrinosolvens* ASCUSBF53 is susceptible to ampicillin, tetracycline, and chloramphenicol, suggesting that should *S. dextrinosolvens* ASCUSBF53 cause an opportunistic infection in a human or animal, i can be readily treated using standard antibiotics.

2.1.7 Antimicrobial Production

S. dextrinosolvens ASCUSBF53 supernatant 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 2.11. 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. 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 *S. dextrinosolvens* ASCUSBF53. The total number of sequences in the PATRIC and Phi-BASE databases is 134,396 and contains 2 entries from *S. dextrinosolvens.* IslandViewer contains 4,065 pathogenicity islands including 1 from *S. dextrinosolvens.*

The analysis in PathogenFinder is database independent and uses a model trained with protein sequences from 886 whole genome sequences. The PathogenFinder model predicts pathogenicity based on matches to proteins found differentially in pathogenic and non-pathogenic bacteria regardless of their annotated function. Therefore, a single hit to a protein found in 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 pathogenic, 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. Characteristics of each database can be found in Table 2.11.

Table 2.11: Characteristics of Databases Used to Assess Virulence and Pathogenicity

Database Name	Number of Entries	Number of *Succinivibrio* Entries	*S. dextrinosolvens* Entries	Contains Redundant Protein ID entries
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	1	1	Yes
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	0	0	Yes
PATRIC_VF	38,791(1,570 non-redundant proteins)	1	1	Yes
Phi-Base	6,780	0	0	No
IslandViewer4	4,065 pathogenicity islands	1	1	No
PathogenFInder	N/A	N/A	N/A	N/A

The alignment process compares all identified *S. dextrinosolvens* ASCUSBF53 genes against all known pathogen-related genes that have been identified across the Bacterial and Fungal kingdoms. To ensure no hits are missed due to codon bias or sequencing error, protein alignments are considered a hit if they have greater than 80% identity over more than 70% query coverage. No genes involved in toxin synthesis, pathogenicity, or virulence were identified in the VFDB database. No pathogenicity islands were detected by IslandViewer. One hit was identified in each Victors, PATRIC_VF, and PhiBase to the same gene, hfq. The hfq gene is found in approximately 50% of all bacteria where it acts as a post transcriptional regulator of various metabolic processes (Sun 2002; Tsui, Leung, and Winkler 1994). It has been implicated as a growth promoter and virulence factor regulator in some pathogens including *Listeria monocytogenes* (Sun, Zhulin, and Wartell 2002), *Yersinia pseudotuberculosis* (Schiano, Bellows, and Lathem 2010), *Shigella sonnei* (Mitobe et al. 2009) and *Salmonella typhimurium* (Sittka et al. 2007) but is not solely responsible for pathogenicity or virulence. There were no other pathogenic or virulent genes detected in the *S. dextrinosolvens* ASCUSBF53 genome that would be regulated by hfq. Additionally, the match to the hfq gene from the PhiBase database was implicated with reduced virulence in *Yersinia pestis* suggesting the variant encoded by the *S. dextrinosolvens* ASCUSBF53 genome

would not confer virulence or pathogenicity even if other virulence or pathogenicity genes were present. Full results can be found in Tables 2.12-2.17.

Table 2.12: Significant Alignments Between Virulence Databases and *S. dextrinosolvens* ASCUSBF53

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
S. dextrinosolvens ASCUSBF53	1	0	1	1	0	1

Table 2.13: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in Victors

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	identity	E-Value
Victors	*Yersinia pseudotuberculosis IP 31758*	hfq	RNA-binding protein Hfq	Translational Regulation	67	80	85	3.00E-25

Table 2.14: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in PATRIC_VF

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	identity	E-Value
PATRIC _VF	*Shigella flexneri 2a*	hfq	RNA-binding protein Hfq	Translational Regulation	66	80	83	4.00E-25

Table 2.15: PathogenFinder Results *S. dextrinosolvens* ASCUSBF53

Gene Matches	Proteins from Pathogens Matched	Proteins from Non-Pathogens Matched	Predicted as Human Pathogen?
4	1	3	No

Table 2.16: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in PathogenFinder

Gene	Genbank Accession Number	Source Organism	Percent Identity
LSU ribosomal protein L14P	CP000378	*Burkholderia cenocepacia* AU 1054	79.5

Table 2.17: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in PhiBase								
Source	Source Organism	Gene	Product	Function	Query Coverage	Identity	E-Value	Phenotype
PhiBase	*Yersinia pestis*	hfq	RNA-binding protein Hfq	Translational Regulation	82	84.06	6E-38	Reduced Virulence

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 *S. dextrinosolvens* ASCUSBF53. In total, these databases encompass 138,461 known pathogen-related genes spanning all microbial taxonomies. Comprehensive alignment of the *S. dextrinosolvens* ASCUSBF53 genome to these databases yielded one hit above the 80% identity, 70% query coverage threshold across three databases. The single hit was to hfq, a post transcriptional regulator. The hfq gene identified by alignment to the Victors, PATRIC_VF, and PhiBase databases is a ubiquitous post transcriptional/translational regulator found in approximately half of all bacteria. The gene in question regulates the expression of general metabolic genes as well as genes involved in pathogenicity and virulence. *S. dextrinosolvens* ASCUSBF53 contained no other pathogenicity or virulence factors that would be under the regulation of hfq. The analysis also included a search of 4,065 pathogenicity islands, 1 of which originated from *S. dextrinosolvens* by the IslandViewer web interface. IslandViewer did not identify any pathogenicity islands. Additionally, database independent analysis using the PathogenFinder web interface was conducted. PathogenFinder identified one hit the *S. dextrinosolvens* ASCUSBF53 however it was below the 80% identity cutoff. Ultimately, PathogenFinder deemed that *S. dextrinosolvens* ASCUSBF53 is not a human pathogen.

2.1.9 Summary of Organism Safety Based on Genomics

S. dextrinosolvens ASCUSBF53 was identified as a strain of *S. dextrinosolvens* by 16S rRNA and whole genome analysis. Examination of the assembly graph of the *S. dextrinosolvens* ASCUSBF53 genome revealed two chromosomes. The smaller of the two chromosomes was analyzed to determine its standing as a chromid or plasmid. No plasmid based origin of replication, relaxases, or mate-pair formation genes were encoded. The chromosome was deemed non-mobile and was thus classified as a chromid. *In vitro* antimicrobial susceptibility testing revealed *S. dextrinosolvens* ASCUSBF53 was susceptible to a broad range of antimicrobial compounds. One antimicrobial resistance gene was identified in the genome, tufA, that contributes to resistance to polyketides. Phenotypic testing confirmed that no antimicrobials were produced by *S. dextrinosolvens* ASCUSBF53 during fermentation. Comparison of the *S. dextrinosolvens* ASCUSBF53 genome to several databases containing known pathogenic-related genes revealed one protein hit, a translational regulator, hfq. Homologues of hfq are found in half of all bacteria and in pathogens as well as non-pathogens. In pathogens, hfq may act to modulate expression of pathogenicity and virulence factors, however no virulent or pathogenic genes were identified in the *S. dextrinosolvens* ASCUSBF53 genome that could come under the regulation of hfq. Based on these analyses, *S. dextrinosolvens* ASCUSBF53 is safe for use as a direct fed microbial.

2.2 Method of Manufacture

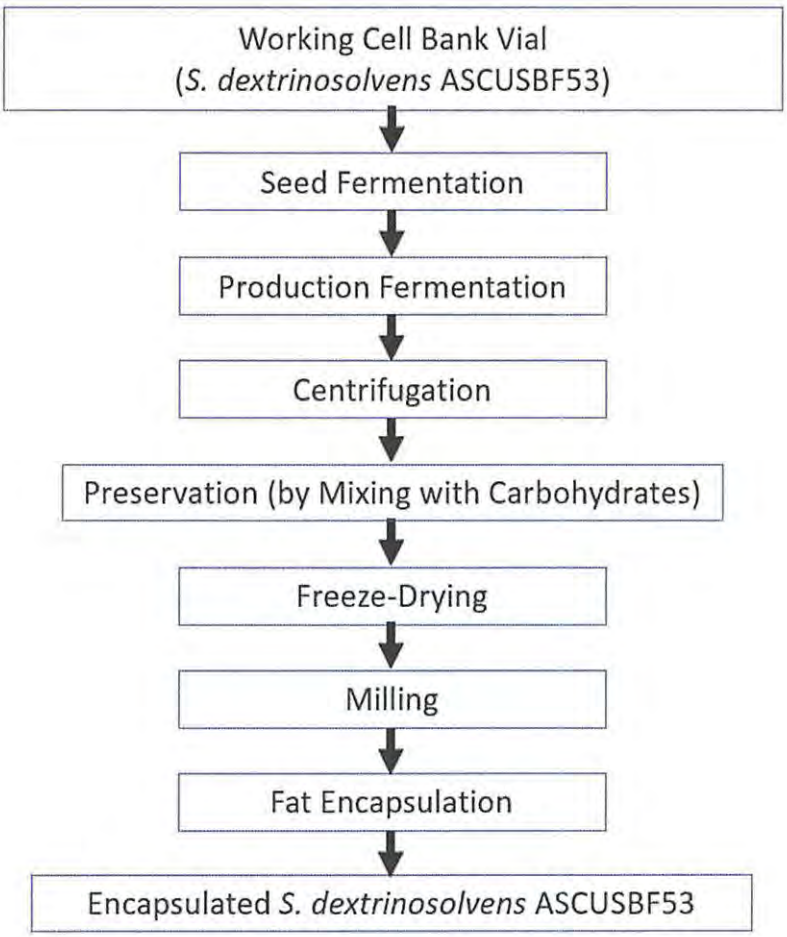
2.2.1 Raw Materials and Processing Aids

The raw materials and processing aids used in the manufacture of fat encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53 are listed in Appendix 009. All raw materials used in the manufacture of *S. dextrinosolvens* ASCUSBF53 have a history of use in the industrial food and feed fermentation processes, and are considered by Native Microbials to be safe and suitable for use in the manufacture of feed ingredients in the U.S.

2.2.2 Manufacturing Process

A schematic overview of the manufacturing process of *S. dextrinosolvens* ASCUSBF53 is provided in Figure 2.4. *S. dextrinosolvens* ASCUSBF53 is produced through a standard anaerobic batch fermentation process. A working cell culture stock is maintained by Native Microbials and used for the seed fermentation. The initial working cell bank vial is propagated through use of anaerobic serum bottle incubated cultures, followed by a series of fermentation steps to the main production fermentor. The biomass is harvested by centrifugation and preserved by the addition of carbohydrates. *S. dextrinosolvens* ASCUSBF53 is then subject to freeze-drying and milling. The notified substance is the unstandardized freeze-dried cell concentrate after milling. In order to standardize and stabilize the microbial strain, the freeze-dried cell concentrate is encapsulated using hydrogenated glycerides or other fat. The resultant fat encapsulated *S. dextrinosolvens* ASCUSBF53 exhibits suitable stability for use as a direct fed microbial in feed but will release under the conditions of the rumen. Details on the manufacturing process are provided in Appendix 010.

Figure 2.4: Schematic Overview of the Manufacturing Process



2.2.3 Production Controls

Commercial manufacture of *S. dextrinosolvens* ASCUSBF53 will be in accordance with current Good Manufacturing Practices (cGMP) and a Hazards Analysis Critical Control Points (HACCP) plan is in place. The requirements of the Food Safety Modernization Act (FSMA) as laid down in 21 CFR §507 will be applied at all stages of the production, processing and distribution.

2.3 Product Specifications and Batch Analyses

2.3.1 Proposed Product Specification for the Cell Concentrate

An appropriate feed-grade specification has been established for the *S. dextrinosolvens* ASCUSBF53 cell concentrate and is presented in Table 2.18. Copies of the methods of analysis are provided in Appendix 007.

Table 2.18: *S. dextrinosolvens* ASCUSBF53 Concentrate Specifications		
Botulinum toxins	Negative/2 g	FDA BAM

Abbreviations: BAM = Bacteriological Analytical Manual

2.3.2 Batch Analyses for the Cell Concentrate

Three batches of *S. dextrinosolvens* ASCUSBF53 cell concentrate representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specification. The results are summarized in Table 2.19 a No botulinum toxins were identified in any of the batches (Appendix 008).

Table 2.19: Analytical Results for 3 Batches of *S. dextrinosolvens* ASCUSBF53 Concentrate					
Parameter	**Unit**	**Specification**	**Analytical Results**		
			Lot 2041	**Lot 2042**	**Lot 2044**
Botulinum toxins*	**Per 2 g**	**Negative**	**Negative**	**Negative**	**Negative**

* Testing done at end of fermentation process

2.3.3 Proposed Product Specifications for the *S. dextrinosolvens* ASCUSBF53 Freeze-dried Powder

Appropriate feed-grade specifications have been established for *S. dextrinosolvens* ASCUSBF53 manufactured as a freeze-dried powder and are presented in Table 2.20. Copies of the methods of analysis are provided in Appendix 012C.

Table 2.20: *S. dextrinosolvens* ASCUSBF53 Product Specifications		
Parameter	**Specification Limits**	**Analytical Method**
Viable cell count	**> 1 x 10^9 CFU/g**	**Internal Method**

Abbreviations: CFU = colony forming units. Internal Method Appendix 012C

2.3.4 Batch Analyses for *S. dextrinosolvens* ASCUSBF53 Freeze-dried Powder

Three batches of *S. dextrinosolvens* ASCUSBF53 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.21 and the Certificates of Analysis are provided in Appendix 013.

Table 2.21: Analytical Results for 3 Batches of *S. dextrinosolvens* ASCUSBF53					
			Analytical Results		
Parameter	Unit	Specification	Lot 2041	Lot 2042	Lot 2044
Viable cell count	CFU/g	> 1 x 10^9 CFU/g	2.23 x 10^9	2.72 x 10^9	1.81 x 10^9

Abbreviations: CFU = colony forming units.

2.3.5 Proposed Product Specifications for the *S. dextrinosolvens* ASCUSBF53 Fat Encapsulate

Appropriate feed-grade specifications have been established for *S. dextrinosolvens* ASCUSBF53 manufactured as a fat encapsulate and are presented in Table 2.22. Copies of the methods of analysis are provided in Appendices 007 and 012.

Table 2.22: *S. dextrinosolvens* ASCUSBF53 Fat Encapsulate Product Specifications		
Parameter	Specification Limits	Analytical Method
Viable cell count	> 2 x 10^7 CFU/g	Internal Method
Coliform	<10 CFU/g	AOAC 2018.13
E. coli	<10 CFU/g	AOAC 2018.13
Salmonella	Negative/25 g	AOAC 2013.01
Listeria	Negative/25 g	AOAC 2013.10

Abbreviations: CFU = colony forming units; AOAC = Association of Official Analytical Chemists. Internal Method Appendix 012C

2.3.6 Batch Analyses for *S. dextrinosolvens* ASCUSBF53 Fat Encapsulate

Three batches of *S. dextrinosolvens* ASCUSBF53 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.23 and the Certificates of Analysis are provided in Appendix 013.

Table 2.23: Analytical Results for 3 Batches of *S. dextrinosolvens* ASCUSBF53 Fat Encapsulate					
			Analytical Results		
Parameter	Unit	Specification	Lot 2041	Lot 2042	Lot 2044
Viable cell count	CFU/g	> 2 x 10^7 CFU/g	9.02 x 10^7	8.18 x 10^7	7.30 x 10^7
Coliform	CFU/g	<10	< 10	< 10	< 10
E. coli	CFU/g	<10	< 10	< 10	< 10
Salmonella	Per 25 g	Negative	Negative	Negative	Negative
Listeria	Per 25 g	Negative	Negative	Negative	Negative

Abbreviations: CFU = colony forming units.

2.3.7 Additional Analytical Data

The levels of heavy metals are also routinely monitored in batches of *S. dextrinosolvens* ASCUSBF53. Three batches of *S. dextrinosolvens* ASCUSBF53 representative of the commercial material were analyzed to verify that the levels of these contaminants fall within acceptable ranges. The results are summarized in Table 2.24 and the Certificates of Analysis from analytical laboratories are provided in Appendix 014. On the basis of the analytical data, no specifications for heavy metals are considered necessary. Based on the level of use, there is no need to identify a specification on these heavy metals based on their insignificant levels and a safety assessment as provided in Part 6.

Table 2.24: Further Analytical Results for 3 Batches of *S. dextrinosolvens* ASCUSBF53					
		Analytical Results			
Parameter	Unit	Lot 2041	Lot 2042	Lot 2044	Analytical Method
Arsenic	ppm	ND	ND	ND	AOAC 2015.01
Cadmium	ppm	ND	ND	ND	AOAC 2015.01
Lead	ppm	ND	ND	ND	AOAC 2015.01
Mercury	ppm	ND	ND	MD	AOAC 2015.01

Abbreviations: AOAC = Association of Official Analytical Chemists. ND= None Detected

2.4 Stability

2.4.1 Shelf-Life Stability Data

Native Microbials guarantees conformity of fat encapsulated *S. dextrinosolvens* ASCUSBF53 to the product specification (see Table 2.22) for a minimum of 12 months when stored in the original, unopened packaging at refrigerated temperature (2 - 10°C). The proposed shelf life is supported through accelerated stability studies in which 3 batches of fat encapsulated *S. dextrinosolvens* ASCUSBF53 representative of the commercial material were stored at 40°C, 50°C, and 60°C, respectively and analyzed through Arrhenius equation regression to represent real-time equivalents, using methods similar to those previously described (Wirunpan, Savedboworn and Wanchaitanawong 2016; King, Lin and Liu 1998) and generally accepted for accelerated shelf-life determination (Tang, 2016). Packaging was done using the same materials as provided in Appendix 06.

2.4.1.1 Accelerated Stability Study at 40°C

The results of the stability study conducted at 40°C for 21 days on *S. dextrinosolvens* ASCUSBF53 are summarized in Table 2.25 and the report is provided in Appendix 15. Over the period evaluated, changes in the viable cell count were observed representing a decay rate plotted in Figure 2.5 for the 3 batches of *S. dextrinosolvens* ASCUSBF53.

Table 2.25: Results of a Stability Study on 3 Batches of *S. dextrinosolvens* ASCUSBF53 Stored at 40°C

Time (Days)	Unit	Analytical Results					
		Lot 2041		Lot 2042		Lot 2044	
		Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	9.02×10^7	5.96×10^6	8.18×10^7	1.16×10^7	7.30×10^7	5.51×10^6
3	CFU/g	2.50×10^7	5.04×10^6	2.48×10^7	5.15×10^6	3.11×10^7	4.29×10^6
7	CFU/g	1.99×10^7	3.40×10^6	2.60×10^7	2.44×10^6	2.41×10^7	1.13×10^6
14	CFU/g	2.64×10^7	7.24×10^6	1.85×10^7	3.78×10^6	1.66×10^7	3.25×10^6
21	CFU/g	1.08×10^7	8.00×10^5	8.97×10^6	1.53×10^6	8.38×10^6	1.68×10^5

Abbreviations: CFU = colony forming units; SD = standard deviation.

2.4.1.2 Accelerated Stability Study at 50°C

The results of the stability study conducted at 50°C for 96 hours on *S. dextrinosolvens* ASCUSBF53 are summarized in Table 2.26 and the report is provided in Appendix 015. Over the period evaluated, changes in the viable cell count were observed representing a decay rate plotted in Figure 2.5 for the 3 batches of *S. dextrinosolvens* ASCUSBF53.

Table 2.26: Results of a Stability Study on 3 Batches of *S. dextrinosolvens* ASCUSBF53 Stored at 50°C

Time (Hours)	Unit	Analytical Results					
		Lot 2041		Lot 2042		Lot 2044	
		Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	9.02×10^7	5.96×10^6	8.18×10^7	1.16×10^7	7.30×10^7	5.51×10^6
8	CFU/g	1.23×10^7	1.37×10^6	1.22×10^7	1.53×10^6	1.03×10^7	2.58×10^6
24	CFU/g	6.46×10^6	3.53×10^5	4.12×10^6	3.70×10^5	4.79×10^6	3.88×10^5
48	CFU/g	4.52×10^6	6.59×10^5	4.06×10^6	9.97×10^5	2.61×10^6	1.42×10^5
96	CFU/g	3.18×10^6	3.93×10^5	2.14×10^6	3.93×10^5	2.83×10^6	1.48×10^5

Abbreviations: CFU = colony forming units; SD = standard deviation.

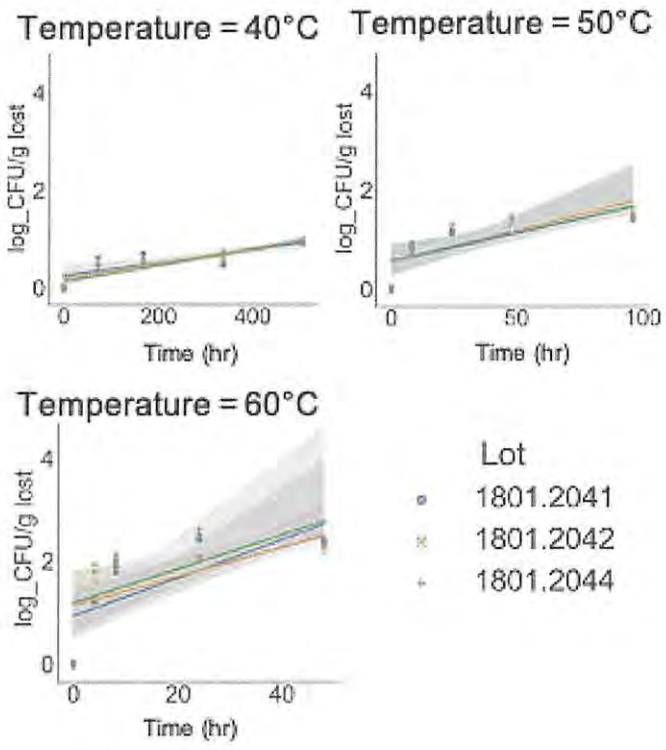
2.4.1.3 Accelerated Stability Study at 60°C

The results of the stability study conducted at 60°C for 48 hours on *S. dextrinosolvens* ASCUSBF53 are summarized in Table 2.27 and the report is provided in Appendix 015. Over the period evaluated, changes in the viable cell count were observed representing a decay rate plotted in Figure 2.5 for the 3 batches of *S. dextrinosolvens* ASCUSBF53.

Table 2.27: Results of a Stability Study on 3 Batches of *S. dextrinosolvens* ASCUSBF53 Stored at 60°C							
		Analytical Results					
		Lot 2041		Lot 2042		Lot 2044	
Time (Hours)	Unit	Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	9.02×10^7	5.96×10^6	8.18×10^7	1.16×10^7	7.30×10^7	5.51×10^6
4	CFU/g	5.25×10^6	4.18×10^5	1.39×10^6	4.46×10^5	1.05×10^6	1.84×10^5
8	CFU/g	1.19×10^6	3.03×10^5	7.53×10^5	2.20×10^5	8.19×10^5	1.54×10^5
24	CFU/g	4.81×10^5	2.55×10^5	7.51×10^5	6.17×10^4	2.06×10^5	2.64×10^4
48	CFU/g	4.06×10^5	5.10×10^4	4.75×10^5	7.00×10^4	3.18×10^5	2.98×10^4

Abbreviations: CFU = colony forming units; SD = standard deviation.

Figure 2.5 Rates 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



2.4.1.4 Shelf Life Prediction

Rates of decay for each lot at each temperature were calculated from the slope of decay over time. As described in the report (Appendix 015), 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 batch was required and the rate data from all 3 lots were demonstrated independently, as they could not be pooled for a combined analysis. The upper-tailed 95% confidence interval for a decay rate was calculated and used to define the shelf life of each lot at 10°C. Lot 2042 had the highest extrapolated decay rate, with a value of -10.9 for the ln[decay rate (log CFU/hr)] which predicted a worst case of 4.31×10^{-4} Log CFU/day, resulting in a minimum shelf life of 1,419 days among the 3 batches of *S. dextrinosolvens* ASCUSBF53. Thus the data confirmed the assigned one year shelf life based on accelerated testing.

2.4.2 In-Feed Stability

As mentioned in Part 1, *S. dextrinosolvens* ASCUSBF53 may be incorporated into the diet of beef cattle as part of the TMR or as top-dressing to individual feeds or the daily ration. The strain is encapsulated with fat to generate a stable product suitable for handling under practical commercial conditions in the U.S. The dry matter intake of beef cattle is optimized by feeding fresh TMR on a twice daily basis. The forage content is typically adjusted to meet the nutrient requirements of the animals on a pen basis. Under the conditions of intended use, *S. dextrinosolvens* ASCUSBF53 may be mixed directly into the TMR or added as a top-dressing at the point of use. On this basis, long-term stability is not relevant, and an in-feed stability study was not conducted.

2.4.3 Homogeneity Data

Due to the highly similar manufacturing process and ensuing encapsulated cell size, the powder attributes, formula, particle size and moisture content (see Appendix 011) of the commercial offering of *S. dextrinosolvens* ASCUSBF53 was noted to be nearly identical to that described in a recent prior submission (AGRN 38) and therefore a separate homogeneity study was deemed unnecessary. Hence we are incorporating by reference the homogeneity study provided in AGRN 38.

2.4.4 Manufacturing Summary

Native Microbials will manufacture a safe stable product for beef cattle meeting cGMP and FSMA compliance. This was demonstrated through batches of product meeting product specifications for contaminants, heavy metals and potency. The product is packaged in moisture protected barrier bags.

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 *S. dextrinosolvens* ASCUSBF53 is to augment normal rumen digestion. As described below, Native Microbials has determined that the technical effect of *S. dextrinosolvens* ASCUSBF53 when fed to beef 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 *S. dextrinosolvens* ASCUSBF53 in the feed of beef cattle are not required as part of this GRAS notice.

S. dextrinosolvens is a prominent member of the rumen of both bovine and sheep and is enriched in animals on high grain diets where it acts to degrade starch and produce volatile fatty acids (VFAs) (Bryant and Small 1956; Wozny et al. 1977a; Hespell 1992; Hippe et al. 1999). Higher abundance of *S. dextrinosolvens* has been associated with more efficient dairy and beef cattle (Elolimy et al. 2018; Hailemariam, Zhao, and Wang 2020; Hernandez-Sanabria et al. 2012). Reduction of *S. dextrinosolvens* has been associated with transportation stress in beef cattle and subsequent weight loss confirming the species as a critical member of a healthy ruminal microbiome (Deng et al. 2017).

The use of this organism is to facilitate the digestion of various carbohydrates of animal feed within the rumen to volatile fatty acids such as succinic acid, lactic acid and acetic acid (see table 2.3). *S. dextrinosolvens* has been found in the rumen in a variety of animals globally (Bryant and Small 1956; Bryant 1959; Wozny et al. 1977a; Wang et al. 2017; Hailemariam et al. 2020; Henderson et al. 2015) and has been assessed as a DFM in both cattle and sheep (Rigobelo et al. 2016; Bello et al. 2019). 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).

The species has been reported to ferment xylan and starch derived from plant material (Hespell et al. 1987; Kozakai et al. 2007). As a commensal microorganism, feeding *S. dextrinosolvens* would have no impact on animal health. Should *S. dextrinosolvens* not act to ferment xylan and starch, 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 less soluble carbohydrates.

2.5.1 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; Wallace *et al.*, 2019). 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; Wallace *et al.*, 2019; Furman *et al.*, 2020; Kumar *et al.*, 2015; Jami *et al.*, 2013; Kittleman *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.*, 2007; Turnbaugh and Gordon, 2009; Turnbaugh *et al.*, 2009). Since then, core microbiomes have been identified in a broad spectrum of environments including agroecosystems, monogastric animals, and ruminants (Shade and Handelman, 2012; Yeoh *et al.*, 2017; Toju *et al.*, 2018; Lowe *et al.*, 2012; Dougal *et al.*, 2013).

For example, there is a core microbiome that appears in the majority of cattle 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 and Mizrahi, 2012; Lima et al, 2015; Deusch, 2017; Huws, 2018; Xue, 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; Belanche *et al.*, 2019; Kumar, 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 (Morais and Mizrah, 2019; Wallace *et al.*, 2019; Furman *et al.*,

2020; Belanche *et al.*, 2012). To better assess the impact of *S. dextrinosolvens* ASCUSBF53 on deleteriously impacting the existing microbiome. If the abundances of core microbiome members are within typically observed ranges, it is likely that rumen fermentation is also operating within normal ranges as well. These studies cover a variety of diets, as diet has the most impact on microbiome composition. In-house data corroborates that no large shifts in the core microbiome beyond observed thresholds are anticipated through feeding a native microorganism, and thus, no detrimental effects of rumen fermentation are expected (Appendix 018). The intent of feeding DFMs, particularly *S. dextrinosolvens* ASCUSBF53, is to improve the nutrient availability from feed. Feeding *S. dextrinosolvens* ASCUSBF53 to beef cattle supplements the existing populations of *S. dextrinosolvens* ASCUSBF53 in the rumen, and ultimately provides additional nutrient availability to the animal. Should *S. dextrinosolvens* ASCUSBF53 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 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, 2016). Should *S. dextrinosolvens* ASCUSBF53 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.

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 *et al.*, 2007, Abu-Tarboush *et al.*, 1996, Higginbotham *and Bath.*, 1993, McGilliard and Stallings, 1997). 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.*, 2009, Zebeli *et al.*, 2012; Hagg , 2008, 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 *S. dextrinosolvens* ASCUSBF53, if the DFM failed to provide improved digestibility, rumen fermentation of treated cattle would be identical to rumen fermentation of untreated cattle. 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 beef cattle (NRC, 2016). Any non-performing *S. dextrinosolvens* ASCUSBF53 or deceased *S. dextrinosolvens* ASCUSBF53 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, *S. dextrinosolvens* ASCUSBF53 will act only to support normal ruminal function of digestion of animal feed. Like other DFMs, while *S. dextrinosolvens* ASCUSBF53 may aid the digestion of feed, the effect is not required for the general well-being and normal performance of beef cattle. Thus, the absence of the anticipated effect of *S. dextrinosolvens* ASCUSBF53 on feed digestion by beef 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 beef cattle (NRC, 2016).

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 *S. dextrinosolvens* ASCUSBF53, 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 *S. dextrinosolvens* ASCUSBF53 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.

PART 3 – TARGET ANIMAL AND HUMAN EXPOSURE

3.1 Target Animal Exposure

3.1.1 Exposure to the Direct Fed Microbial Strain

As mentioned in Part 1, *S. dextrinosolvens* ASCUSBF53 is intended for use as a source of viable microorganisms in feed for beef cattle. The microbial strain will be delivered as a fat encapsulated direct fed microbial to beef cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing *S. dextrinosolvens* ASCUSBF53 may be incorporated into the diet of beef cattle include as part of the feedlot TMR, as top-dressing on feeds or the daily ration, and as a component of a feed supplement. The product will be incorporated into beef cattle feed at the recommended use level of $1x10^8$ CFU of *S. dextrinosolvens* ASCUSBF53/hd/day. As mentioned in Part 2.2, the fat encapsulated product is comprised of approximately 30% sodium sulfate, 50% hydrogenated glycerides and 20% freeze-dried *S. dextrinosolvens* ASCUSBF53 powder. Thus, under the conditions of intended use, beef cattle will be exposed to maximum 1 g of the *S. dextrinosolvens* ASCUSBF53.

3.1.2 Exposure to the Other Components of the Fat Encapsulated Product

At the intended intake of $1x10^8$ CFU *S. dextrinosolvens* ASCUSBF53/hd/day, the animal will be exposed to up to 5 g of the notified substance (min. $2x10^7$ CFU/g). The product is comprised of approximately 30% sodium sulfate, 50% hydrogenated glycerides and 20% freeze-dried *S. dextrinosolvens* ASCUSBF53 powder (see Appendix 010). As mentioned in Part 2, the amount of hydrogenated glycerides, sodium sulfate, and freeze-dried *S. dextrinosolvens* ASCUSBF53 powder is adjusted for each batch to standardize the viable cell count. These encapsulation ingredients are acceptable for use in beef cattle feed and comply with the corresponding ingredient definitions in the AAFCO Official Publication (AAFCO 2020; ingredient definitions 33.19 and 57.106 - see Appendix 010). Under these conditions of use, the animal will be exposed up to a maximum of 2.5 g of hydrogenated glycerides and 1.5 g of sodium sulfate. Considering that the typical dry matter intake by the beef cattle will be about 20 kg/hd/day, the contribution of hydrogenated glycerides to the beef ration is expected to be no more than 0.006% DM. While the fat concentration of a typical beef diet is reported to be relatively low (approximately 2.5% DM), supplemental fats can be added to achieve a total ration content of around 6% DM (MSD Veterinary Manual, 2019). On this basis, the use of hydrogenated glycerides or similar acceptable fat source as an encapsulating aid in the manufacture of fat encapsulated *S. dextrinosolvens* ASCUSBF53 will have a negligible impact on the total fat intake by beef cattle under the conditions of use. Similarly, an intake of 1 g/hd/day of sodium sulfate will provide beef cattle with approximately 0.48 g of sodium/hd/day, representing less than 0.004% of the DM intake. The maximum tolerable levels of sodium chloride set by the National Research Council (NRC) for beef cattle is 3% of DM intake, equivalent to around 1% DM of sodium. Thus, the use of sodium sulfate as an encapsulating agent in the manufacture of fat encapsulated *S. dextrinosolvens* ASCUSBF53 is not expected to have any significant impact on the overall sodium intake by beef cattle under the intended conditions of use. Another element of interest is sulfur. The use of *S. dextrinosolvens* ASCUSBF53 would provide approximately 1 g of sodium sulfate or 0.34 g of sulfur per day. The NRC (2005) has suggested that Total Mixed rations (grain based) of cattle diets should be at a maximum tolerable level of 0.3% sulfur (60 g/hd/day), as such this ingredient would provide an insignificant amount of the total sulfur in the diet of the beef cattle.

3.1.3 Background Exposure to the Microorganism

As mentioned in Part 2, the strain was isolated from the rumen content of a healthy steer and in this respect, the fat encapsulated *S. dextrinosolvens* ASCUSBF53 powder will contribute to the native population of *Succinivibrio* species in the gut of the animal (see Part 6.4). *S. dextrinosolvens* is part of the rumen microflora (Seshadri 2018). Although it is routinely isolated from rumen content of cow and sheep (Gomez-Alarcon 1982, Hespell 1992, Bryant 1956), *S. dextrinosolvens* has been detected in feces of swine, canine, and human (Tanner 2014; Xu 2019; Drasar and Roberts, 2020). Thus, while not present to a significant or intentional degree in feedstocks, background exposure by feedlot cattle to *S. dextrinosolvens* from the environment is likely to be significant.

3.2 Human Exposure

S. dextrinosolvens ASCUSBF53 is intended for use as a supplemental source of viable microorganisms in the feed of beef cattle. As mentioned in Part 2.1, the strain was isolated from the rumen content of a healthy steer and in this respect, *S. dextrinosolvens* ASCUSBF53 will contribute to the native ruminal population of *Succinivibrio* species (see Part 6). No transfer of viable *S. dextrinosolvens* ASCUSBF53 from the rumen to edible tissues is anticipated.

The strain has been unambiguously characterized as *S. dextrinosolvens* 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). As a consequence, there should be no transfer of pathogenicity or toxigenicity to edible tissues through the use of *S. dextrinosolvens* ASCUSBF53 as a source of viable microorganisms in the feed of beef cattle.

No withdrawal period is considered necessary on the basis that *S. dextrinosolvens* ASCUSBF53 is native to the rumen of beef cattle and as detailed in Part 6, and the strain has been shown to have no pathogenic or toxigenic properties.

PART 4 – SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with *S. dextrinosolvens* ASCUSBF53.

PART 5 – EVIDENCE BASED ON COMMON USE BEFORE 1958

Not applicable.

PART 6 – NARRATIVE

The conclusion that *S. dextrinosolvens* ASCUSBF53 fat encapsulated powder, as described herein, is GRAS under the conditions of intended use as a direct fed microbial in feed for 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 *Succinivibrio* species.

As mentioned in Part 1.3, *S. dextrinosolvens* ASCUSBF53 will be provided to cattle either alone or in combination with other direct fed microbials. The strain was isolated from the rumen content of a healthy Angus steer and is intended as a source of commensal microorganisms. In this respect, *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 for use as a direct fed microbial for cattle is evaluated according to the guidelines developed by (Pariza *et al.* 2015). These guidelines are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial cultures for consumption by humans and animals (AAFCO, 2020). In accordance with these guidelines, the safety of a microorganism without an extensive history of use in food or feed is primarily addressed by evaluating the 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 *S. dextrinosolvens* ASCUSBF53 are detailed in Part 2. The microbial has been unambiguously characterized as *S. dextrinosolvens* (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 *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* (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). Following the decision tree established by (Pariza *et al.* 2015), these data are pivotal to the safety evaluation of *S. dextrinosolvens* ASCUSBF53 and are summarized below. The Pariza decision tree that outlines the safety evaluation is provided in Appendix 016.

6.1 Functionality

The microbial population of the rumen plays an important role in the utilization of feed by cattle. Manipulation of rumen microbiota by dietary supplementation with sources of viable microorganisms is common practice in the beef cattle industry in the U.S. in order to facilitate fermentation and contribute to the general digestive health of the animal (Chaucheyras-Durand and Durand 2010; Abd El-Tawab et al. 2016; Yoon and Stern 1995). 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 (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, *S. dextrinosolvens* ASCUSBF53 was isolated from the rumen content of a healthy steer and is expected to contribute in the same way as other bacteria to digestion and metabolism in the ruminal environment.

In particular, *S. dextrinosolvens* ASCUSBF53 was shown to utilize various carbon sources including simple carbohydrates (e.g., glucose and fructose) as well as reducing sugars derived from plant materials such as xylose and arabinose, sugar alcohols such as arabitol and sorbitol, and starch (see Part 2.1). Similar phenotypes are reported in the published literature for other *S. dextrinosolvens* strains (Bryant 2015; Bryant and Small 1956). Additionally, some strains of the species are known to assimilate nitrogen and ferment pectin (Patterson and Hespell 1985; Dehority 1969; Hailemariam, Zhao, and Wang 2020; Bryant 2015). *In vitro* experiments have shown that *S. dextrinosolvens* is capable of degrading xylans derived from wheat straw, larchwood, and oats (Hespell, Wolf, and Bothast 1987). The species has a demonstrated ability to colonize and aid in the digestion of corn silage in vivo (Kozakai et al. 2007). Thus, the microorganism has the potential to support digestion by aiding fermentation of forages and partially degraded digesta in the rumen.

Similar to other *S. dextrinosolvens* strains, *S. dextrinosolvens* ASCUSBF53 has been shown to utilize a range of monosaccharides including glucose to produce relatively high levels of succinate, lactate, and acetate (O'Herrin and Kenealy 1993; Bryant and Small 1956; Russell and Hespell 1981). Volatile fatty acids (VFAs) are the main source of energy in ruminants and are produced predominantly through microbial fermentation of feed in the rumen (Bergman 1990; Council and Others 2007). The three major VFAs produced by anaerobic microbial fermentation in the rumen are acetate, propionate and butyrate with the relative ratios largely depending on the nature of the feed. The VFAs are readily absorbed and utilized by ruminants accounting for up to 80% of their maintenance energy requirements. Butyrate in particular acts as the major energy source for epithelial cells in ruminants and is recognized to play an important role in maintaining colonic health in the animal. Studies have also linked butyrate to the development of rumen papillary and calf gastrointestinal tracts (Weigand, Young, and McGilliard 1975; Górka et al. 2018). A review of 8 studies that evaluated relationships between residual feed intake (RFI) and VFA production concluded that there was no consistent relationship between more efficient cattle and VFA concentration (Kenny et al. 2018). Similarly, a number of other studies in the published literature indicate that butyrate can support the general production performance of the animals (Rook and Balch 1961; Huhtanen, Miettinen, and Ylinen 1993; Miettinen and Huhtanen 1996). The role of VFAs as energy sources for cattle also is supported by the existing food additive listing for the ammonium or calcium salts of isobutyric acid, iso-valeric acid, 2-methylbutyric acid and n-valeric acid as sources of energy in cattle feeds under 21 CFR §573.914.

It is also worth noting that *S. dextrinosolvens* is a succinate producer (Bryant and Small 1956; Bryant 2015; Hespell 1992). Succinate is a precursor for propionate, which is an important VFA for rumen digestion and animal growth (Castillo et al. 2004; O'Herrin and Kenealy 1993; Clemmons et al. 2020). Clemmons (2020) found a significantly higher succinate concentration in rumen fluid of more efficient cattle than the less efficient cattle, suggesting that succinate may be an important metabolite in nutrient

conversion. It has also been reported that a significantly greater abundance of succinate- and propionate- producing bacteria were observed in more efficient cattle (Myer et al. 2015). Therefore, supplementing *S. dextrinosolvens* ASCUSBF53 as a DFM has the potential to improve succinate production.

Direct infusion of VFAs into the rumen has been shown to improve feed digestibility. For example, (Ribeiro et al. 2009) directly infused propionate into the rumen of 7 months old young bulls daily for 16 days. The bulls were fed with a high forage (80:20 forage:concentrate) or an elevated concentrate (60:40 forage:concentrate) diet. Over the entire period, the bulls on the elevated concentrate diet with propionate infusion had significantly less total digestible nutrient (TDN) intake compared to those on the same diet without propionate infusion. On day 15, regardless of diet, a significantly higher rumen pH and lower accumulation of ammonium in the rumen fluid were observed in propionate infused animals 4-hours post feeding. While a higher rumen pH suggests a more stable rumen fermentation environment, a lower accumulation of ammonium and TDN intake reflects a more efficient utilization of dietary nutrients (Ribeiro et al. 2009; Russell 2002). Microbial protein fermentation by rumen microorganisms, positively correlated with TDN intake, leads to ammonium accumulation and can result up to 25% dietary nitrogen loss (Russell 2002; Galyean and Tedeschi 2014; Bach, Calsamiglia, and Stern 2005). A consistent reduction in TDN intake and ruminal ammonium suggests that propionate infusion improves nutrients, particularly nitrogen turn-over in ruminants.

Strain-specific degradation of nitrogen containing compounds and assimilation of the resulting nitrogenous compounds and/or environmental ammonia has been observed in *S. dextrinosolvens* (Hailemariam, Zhao, and Wang 2020; Patterson and Hespell 1985; Wozny et al. 1977b). Some members of the species have both the ability to degrade urea through ureases and possess genes encoding for the nitrogen assimilation enzymes glutamine synthetase and glutamine dehydrogenase, while others possess only the genes encoding for the assimilation enzymes (Hailemariam, Zhao, and Wang 2020; Patterson and Hespell 1985). Nitrogen assimilation in the rumen is important to sustain the protein requirements of rumen microbiota, increase the amount of microbial derived protein available for the host, and has the potential to reduce the environmental pollution that results from excreting urea and ammonia in ruminant waste (Pengpeng and Tan 2013; Hobson and Stewart 2012; Walker, Newbold, and Wallace 2005). Estimates put 40-95% of the total nitrogen utilized by bacteria as ammonia derived, and since ruminants can derive as much as 80% of their protein from their resident rumen microbiota, microbial nitrogen assimilation is critical to fulfill host nutritional requirements (Clark, Klusmeyer, and Cameron 1992; Storm and Ørskov 1983; Walker, Newbold, Wallace 2005). Studies have demonstrated that high ruminal concentrations of ammonia and subsequent high plasma ammonia levels have a potentially negative effect on feeding habits of ruminants resulting in the animals eating fewer meals and having shorter feeding times (Sinclair, Sinclair, and Robinson 2000; Conrad, Baile, and Mayer 1977; Sinclair et al. 2012). In some cases the supplementation of slow release urea in ruminant diets has resulted in improvements in milk yield, protein content, and/or milk fat in dairy cows, while meta analyses of studies testing the effect of slow release urea supplemented to beef cattle demonstrated increases in both feed efficiency and live weight gain (Highstreet et al. 2010; Sinclair et al. 2012; Salami et al. 2020). This suggests that urea degradation and nitrogen assimilation by *S. dextrinosolvens* and other microbes

may play a role in feed intake and efficiency.

Taken together, these examples of the potential functionality of *S. dextrinosolvens* in the rumen support the proposed role of *S. dextrinosolvens* ASCUSBF53 as a source of viable microorganisms in the diet to support the production of VFAs and feed digestibility. While *S. dextrinosolvens* ASCUSBF53 may contribute to the native population of *Succinivibrionaceae* 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 beef cattle. Should *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 was required for the safety evaluation (see Part 2.5).

6.2 Identity

The family *Succinivibrionaceae* includes the genera *Anerobiospirillum, Ruminobacter, Succinatimonas, Succinimonas,* and *Succinivibrio*. The family clusters within the gamma-subclass of the class *Proteobacteria,* also known as the gammaproteobacteria (Stackebrandt and Hespell 2006; Hippe et al. 1999). With the exception of *Anerobiospirillum*, which was isolated from domesticated dogs, *Succinivibrionaceae* species are native to the gastrointestinal tract of ruminants (Hippe et al. 1999). *Succinivibrionaceae* as a family shares 84.6-88.5% 16S rRNA similarity to species in neighboring families within gammaproteobacteria. Amongst type strains of species within *Succinivibrionaceae,* a maximum of 93% 16S rRNA similarity is observed and the species can be identified unambiguously by 16S rRNA sequencing (Stackebrandt and Hespell 2006). The closest phylogenetic neighbor to the *Succinivibrio* in the family is the genus *Anerobiospirillum*. *S. dextrinosolvens* is currently the only species with standing nomenclature in the genus *Succinivibrio* (Bryant 2015).

6.3 Literature Search

A comprehensive literature search was conducted in order to identify all publicly available information pertaining to the safety of *S. dextrinosolvens* for the intended use as a source of viable cells for beef cattle. Details of the search strategy are provided in Appendix 017. Results from the literature search form the basis of the safety assessment found in Parts 6.4, 6.5, 6.6.

6.4 Natural Occurrence

6.4.1 Prevalence in Animals

S. dextrinosolvens is routinely isolated from rumen of cattle and sheep (Bryant and Small 1956; Bryant 1959; Wozny et al. 1977b; Wang et al. 2017; Hailemariam, Zhao, and Wang 2020) and has been found in swine colons (Li et al. 2012). While there is little research to support this, it is speculated that *S. dextrinosolvens may* be present at low levels in the human gut (Hespell 1992). A single isolate of a *Succinivibrio* species was found in a human during an astronaut dietary study (Holdeman, Good, and Moore 1976). A *Succinivibrio* species was also identified in a study regarding advanced periodontitis and oral bone loss, though no connection between the health condition and the isolate was drawn (Tanner et al. 1979). A total of 4 different strains of *S. dextrinosolvens* have been isolated, sequenced, and analyzed

in the JGI genome portal to date (https://genome.jgi.doe.gov/portal/), and 6 strains were found in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

In cattle being fed a standard diet, the abundance of *S. dextrinosolvens* in the rumen was found to be approximately 0.81% (Stevenson and Weimer 2007). However, it has been reported that *S. dextrinosolvens* is often found in greater abundance in animals on a high-starch diet (Bryant 1959; Bryant 1970). Internal studies have shown that this species can comprise up to 20% of the rumen population. Similarly, The Global Rumen Census found that members of the *Succinivibrionaceae* family were more abundant in animals that were fed a high-concentrate or high-starch diet (Henderson et al. 2015).

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 components of feed. The fermentation process converts nearly all dietary carbohydrates to VFAs, 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; Wallace, Onodera, and Cotta 1997; Broudiscou and Jouany 1995; Weigand, Young, and McGilliard 1975; Górka et al. 2018; Leng, Steel, and Luick 1967; Young 1977; Huws et al. 2018; Bach, Calsamiglia, and Stern 2005; Edwards et al. 2008). Kristensnen et al. (2004) found that rumen epithelium absorbs and metabolizes butyrate primarily, consistent with the observation of butyrate infusion significantly improved the development of rumen papillae in weaning calves. Approximately only 10% of the propionate absorbed is metabolized by the rumen epithelium and the rest are used to support the function of other organs (Kristensen and Harmon 2004). Both acetate and propionate also significantly stimulate rumen epithelial cell proliferation, indicating their roles in rumen epithelial reorganization upon diet changes (Baldwin et al. 2004; Steele et al. 2011).

Bacteria catabolism also plays an important role in animal nutrient cycling. Hoogenraad et al (1969) 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 freeze-dried whole cells of either bacteria were quickly digested by 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, Da Silva Cabral, and Cacite 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; Belanche et al. 2019; Johnson and Johnson 1995; Bruic 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 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 and colleagues (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 microbiome significantly, despite confirmation of colonization of the DFM strain in rumen (Schofield et al. 2018). In another study, Fomenky and colleagues (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. These studies demonstrated that DFMs could promote better microbial interactions and improve the overall rumen feed digestibility without significantly changing microbial community structure.

S. dextrinosolvens is one of the representative species of the genus *Succinivibrio* (Hespell 1992; Bryant 2015). First isolated from cattle rumen fluid, *S. dextrinosolvens* is particularly enriched in rumen fluid of animals on a diet containing a large amount of starch or rapidly fermentable carbohydrates (Hespell 1992). This is consistent with the isolation pattern of *S. dextrinosolvens* ASCUSBF53. Studies reported the relative abundance of *S. dextrinosolvens* in dairy cow rumen microbiome ranges from 0.5% to 1% (Stevenson and Weimer 2007; Tajima et al. 2001). Similarly, the Hungate1000 project isolated 3 *S. dextrinosolvens* strains (https://genome.jgi.doe.gov/portal/TheHunmicrobiome/TheHunmicrobiome.info.html), representing 0.7% of the global ruminant rumen microbial community (Seshadri et al. 2018). However, *S. dextrinosolvens* can proliferate in the rumen of cattle on a high concentrate diet and can comprise as high as 18% of the total number of bacterial cells in the rumen microbiome (Bryant and Small 1956; Petri et al. 2013). Thus, although it has not been used as a DFM in ruminants, *S. dextrinosolvens* is a common commensal rumen microorganism.

Native Microbials conducted a series of experiments in order to better understand the rumen composition of beef cattle as well as the impacts of administering native rumen microorganisms, including *S. detrinosolvens* ASCUSBF53. In one survey experiment (Clemmons et al. 2019a, Clemmons et al. 2019b), cattle fed a high-grain diet were followed over 10 weeks to determine how the microbiome changed and adapted in response to diet changes. Three follow-up studies were conducted to determine the impacts of daily supplementation of *S. dextrinosolvens* ASCUSBF53 in conjunction with other native rumen microorganisms on rumen microbiome composition. In all of the experiments, the typical

abundance of *S. dextrinosolvens* in the rumen of cattle was found to vary from 1.3% to 19.99% (average 8.84%) of the bacterial population. General observations indicated that all animals were in good health. *S. dextrinosolvens* ASCUSBF53 inoculation was not observed to have a significant impact on the ruminal microbial community. Taken together, these studies provide corroborative experimental evidence that *S. destrinosolvens* is naturally abundant in the rumen of feedlot cattle and not associated with any health concerns.

6.4.3 Environmental Occurrence

S. dextrinosolvens occurs extensively in the gastrointestinal tracts of animals, primarily in the rumens of cows and sheep (Bryant and Small 1956; Bryant 1959; Wozny et al. 1977b; Wang et al. 2017; Hailemariam, Zhao, and Wang 2020), as well as the colons of pigs (Li et al. 2012). Bacteria in the *Succinivibrio* genus have also been isolated from manure sludge from a Korean swine farm (Han et al. 2011).

Occurrences of *S. dextrinosolvens* being isolated in environmental samples that are not associated with animals are not well documented. Furthermore, the entry regarding *Succinivibrio* in Bergey's Manual of Systematics of Archaea and Bacteria only reported isolations from mammalian samples (Bryant 2015).

6.4.4 Section Summary

S. dextrinosolvens is readily found in the rumens of cattle and sheep. It is a common, ruminant commensal organism. Supplementation of the diet with *S. dextrinosolvens* ASCUSBF53 will not negatively impact the function of the rumen nor negatively impact the well-being of the animal.

6.5 History of Use in Manufacture of Food and Feed Ingredients

S. dextrinosolvens has been previously used as an additive in feed. *S. dextrinosolvens*, in conjunction with several other microbes, was administered to dairy cattle as a DFM (Bello et al. 2019). While no improvements to milk production were reported, no ill effects of the microbial supplementation were found in this study. It has also been used as a feed supplement for sheep, in efforts to reduce the amount of *E. coli* present in their gastrointestinal tracts (Rigobelo et al. 2016). No adverse effects were reported.

6.6 Toxigenicity and Pathogenicity

The family *Succinivibrionaceae* consists of 5 distinct genera, including *Succinivivibrio*. Members of the *Anaerobiospirillum* genus of the *Succinivibrionaceae* family have been identified in a number of clinical reports, however, these bacteria are considered to be uncommon causative agents and nearly all cases involving *Anaerobiospirillum* species were reported in people with additional health problems, suggesting that these infections are opportunistic (Tee et al. 1998; Kelesidis et al. 2010; Decroix et al. 2016; Epstein et al. 2017; Schaumburg et al. 2017; Madden et al. 2019). There have been no reported infections confirmed to be caused by other members of the *Succinivibrionaceae* family in the genera *Ruminobacter*, *Succinatimonas,* and *Succinimonas*. Furthermore, no published data was found regarding common pathogenic elements in the genomes across the genera in the *Succinivibrionaceae* family.

At the genus level, *Succinivibrio* have been cited in two cases of bacteremia. The first suspected case was in a man that arrived at a hospital in a non-responsive state and suffering from heat stroke, numerous external injuries, and gastrointestinal hemorrhaging (Southern 1975). The patient's blood was diagnosed with bacteremia and an organism identified as *S. dextrinosolvens* was cultured from the blood. The patient died 60 hours after being admitted to the hospital, and the role of bacteremia in the death of the patient was uncertain, as there was little evidence of residual infection in the blood.

The second case reported was in a man suffering from gastrointestinal bleeding and an esophageal hernia (Porschen and Chan 1977). He underwent surgery, and then an organism later identified as *S. dextrinosolvens* was cultured from blood samples using agar plates. The authors speculated that the bacteria found in the blood originated in the gastrointestinal tract, and no signs of sepsis was observed after the bacteremia was identified. It should also be noted that in both cases of suspected *S. dextrinosolvens* infection the identifications of the causative organisms were made using morphology data, as well as metabolic and antimicrobial susceptibility profiles. No infections of *S. dextrinosolvens* have been confirmed using molecular or genetic sequencing-based methods.

6.6.1 Section Summary

Overall, the available information indicates that *S. dextrinosolvens* is an organism abundant in the rumens of cattle and sheep. Few clinical cases were suspected to be caused by *S. dextrinosolvens* though none were confirmed by unambiguous genetic methods. As indicated in Part 2.1.8, interrogation of the whole genome sequence of *S. dextrinosolvens* ASCUSBF53 did not reveal the presence of any significant genes that encode for virulence factors or protein toxins.

6.7 Studies in Target Animals

The determination that *S. dextrinosolvens* ASCUSBF53 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.

Two investigative studies in which cattle were inoculated with *S. dextrinosolvens* ASCUSBF53 were conducted by Native Microbials to corroborate the target animal safety determination. These unpublished studies, summarized in turn below, were designed primarily to assess the potential value of *S. dextrinosolvens* ASCUSBF53 and other microorganisms as potential direct fed microbials. In these studies, *S. dextrinosolvens* ASCUSBF53 was administered via ruminal cannulation or in feed in combination with at least one other microorganism. Overall, the study findings provide corroborative evidence that *S. dextrinosolvens* ASCUSBF53 is well-tolerated and without adverse effects but are of limited relevance to the assessment of safety.

6.7.1 Study BUS1801 (Unpublished Study Report – Appendix 019)

In the first study, 16 Angus heifers were individually housed for a total of 3.5 months at the (b) (4) (b) (4) Animals underwent ruminal cannulation surgery, and the project began two months later after cattle recovered from fistulation. The project consisted of two (2) grain challenge periods within a cross-over design utilizing 16 heifers; $n = 8$ treatment. An additional heifer was retained

for replacement in the event of animal illness or deleterious contingencies related to the project. Throughout the project, microbes/controls were administered via cannula. The control consisted of a buffer solution, and the treatment consisted of the buffer solution containing a selection of microorganisms including *S. dextrinosolvens* ASCUSBF53. Feed intake and gain were measured, ruminal fermentation analyzed via volatile fatty acid (VFA) analysis, ruminal parameters were measured (pH, temperature, and dissolved CO2), and finally, blood analyses were completed (e.g. temperature, lactate, CO2). Weekly, ruminal samples were shipped to Native Microbials for microbiome analysis.

No adverse effects were reported for any of the variables measured over the duration of the study. Overall, the findings of the study corroborate the safety of *S. dextrinosolvens* ASCUSBF53 for cattle.

6.7.2 Study BUS1901 (Unpublished Study Report – Appendix 020)

In the second study, 75 steers were blocked and allocated to 3 treatment groups (25 steers/treatment). Cattle were administered test article containing either a low dose of product (treatment group 1), a high dose of product (treatment group 2) or no product (treatment group 3; control) once daily via feed. The microorganisms fed to the steers in treatment groups 1 and 2 included *S. dextrinosolvens* ASCUSBF53. The study ran for 171 days, and observations included weight, feed intake, rumen pH, general health and clinical evaluation.

Overall, general health was within normal limits across all treatment groups. There were no adverse events attributable to or consistent with a specific test article. Health events were not outside of normal limits for cattle transitioning to high concentrate diets in the feed yard. Overall, the findings of the study corroborate the safety of *S. dextrinosolvens* ASCUSBF53 for cattle.

6.8 Summary and Critical Evaluation of Target Animal Safety

S. dextrinosolvens is a commensal bacteria in the gut of humans and animals. It occurs widely in the rumen of various ruminant species. No reports of toxigenicity or pathogenicity associated with *S. dextrinosolvens* were identified in the published literature. Native Microbials has conducted an assessment of *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 should not be associated with any safety concerns for 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 *S. dextrinosolvens* ASCUSBF53 from the rumen to edible tissues is anticipated under the conditions of intended use as a direct fed microbial in the feed of cattle. Furthermore, the strain has been unambiguously characterized as *S. dextrinosolvens* 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 *S. dextrinosolvens* and its natural occurrence in the rumen of animals. Taken

together, these data indicate that *S. dextrinosolvens* ASCUSBF53 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 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.

PART 7 – LIST OF SUPPORTING DATA AND INFORMATION IN YOUR GRAS NOTICE

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

TO
Dr. Mallory Embree
Ascus BioSciences
6450 Lusk Blvd.
Suite E109
San Diego, Ca 92121

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Succinivibrio spp. **(Ascusbbf_154B)**	**NRRL B-67550**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

☐ [1]a scientific description

☒ [1]a proposed taxonomic designation

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on **February 07, 2018** (date of the original deposit)[2]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I. above, was received by this International Depositary Authority on _____ (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	Agricultural Research Culture Collection (NRRL) International Depositary Authority	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	1815 N. University Street Peoria, Illinois 61604 U.S.A.	Date: **February 07, 2018** **Travis W. Adkins**

[1] Mark with a cross the applicable box.
[2] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

TO
Dr. Mallory Embree
Ascus BioSciences
6450 Lusk Blvd.
Suite E109
San Diego, Ca 92121

VIABILITY STATEMENT

issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: **Dr. Mallory Embree** **Ascus BioSciences** Address: **6450 Lusk Blvd.** **Suite E109** **San Diego, Ca 92121**	Depositor's taxonomic designation and accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY ***Succinivibrio spp.* (Ascusbbf_154B)** **NRRL B-67550** Date of: **February 07, 2018** ☒ Original Deposit ☐ New Deposit ☐ Repropagation of Original Deposit

III. (a) VIABILITY STATEMENT

Deposit was found:
☒ [2]Viable ☐ [2]Nonviable on _____ (Date)
International Depositary Authority's preparation was found viable on **February 07, 2018** (Date)[3]

III. (b) DEPOSITOR'S EQUIVALENCY DECLARATION

Depositor determined the International Depositary Authority's preparation was
☐ [2]Equivalent
☐ [2]Not equivalent to deposit on NA (Date)

Signature of Depositor

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST WAS PERFORMED (Depositors/Depositary)[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Agricultural Research Culture Collection (NRRL) International Depositary Authority Address: 1815 N. University Street Peoria, Illinois 61604 U.S.A.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: **February 07, 2018** **Travis W. Adkins**

[1] Indicate the date of the original deposit or when a new deposit has been made.
[2] Mark with a cross the applicable box.
[3] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
[4] Fill in if the information has been requested.

Appendix 002: Supplementary Methods and Results for *S. dextrinosolvens* ASCUSBF53 In Vitro Biochemical Assays

Objectives:

The objective of this work was to assess the carbohydrate fermentation capabilities and metabolite production of *S. dextrinosolvens* ASCUSBF53 through in vitro assays.

Methods:

Carbohydrate fermentation of *S. dextrinosolvens* ASCUSBF53 was qualitatively measured using the API 50CH carbon panel ((b) (4)). Results can be found in Table 1. *S. dextrinosolvens ASCUSBF53* cells were grown to late exponential phase and recovered by centrifugation at (b) (4). Cells were resuspended and (b) (4) (wt/vol (b) (4) added as a pH indicator for acidification of carbohydrates (Avgustin et al. 1997).

Metabolite production of *S. dextrinosolvens* ASCUSBF53 fermentation run (b) (4) was measured at (b) (4) hours using an (b) (4). The column used was a (b) (4) with (b) (4) operated at (b) (4). The mobile phase was (b) (4)nL (b) (4) at a flow rate o (b) (4)nL/min. Pure standards were used at varying concentrations to generate a standard curve.

Results:

S. dextrinosolvens ASCUSBF53 was assessed for fermentation of 50 carbon sources. Carbon source fermentation data is shown below in table 1. Metabolite production at each fermentation time point can be found in table 2.

Table 1. Carbon Source Fermentation by *S. dextrinosolvens* ASCUSBF53

Carbon Source	Growth	Carbon Source	Growth
No Carbon Control	No Growth	(b) (4)	No Growth
Glycerol	No Growth		No Growth
Erythritol	No Growth		Growth
D-Arabinose	No Growth		No Growth
L-Arabinose	Growth		No Growth
D-Ribose	Growth		No Growth
D-Xylose	Growth		No Growth

L-Xylose	No Growth	(b) (4)	No Growth
D-Adonitol	No Growth		No Growth
Methyl-BD-xylopyranoside	No Growth		No Growth
D-Galactose	Growth		No Growth
D-Glucose	Growth		Growth
D-Fructose	Growth		Growth
D-Mannose	No Growth		Growth
L-Sorbose	No Growth		Growth
L-Rhamnose	No Growth		No Growth
Dulcitol	No Growth		No Growth
D-Melezitose	No Growth		No Growth
D-Raffinose	No Growth		No Growth
Starch	Growth		No Growth
Glycogen	No Growth		Growth
Xylitol	No Growth		No Growth
Gentiobiose	No Growth		No Growth
D-Turanose	No Growth		No Growth
D-Lyxose	No Growth		

Table 2. Metabolite Production by *S. dextrinosolvens* ASCUSBF53 on Complex Media with (b) (4)

	(b) (4)								
Fermentation Time (hrs)	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**

(b) (4)

Conclusions:

In vitro assays demonstrate that *S. dextrinosolvens* ASCUSBF53 grows on a variety of monosaccharides, disaccharides, and sugar alcohols including (b) (4) When grown on (b) (4) S. dextrinosolvens ASCUSBF53 produces succinate, lactate, and acetate as major fermentation products.

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

References

Avgustin, G., R. J. Wallace, and H. J. Flint. 1997. “Phenotypic Diversity among Ruminal Isolates of Prevotella Ruminicola: Proposal of Prevotella Brevis Sp. Nov., Prevotella Bryantii Sp. Nov., and Prevotella Albensis Sp. Nov. and Redefinition of Prevotella Ruminicola.” *International Journal of Systematic Bacteriology*. https://doi.org/10.1099/00207713-47-2-284.

Objectives

The objective of this work was to determine the identity of *S. dextrinosolvens* ASCUSBF53 using genomic methods.

Methods

For 16S sequence analysis, the 16S gene was amplified from *S. dextrinosolvens* ASCUSBF53 the 27F/534R primers and sequenced using an (b) (4) (Stackebrandt and Goodfellow 1991; Muyzer, de Waal, and Uitterlinden 1993; LANE and J 1991). The resulting sequence was quality trimmed and compared to NCBI databases (excluding "uncultured" and environmental samples) to establish the identity of the strain. The NCBI databases were queried on January 6, 2021.

Genomic DNA was isolated from a pure culture of *S. dextrinosolvens* ASCUSBF53 by a modified Sambrook phenol-chloroform extraction/purification protocol (Jain et al. 2018). Short read sequencing libraries were prepared using the (b) (4)) by manufacturer's recommended protocol and the resulting libraries were sequenced (b) (4). In parallel, long read libraries were prepared from the same extracted DNA using the (b) (4) (b) (4)) using a modified version of the protocol outlined by (Jain et al. 2018) and (b) (4). Full details of the genome assembly can be found in appendix 003c. (b) (4) was used to generate the alignments for whole genome average nucleotide identity (ANI) (Kurtz et al., 2004).

Results

Table 1: 16S Matches with Standing Nomenclature to *S. dextrinosolvens* ASCUSBF53

Genus species (GenBank accession #)	Percent Match	Percent Coverage
Succinivibrio dextrinosolvens CA76 (AB849336)	99.8%	99%
Succinivibrio dextrinosolvens CG79 (AB849335)	99.8%	99%
Succinivibrio dextrinosolvens Z6 (CP047056)	99.2%	100%
Succinivibrio dextrinosolvens CA79 (AB849334)	100%	97%
Succinivibrio dextrinosolvens 0554 (NR_026476)	100%	97%

Whole genome average nucleotide identity (ANI) was used to confirm the 16S identification. Genomes for ANI comparison were selected based on 16S similarity to *S. dextrinosolvens* ASCUSBF53. As shown in Table 2, the *S. dextrinosolvens* ASCUSBF53 genome most closely matched *Succinivibrio dextrinosolvens* DSM 3072. The top 2 matches *S. dextrinosolvens* ASCUSBF53 meet the 95% identity cutoff for defining a species (Yoon et al. 2017; Goris et al. 2007; Richter and Rosselló-Móra 2009).

Table 2. Average Nucleotide Identity (ANI) by (b) (4)

Genus species (assembly)	ANI (%)	Coverage (%)
Succinivibrio dextrinosolvens DSM 3072 (GCA_900167015)	97.9	74.9
Succinivibrio dextrinosolvens H5 (GCA_000702045)	96.8	72.9
Succinivibrio dextrinosolvens ACV-10 (GCA_900116345)	88.3	43.0
Succinivibrio dextrinosolvens 22B (GCA_900114195)	87.9	35.0
Succinivibrio dextrinosolvens Z6 (GCA_011065405)	87.9	31.7
Anaerobiospirillum succiniciproducens DSM 6400 (GCA_000482845)	82.4	0.64
Anaerobiospirillum thomasii NCTC13093 (GCA_900445225)	82.3	0.62
Succinatimonas hippei YIT12066 (GCA_000188195)	82	0.22

Conclusions

Whole genome ANI and 16s comparisons suggest that *S. dextrinosolvens* ASCUSBF53 represents a strain of *S. dextrinosolvens*. The genomic data in this Appendix should be used along with the phenotypic data from Appendix 002 to confirm this assessment.

Documentation

The full list of 16S hits and alignments can be found on the Native Microbials drive under:

(b) (4)

The details of the ANI analysis can be found on the Ascus drive under:

(b) (4)

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

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BLASTN 2.11.0+
Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

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Query=
Length=490

Sequences producing significant alignments:	Score (Bits)	E Value	Max Ident
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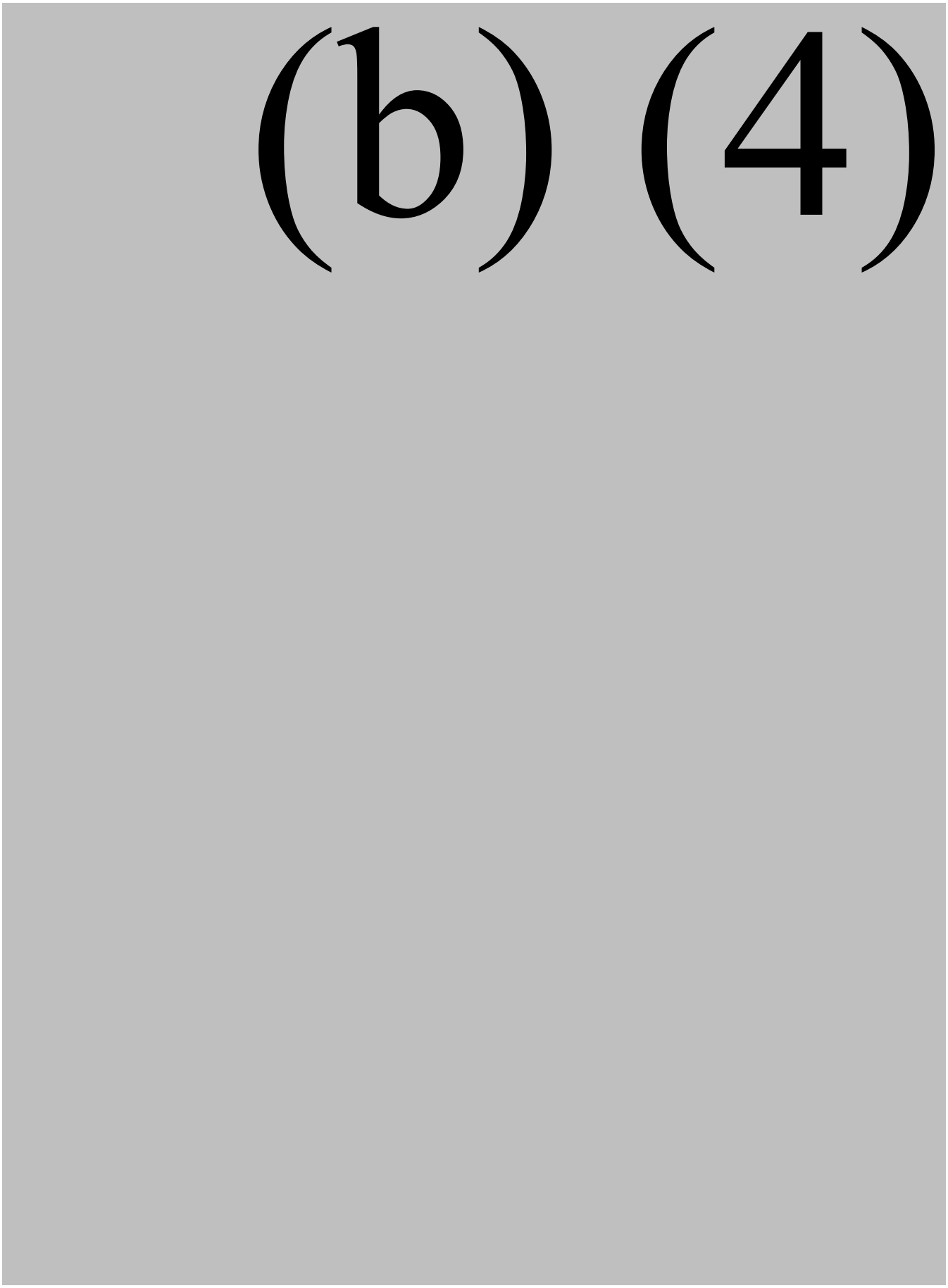
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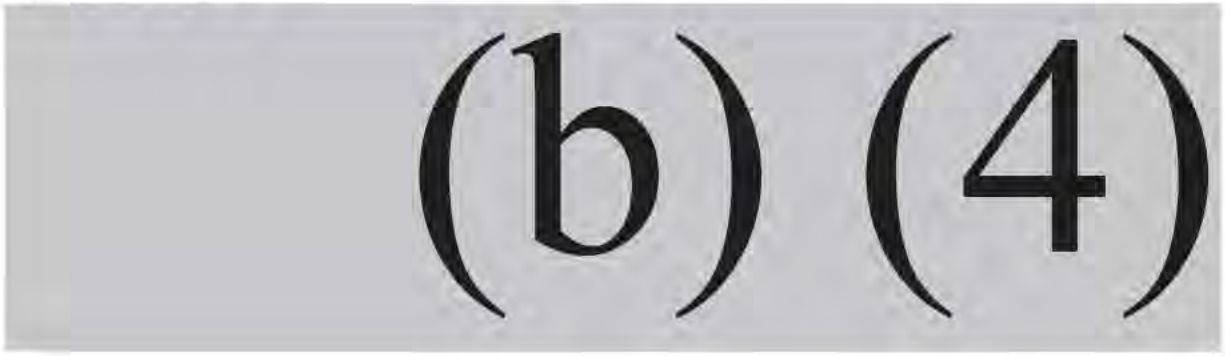
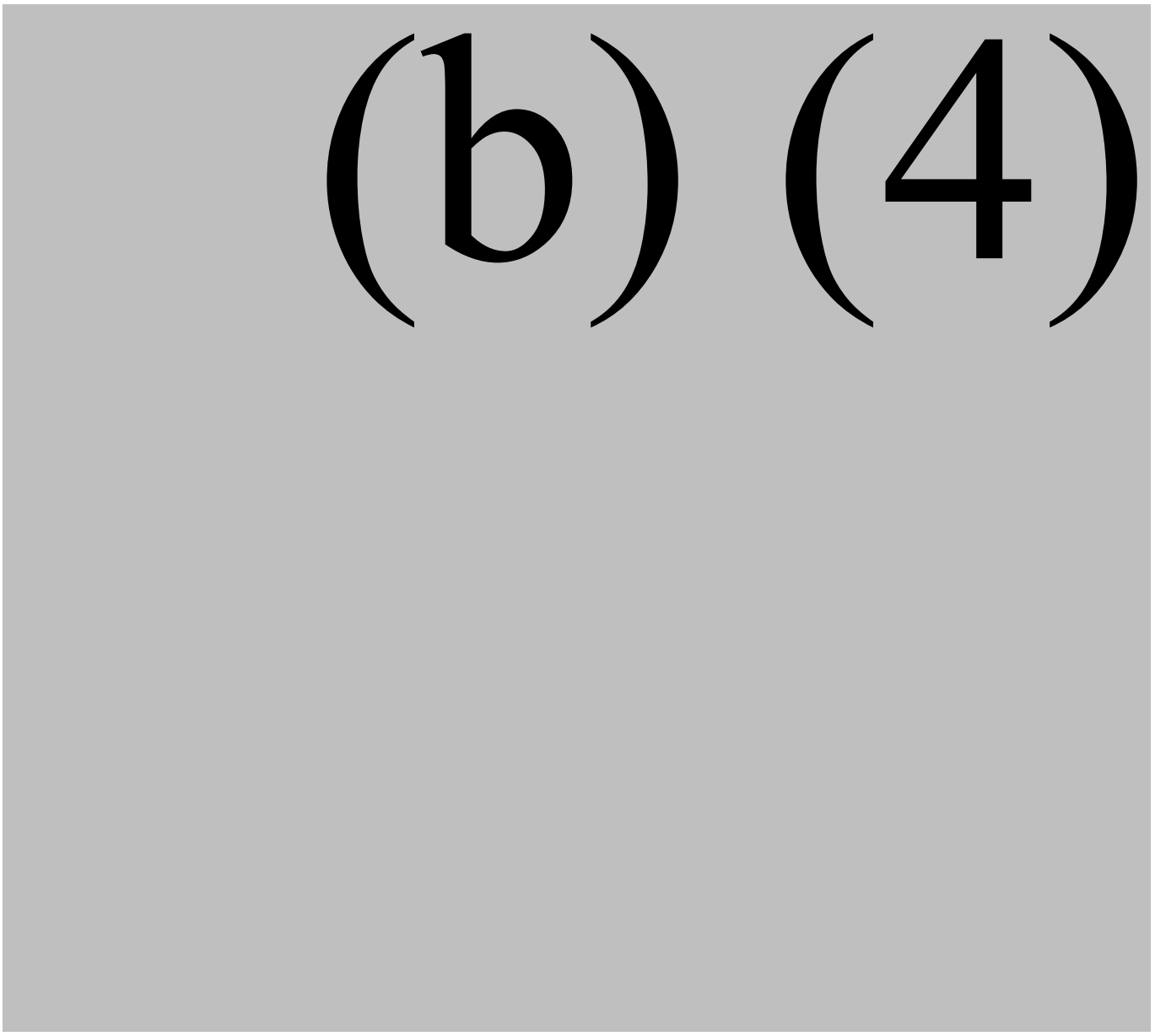
Appendix 003C: Supplementary Whole Genome Analysis Methods and Read Quality Metrics for *Succinivibrio dextrinosolvens* ASCUSBF53

The *S. dextrinosolvens* ASCUSBF53 genomic DNA was extracted and sequenced as described in the main text of the dossier. This appendix contains details about the assembly methods used, the protocol for (b) (4) library preparation, (b) (4) quality metrics for the (b) (4) reads respectively, metrics generated by (b) (4) for the completed assembly, and a visualization of the assembly graph generated by (b) (4)

Assembly Pipeline in Detail

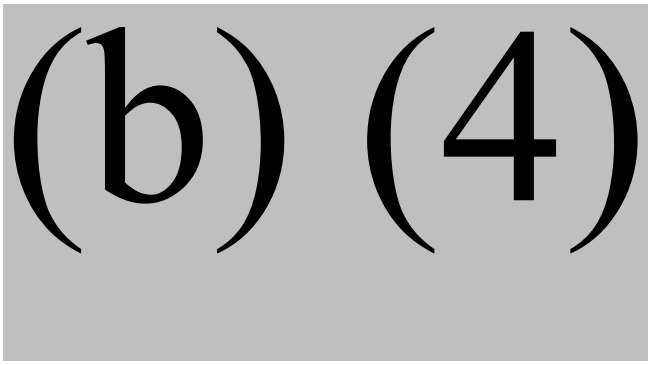


(b) (4) **Protocol as Provided by the Manufacturer**

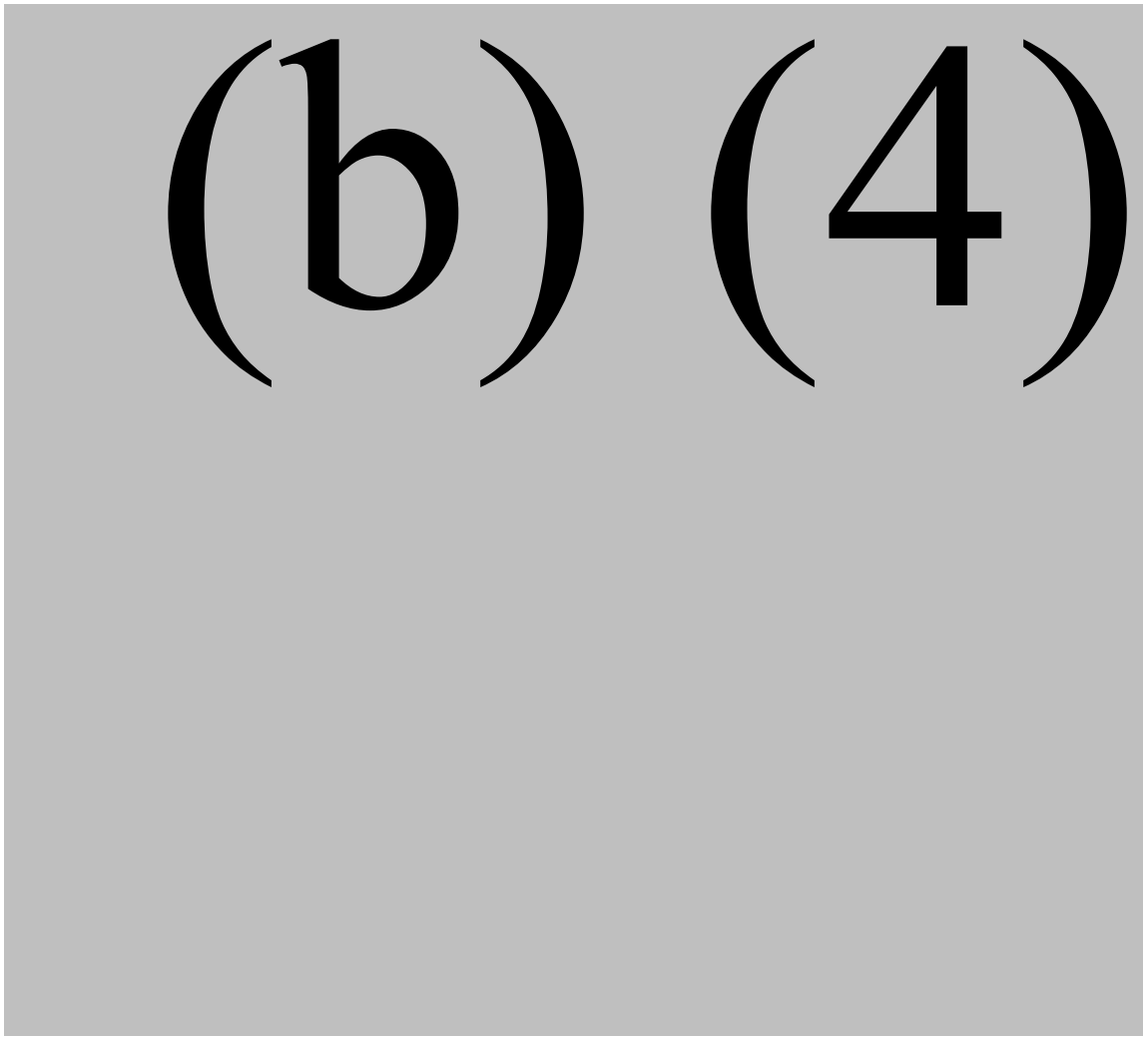


Full Protocol (b) (4)

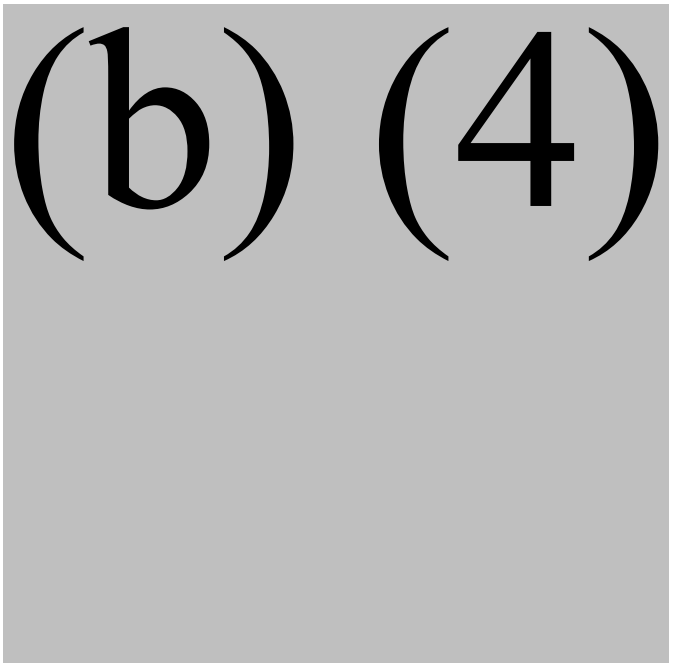
Quality Metrics of (b) (4) **Reads as Generated by** (b) (4)



Read distribution as related to quality score

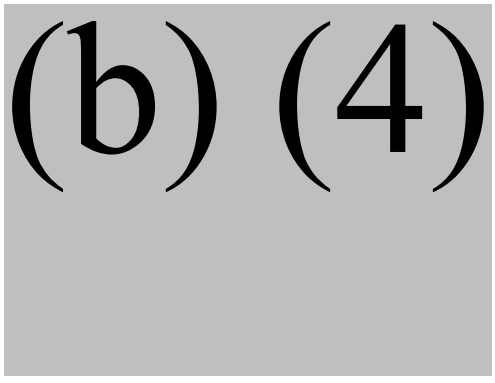


Metrics for *S. dextrinosolvens* ASCUSBF53 Oxford Nanopore reads as generated by NanoStat

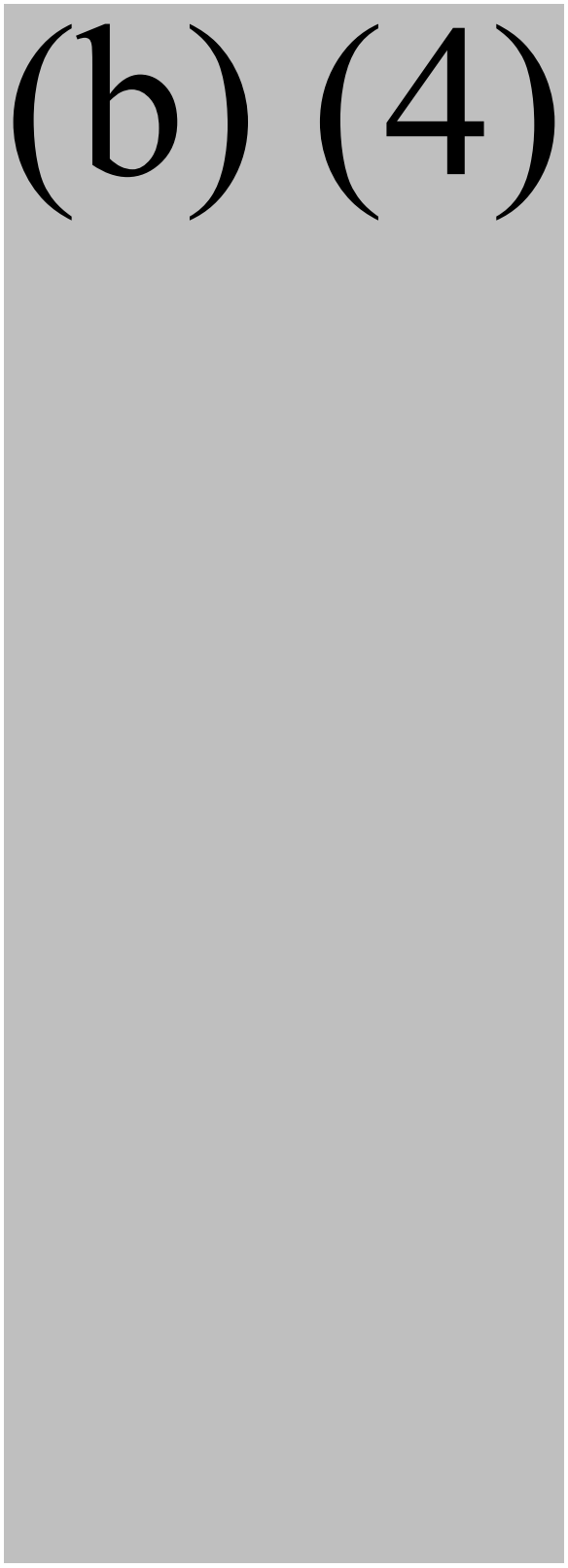


Number, Percentage, and Megabases of Reads Above Quality Cutoffs

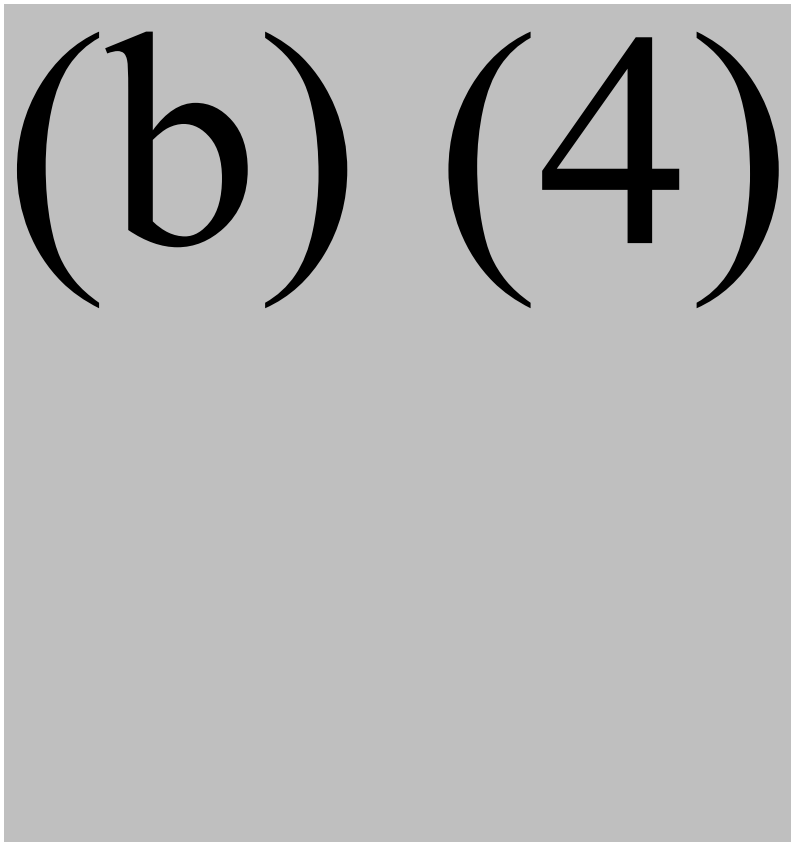
Longest Reads in Base Pairs (bp)



Assembly Statistics as reported by (b) (4)



Assembly Graph as Visualized by (b) (4)



References

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(b) (4)

Characterization of Native Microbials *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53) Production Strain: Antibiotic Susceptibility Profile

Approvers:

(b)(6)

12/28/2020

Date

12/28/2020

Date

DocuSigned by:
Kevin Korth
869AE3638AA543B...

12/28/2020

Kevin Korth
Regulatory

Date

Table of Contents

Title: Characterization of Native Microbials *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53) Production Strain: Antibiotic Susceptibility Profile

1 OBJECTIVE

To determine the Susceptibility Profile of *Succinivibrio dextrinosolvens* (Beef-53) production strain to European Food Safety Authority recommended antimicrobials.

2 STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI documents VET01 and M11 to the extent to which it is applicable as detailed in the protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) may be referenced for determining non-wildtype minimal inhibitory concentration (MIC) values. Procedures for the susceptibility were designed to follow those in European Food Safety Authority (EFSA) *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA Panel on Additives and Products or Substances used in Animal Feed [FEEDAP] Rychen et al., 2018) as applicable and as detailed in the protocol.

3 STUDY SITE

Antimicrobial susceptibility testing was performed at Native Microbials Inc.

4 MATERIALS AND METHODS

4.1 Isolate

A production strain of *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53) was procured from the 20Sep20 Commerical Working Cell Bank. The culture was streaked onto both Brucella agar and Mueller Hinton agar to verify that the organism is viable, pure, and morphologically typical of the purported species and to verify growth on the selected media.

4.2 Susceptibility Profile

4.2.1 Procedure

The procedures listed in the protocol “Agar-Dilution Susceptibility Testing of Anaerobes” (Appendix A) were written to comply with CLSI document VET01 entitled *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals* and CLSI Document M11 entitled *Methods of Antimicrobial Susceptibility Testing of Anaerobic Bacteria*.

4.3 Media

MIC agar plates for use in an agar dilution method were prepared by Native Microbials with antimicrobials and doubling dilution concentrations. The media for MIC testing was Brucella Broth. Stock solution concentrations and media recipes are captured in Appendix B.

4.4 Incubation and Interpretation of Susceptibility Tests

MIC agar plates were incubated and interpreted according to Native Microbials internal protocol "Agar-Dilution Susceptibility Testing of Anaerobes" (Appendix A).

(b) (4)

4.5 Quality Control

Reference Strain *Escherichia coli* (ATCC 25922) was tested on each agar dilution plate to ensure proper quality control (QC) of the MIC tests. Available CLSI (CLSI, 2020) and EUCAST ("Routine and Extended Internal Quality Control for MIC Determination and Disk Diffusion as Recommended by EUCAST, Version 10.0"; EUCAST, 2020) acceptable QC ranges for each antimicrobial were referenced (Table 3).

With each test, all growth was verified to be of one morphology and of the correct colony morphological features as considered typical of the strain.

Table 1. EFSA Gram Positive Breakpoints, EUCAST Gram-Positive Anaerobic Breakpoints and CLSI Anaerobes Breakpoints.

	EFSA Gram-Negative		EUCAST Gram-Negative Anaerobes		CLSI Anaerobes		
Antibiotic	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**I**	**R >**
Ampicillin	1	1	0.5	2	0.5	1	2
Vancomycin	Not Required		-	-			
Gentamicin	2	2	-	-			
Kanamycin	8	8					
Streptomycin	16	16					
Erythromycin	Not Required		-	-			
Clindamycin	Not Required		4	4	2	4	8
Tetracycline	8	8			4	8	16
Chloramphenicol	Not Required		8	8	8	16	32

5 DISPOSITIONS

All agar dilution plates were discarded after their expiration. The isolate and all subcultures were discarded after autoclaving. No retention cultures were created or maintained from this study.

6 RESULTS

MIC results of the *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53) isolate and breakpoints interpretations are presented in Table 2. Photographs of agar dilution plates are shown in Appendix C. The isolate would be considered wild-type or susceptible according to all three criteria (EFSA, EUCAST, and CLSI) to Ampicillin. The isolate would be considered susceptible to Tetracycline according to EFSA and CLSI breakpoints. The isolate would be considered sensitive to Chloramphenicol according to EUCAST and CLSI. The isolate would be considered non-wildtype or non-susceptible, against Gentamicin, Kanamycin and Streptomycin according to EFSA. The isolate is considered non-susceptible to Clindamycin per EUCAST and CLSI.

However, consideration must be given that some classifications set forth by EFSA are for typical Gram-Negative organisms and are not applicable to *Succinivibrio dextrinosolvens* due to its anaerobic nature. EUCAST provides a breakpoint of "-" for Gentamicin and Erythromycin (Table 1) indicating that the species is a poor target for therapy with these antibiotics. CLSI refrains from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. It is well documented that aminoglycosides are hindered by anaerobic growth. Active electron transport is required for aminoglycoside uptake into cells, so the class inherently lacks activity against anaerobic bacteria (Kislak, 1973; Martin, Gardner, and Washington, 1972; Ramirez and Tolmasky, 2010). Susceptibility to aminoglycosides and macrolides decreases significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al., 2016). EFSA, EUCAST and CSI refrain from providing breakpoints for gram-negative organisms to Vancomycin. Gram-negative organisms' outer membranes are impermeable to large glycopeptide molecules, conferring an intrinsic resistance to the entire class of glycopeptide antibiotics, including Vancomycin (Antonoplis et al., 2019).

Table 2. Minimal Inhibitory Concentrations for *Succinivibrio dextrinosolvens* ASCUSBF53 and Sensitivity Interpretation

Antibiotic	Range Tested (ug/mL)	Succinivibrio dextrinosolvens	Interpretation		
			EFSA	EUCAST	CLSI
Ampicillin	(b) (4)	< 0.5	S	S	S
Vancomycin		> 32			
Gentamicin		16	R	-	
Kanamycin		16	R		
Streptomycin		32	R		
Erythromycin		16		-	
Clindamycin		> 32		R	R
Tetracycline		0.25	S		S
Chloramphenicol		< 0.5		S	S

MIC results of the QC strain ATCC 25922 *Escherichia coli* are presented in Table 3. ATCC 25922 performed within the expected range for Ampicillin, Tetracycline and Chloramphenicol. When compared to QC ranges for the aminoglycosides, Gentamicin, Kanamycin and Streptomycin, it appears to be out of specification. However, ATCC 25922 is a facultative

anaerobe and in this testing, was grown in an anaerobic environment. The QC range provided by CLSI and EUCAST are for aerobic growth of ATCC 25922. For the reasoning provided above, these results are to be expected and are not indicative of a failure in the agar dilution plates.

The MIC results for the quality control organism is within the expected values, knowing that aminoglycosides (gentamicin, kanamycin and streptomycin) and macrolides (erythromycin) have reduced efficacy in anaerobic conditions.

Table 3. Minimal Inhibitory Concentrations for QC Strain ATCC 25922

Antibiotic	ATCC 25922	CLSI QC Ranges (µg/mL)	EUCAST QC Range (µg/mL)
Ampicillin	(b) (4)	2-8	2-8
Vancomycin			
Gentamycin		0.25 - 1	0.25 - 1
Kanamycin		1 -4	
Streptomycin			
Erythromycin			
Clindamycin			
Tetracycline		0.5 - 2	
Chloramphenicol		2-8	2-8

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Appendix A. Agar-Dilution Susceptibility Testing of Anaerobes

1 General Considerations

1.1 The procedures described herein are designed to follow those described in Clinical & Laboratory Standards Institute (CLSI) document M11: Anaerobic Bacteria Antimicrobial Susceptibility.

1.2 Agar-dilution method is considered the standard method of antimicrobial susceptibility testing of anaerobic bacteria by CLSI.

1.2.1 Anaerobic organisms commonly require complex nutritional formulations for growth. Organisms to be assayed using this method need to be tested for growth on Mueller-Hinton Agar or Supplemented Brucella Agar. Supplements should not be used unless necessary for the growth of the organism. The use of other media is not recommended due to potential interference between antibiotics and media components (e.g. p-aminobenzoic acid, thymidine, glycine, divalent cations).

1.3 Unless otherwise noted, perform all work in an anaerobic chamber using degassed supplies.

1.4 Organisms will be grown on pre-reduced agar as appropriate for the particular strain (Reinforced Clostridial Agar, Tryptic Soy Agar, etc.). Organisms that are more aerotolerant may be grown on non-reduced agar.

1.4.1 To reduce media for testing, place agar plates or liquid media into an anaerobic chamber overnight. A reducing agent may be added to liquid media to expedite oxygen removal. An anaerobic indicating dye may be used in both agar or liquid media to provide a visual cue for reduced media.

2 Media Preparation

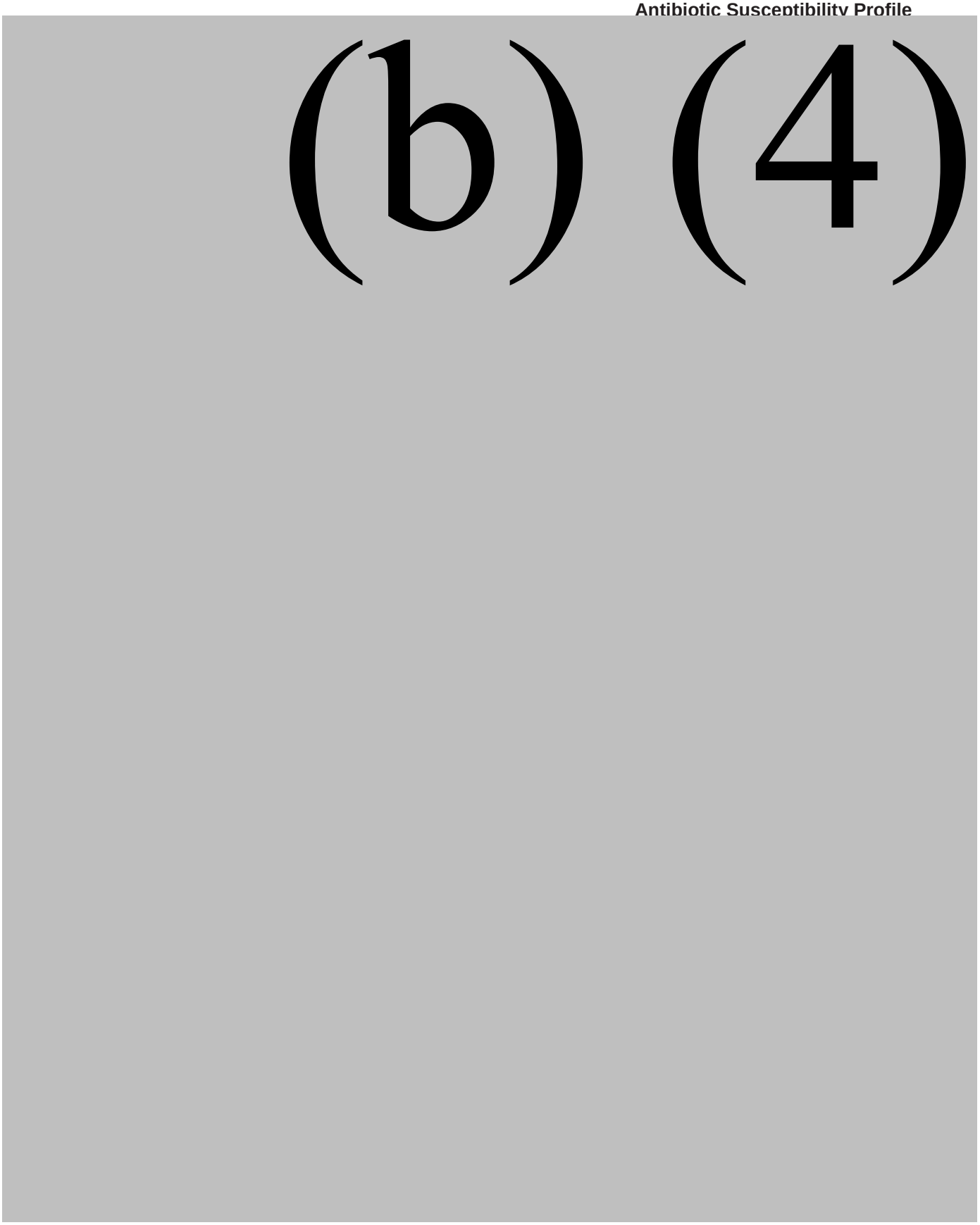
(b) (4)

(b) (4)

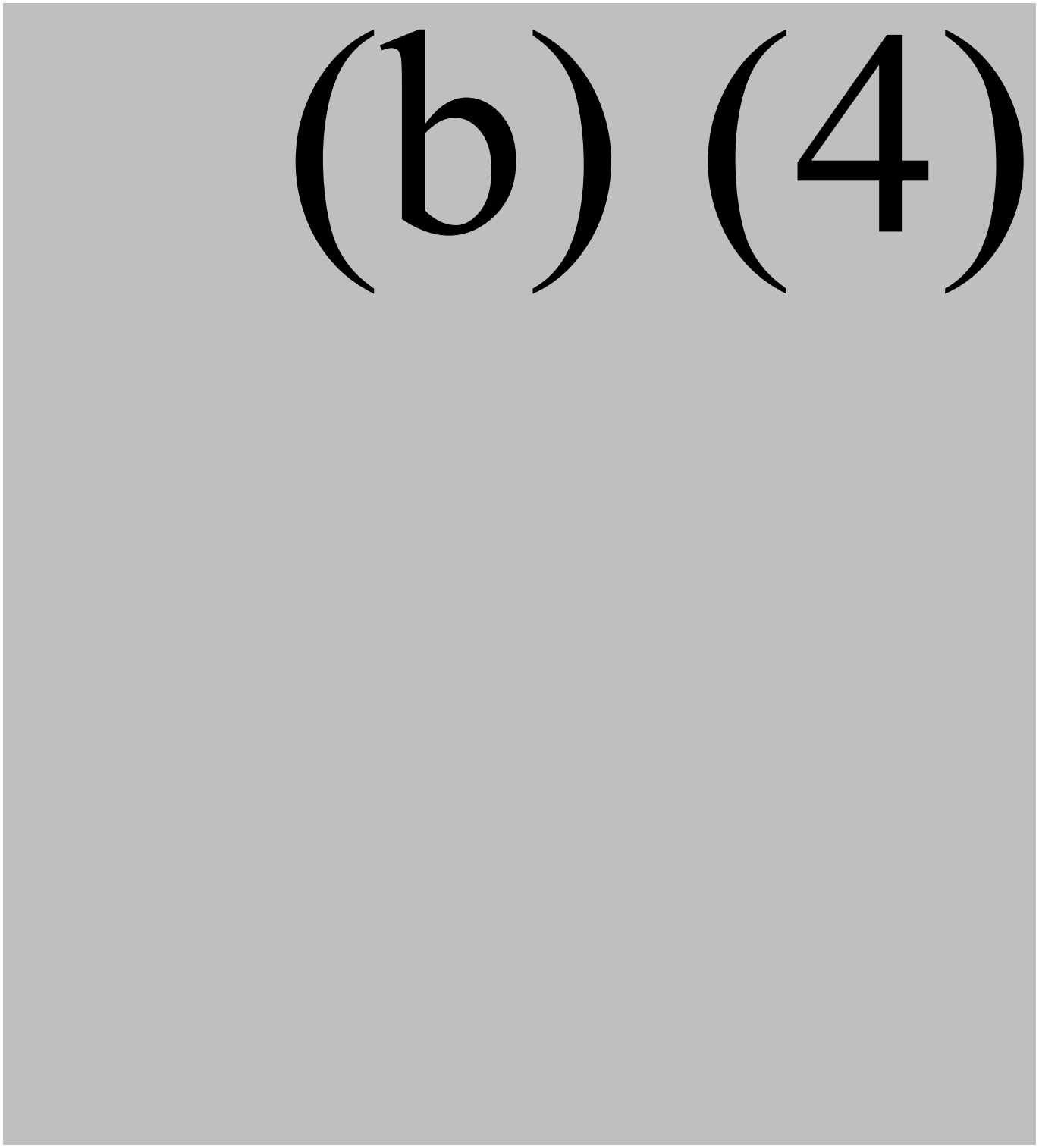
Table 1. Preparation of Dilutions of antimicrobial agents for use in agar dilution susceptibility tests.

Antimicrobial concentration (µg/mL) in stock	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (µg/mL)	Final Concentration in Agar (µg/mL)
(b) (4)				–
				–
				–
				–
				512
				256
				128
				64
				32
				16
				8
				4
				2
				1
				0.5
				0.25
				0.125
				0.06
				0.03
				0.015
				0.008
				0.004

(b) (4)


(b) (4)

Appendix B. Raw Data



Antibiotic Sources

(b) (4)

Strain Sources

(b) (4)

Antibiotic Concentration Calculations

(b) (4)

(b) (4)

Streptomycin

(b) (4)

Chloramphenicol

(b) (4)

Tetracycline

(b) (4)

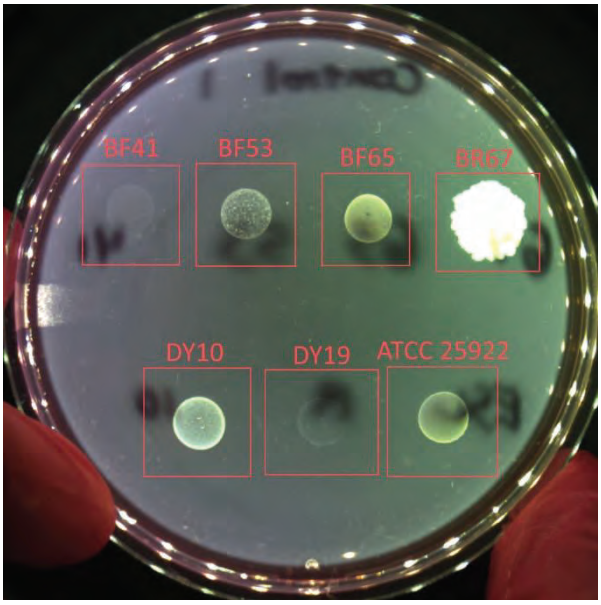
Appendix C. Agar Dilution Data and Photos

Table C-1. Agar Dilution Antibiotic Results and Susceptibility Photos: Ampicillin

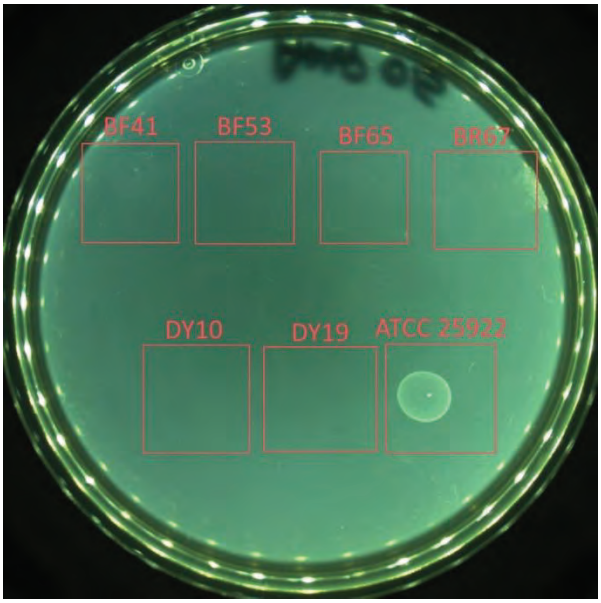
	Ampicillin Concentration (µg/mL)									
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	NG	NG	NG	NG	NG	NG	NG
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	NG	NG	NG	NG	NG	NG

G = Growth
NG = No Growth

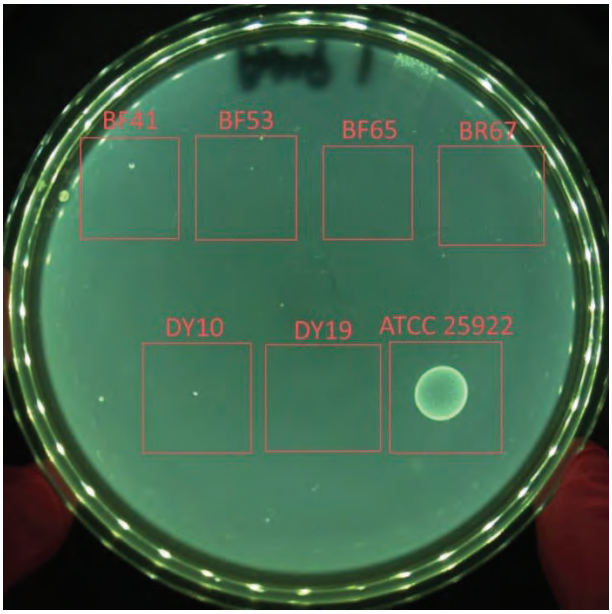
Agar Dilution Antibiotic Susceptibility Photos: Ampicillin



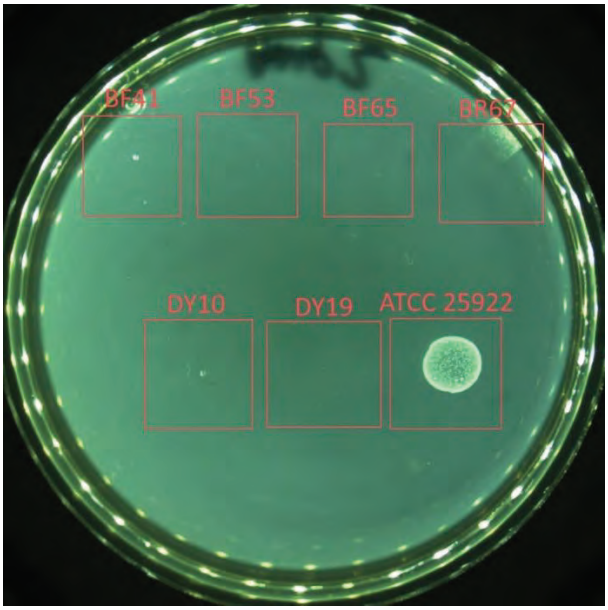
0 µg/mL Ampicillin



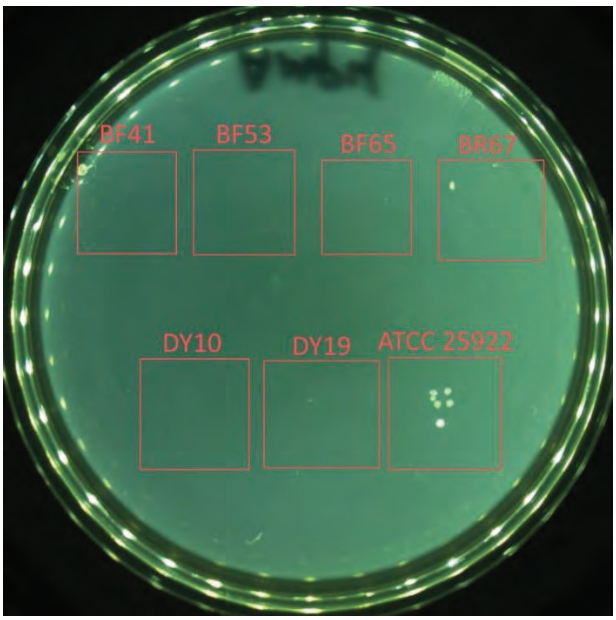
0.5 µg/mL Ampicillin



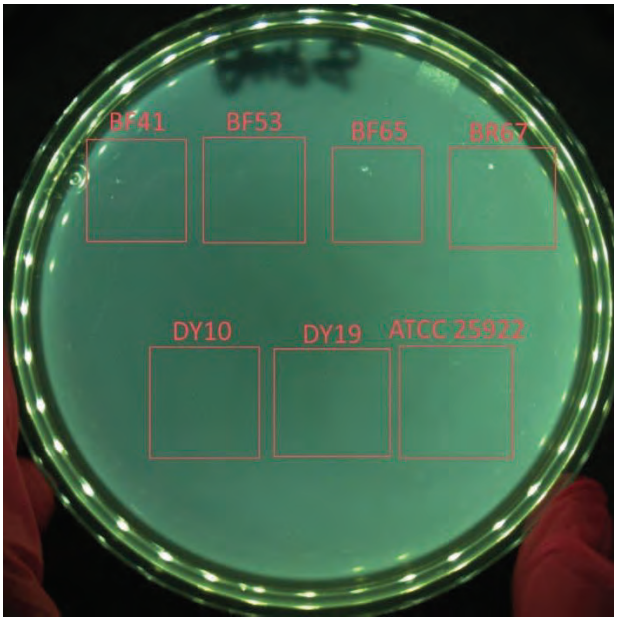
1 µg/mL Ampicillin



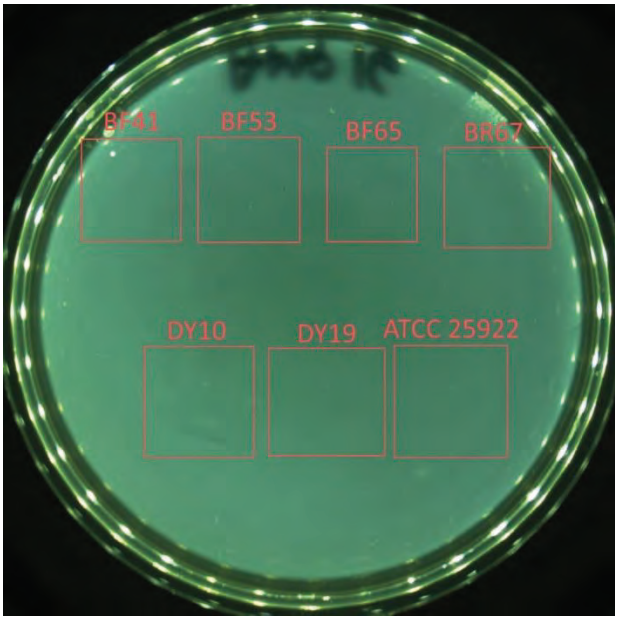
2 µg/mL Ampicillin



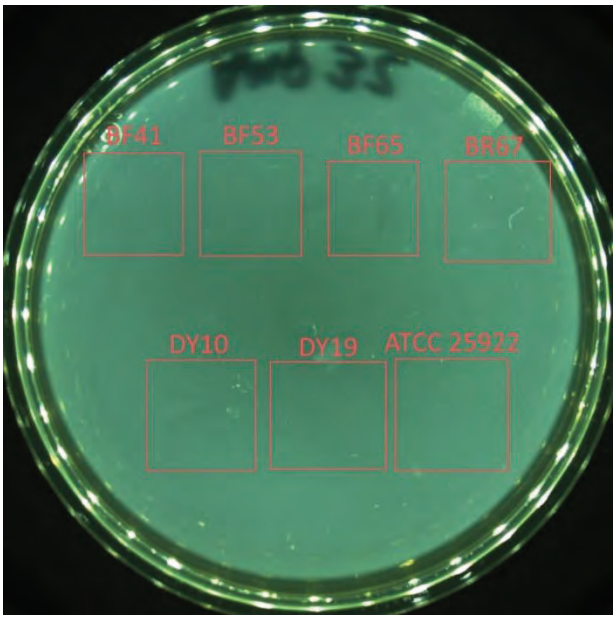
4 µg/mL Ampicillin



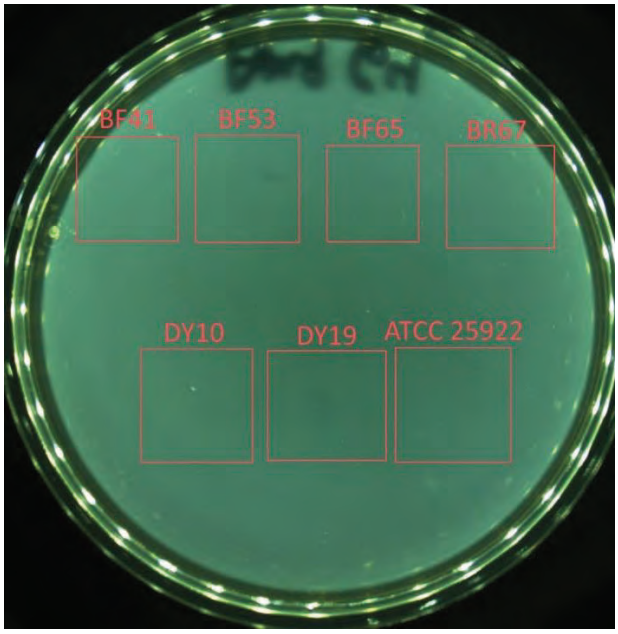
8 µg/mL Ampicillin



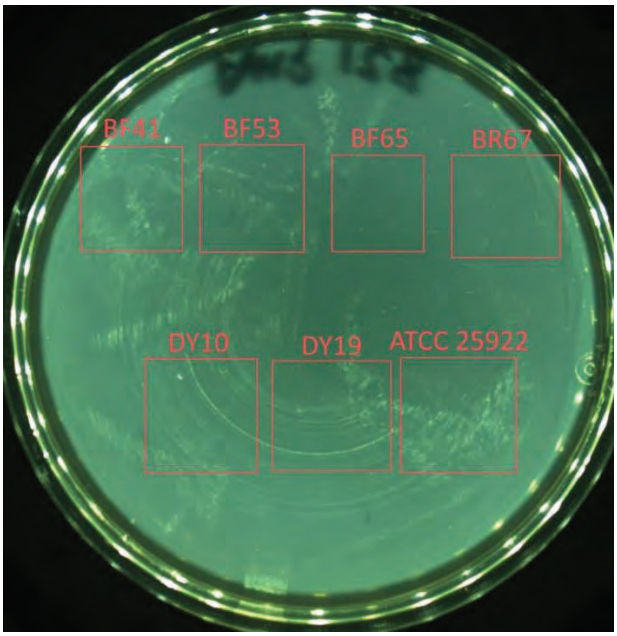
16 µg/mL Ampicillin



32 µg/mL Ampicillin



64 µg/ml Ampicillin



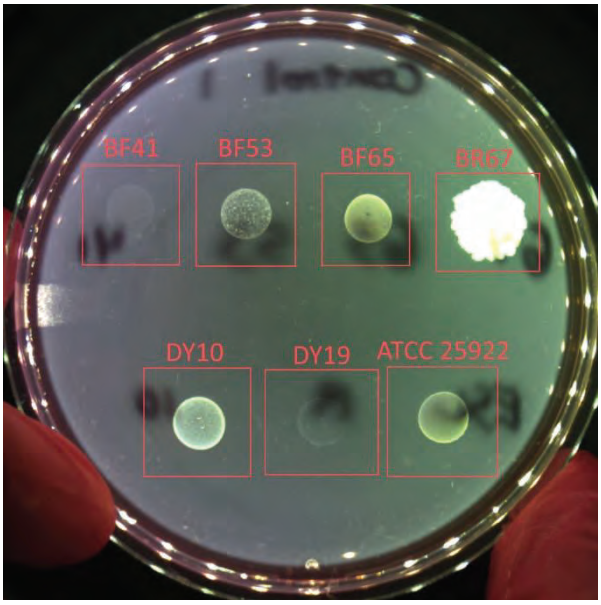
128 µg/ml Ampicillin

Table C-2. Agar Dilution Antibiotic Results and Susceptibility Photos: Chloramphenicol

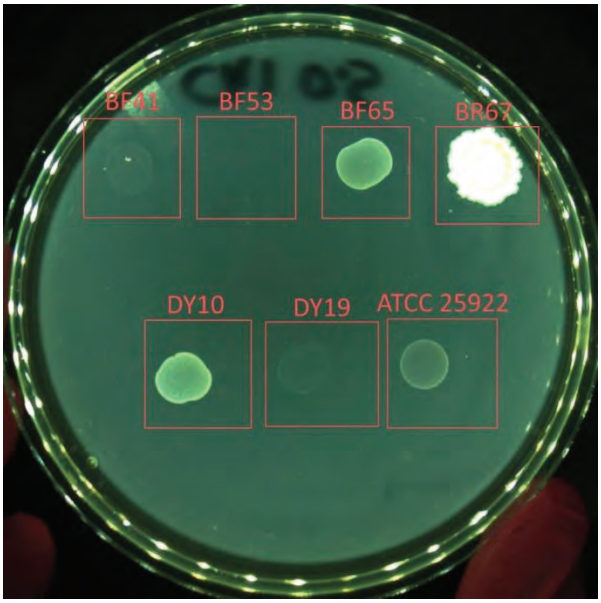
	Chloramphenicol Concentration (µg/mL)								
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	NG	NG	NG	NG	NG	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	NG	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	NG	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	NG	NG	NG	NG

G = Growth
NG = No Growth

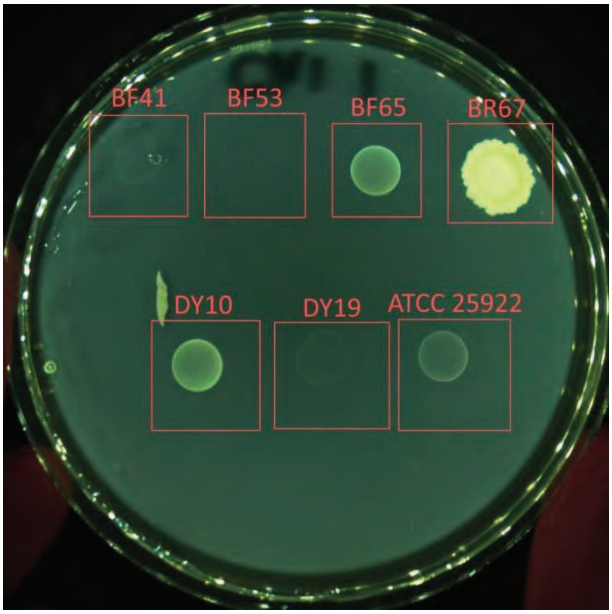
Agar Dilution Antibiotic Susceptibility Photos: Chloramphenicol



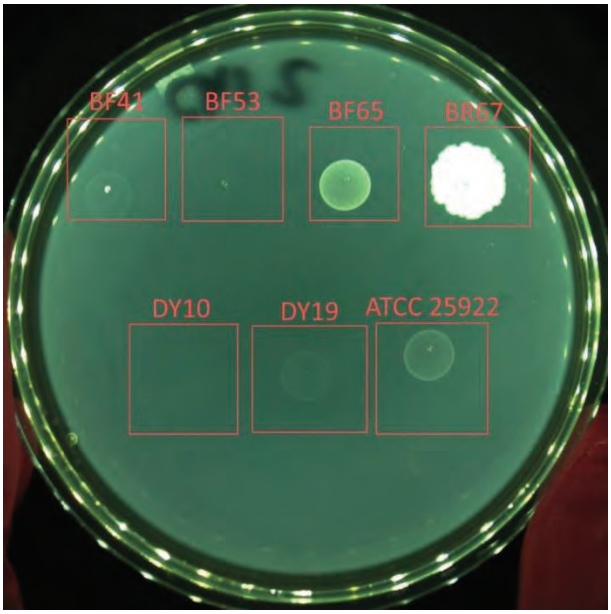
0 µg/mL Chloramphenicol



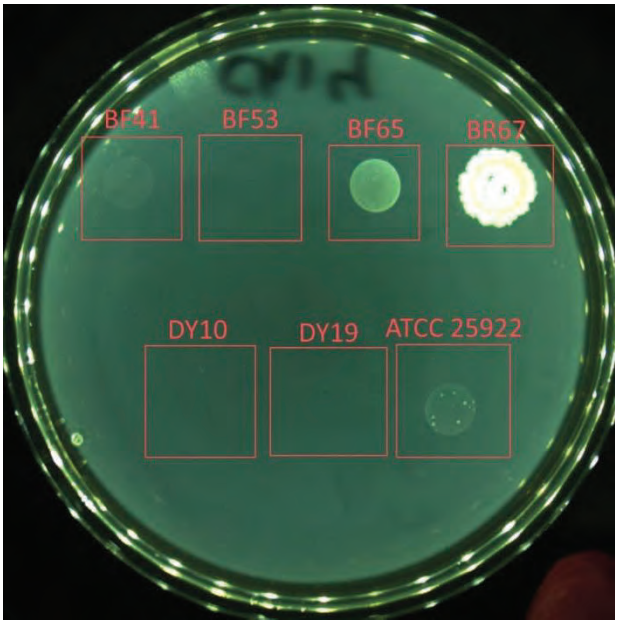
0.5 µg/mL Chloramphenicol



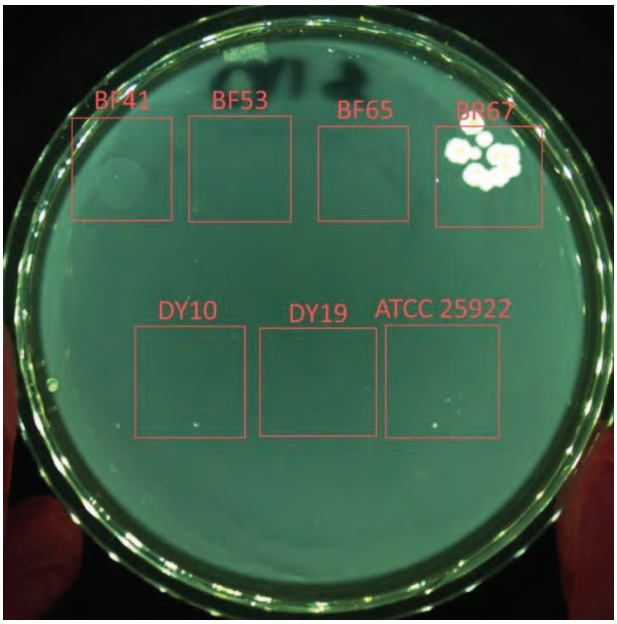
1 µg/mL Chloramphenicol



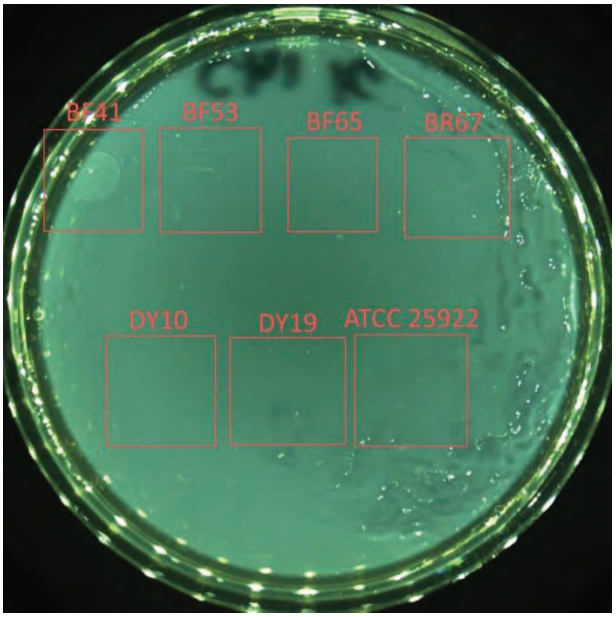
2 µg/mL Chloramphenicol



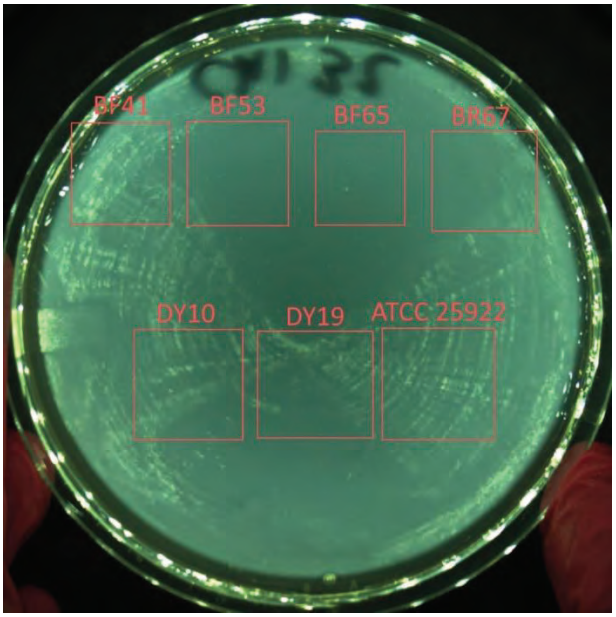
4 µg/mL Chloramphenicol



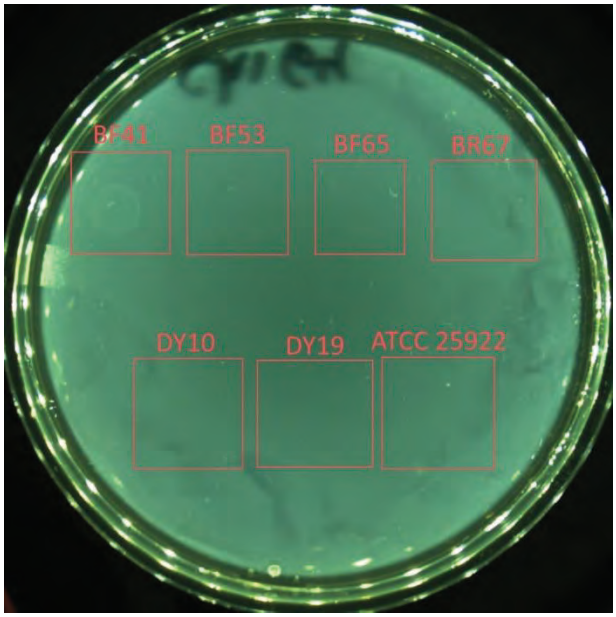
8 µg/mL Chloramphenicol



16 µg/mL Chloramphenicol



32 µg/mL Chloramphenicol



64 µg/ml Chloramphenicol

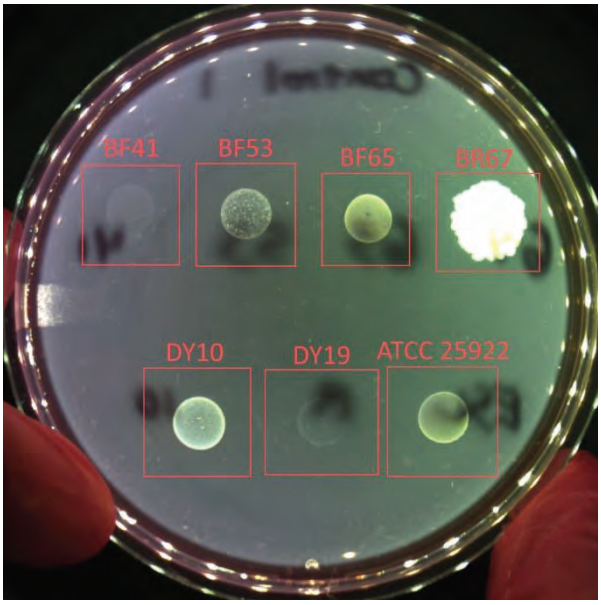
Table C-3. Agar Dilution Antibiotic Results and Susceptibility Photos: Clindamycin

	Clindamycin Concentration (µg/mL)											
Organism	**0 (Control)**	**0.03125**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	G	G	G	G
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	NG	NG	NG	NG	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	G	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	G	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G	G	G	G

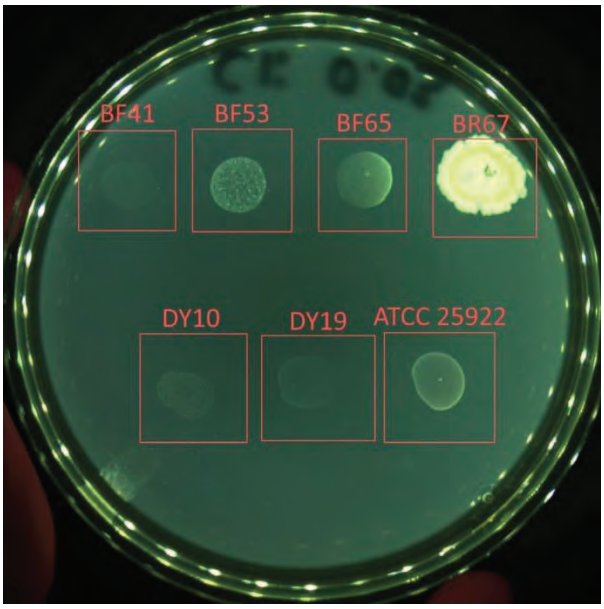
G = Growth

NG = No Growth

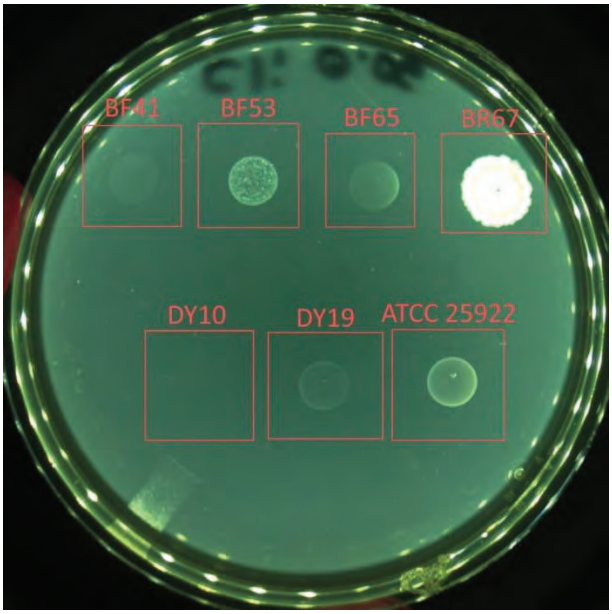
Agar Dilution Antibiotic Susceptibility Photos: Clindamycin



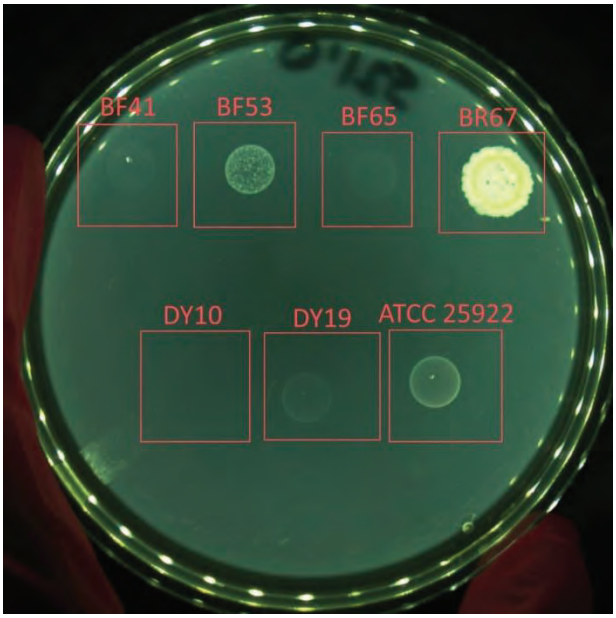
0 µg/mL Clindamycin



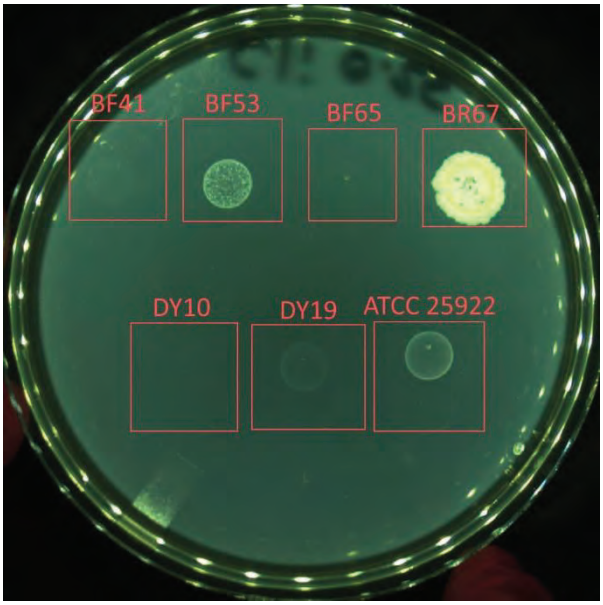
0.03125 µg/mL Clindamycin



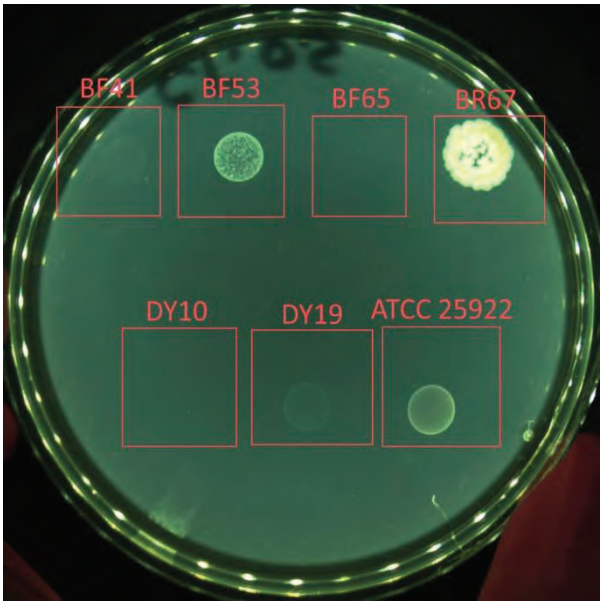
0.0625 µg/mL Clindamycin



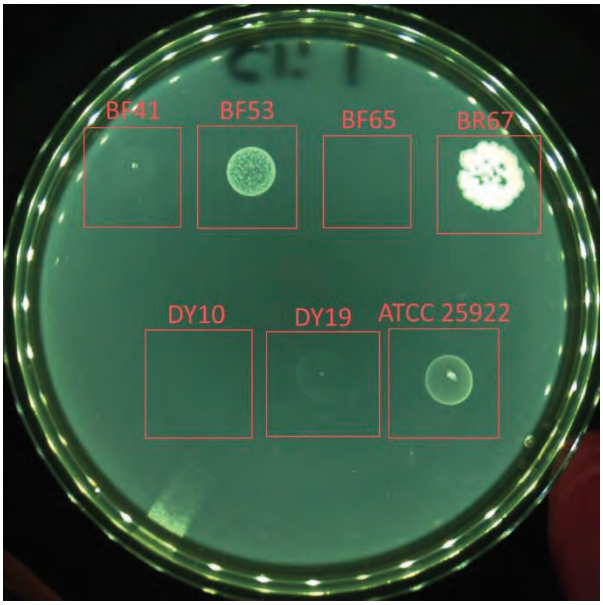
0.125 µg/mL Clindamycin



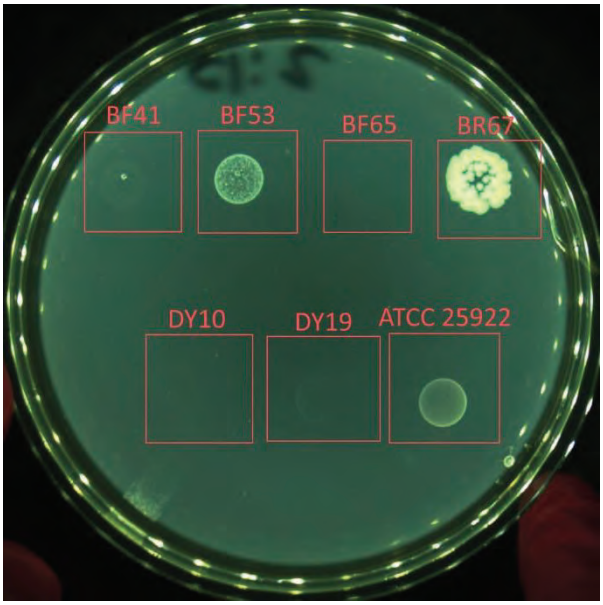
0.25 µg/mL Clindamycin



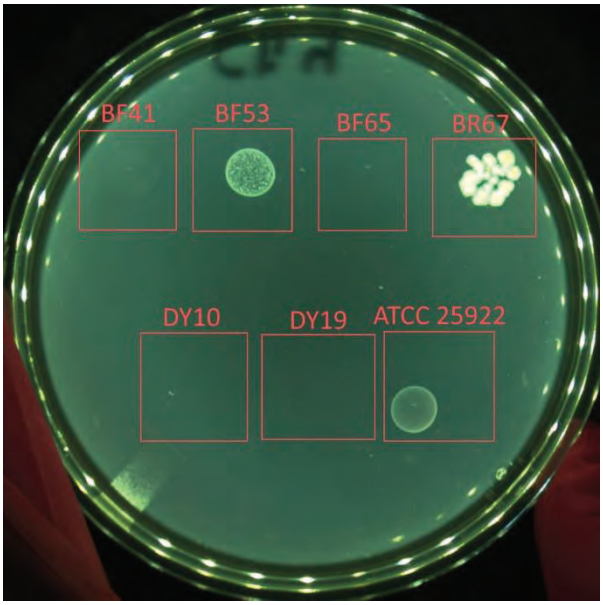
0.5 µg/mL Clindamycin



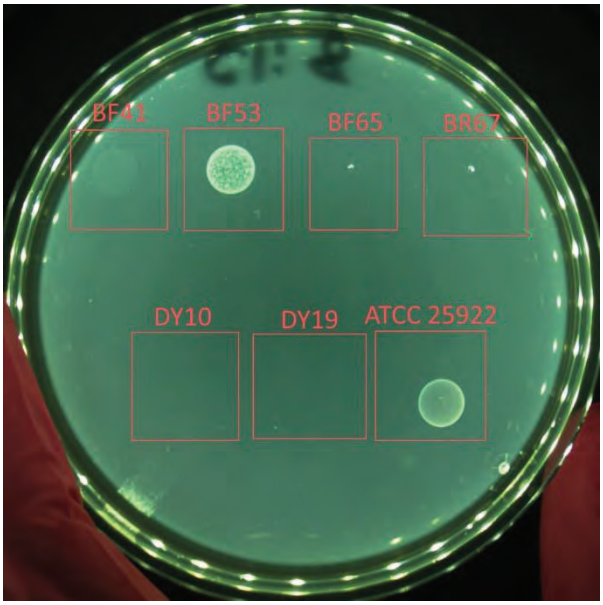
1 µg/mL Clindamycin



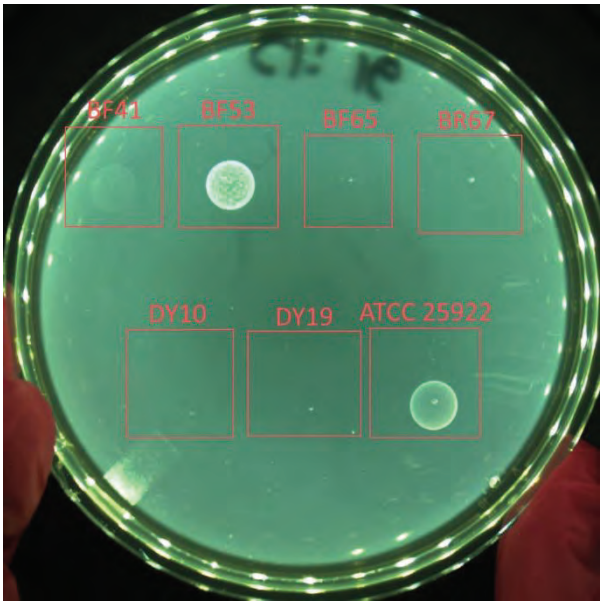
2 µg/mL Clindamycin



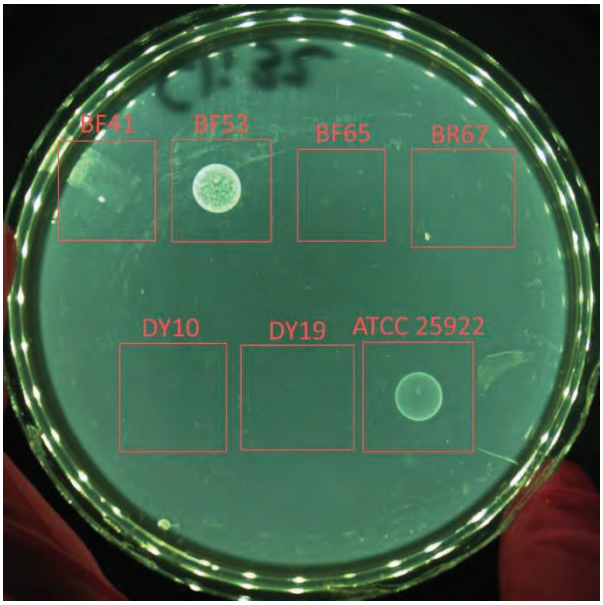
4 µg/mL Clindamycin



8 µg/mL Clindamycin



16 µg/mL Clindamycin



32 µg/mL Clindamycin

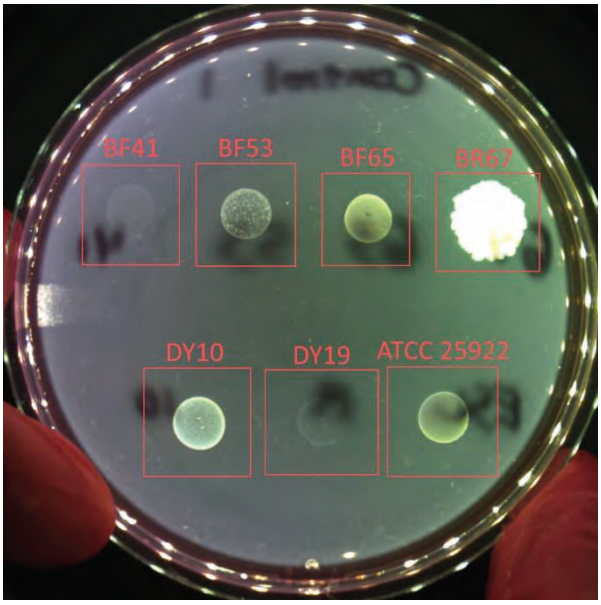
Table C-4. Agar Dilution Antibiotic Results and Susceptibility Photos: Erythromycin

	Erythromycin Concentration (µg/mL)								
Organism	**0 (Control)**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	G
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G

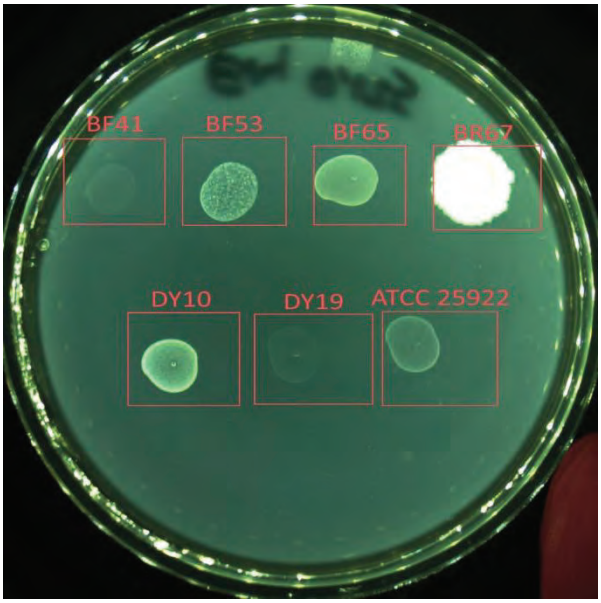
G = Growth

NG = No Growth

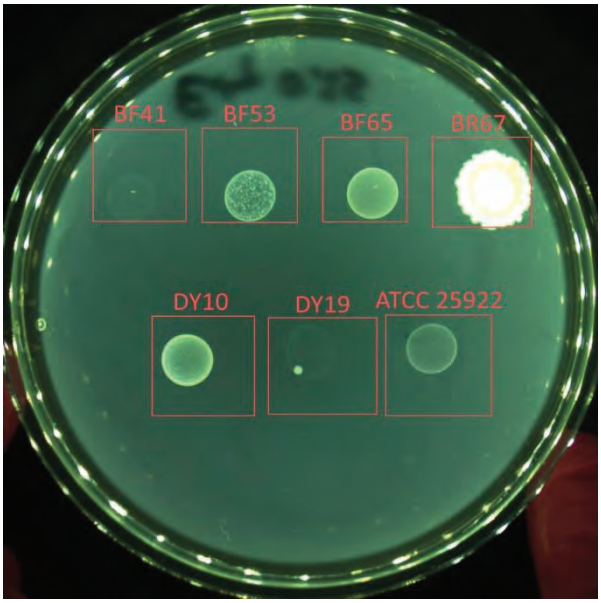
Agar Dilution Antibiotic Susceptibility Photos: Erythromycin



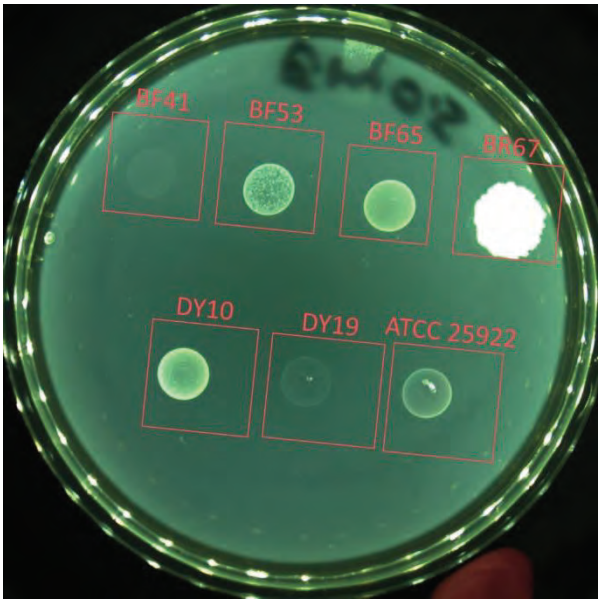
0 µg/mL Erythromycin



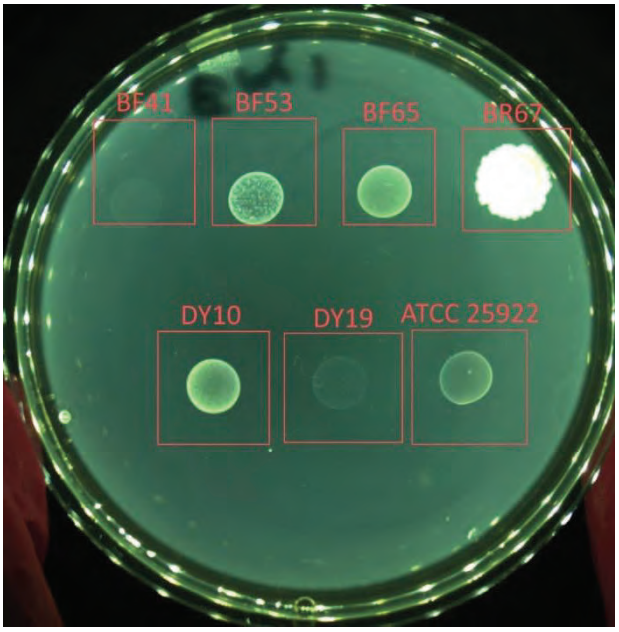
0.125 µg/mL Erythromycin



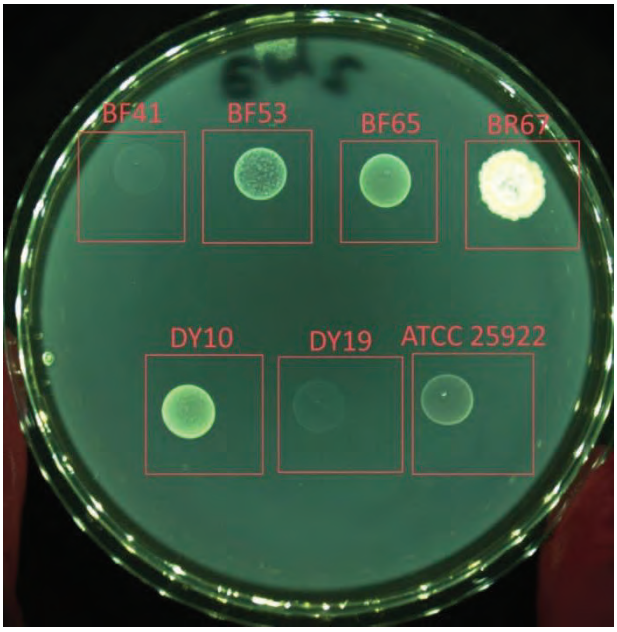
0.25 µg/mL Erythromycin



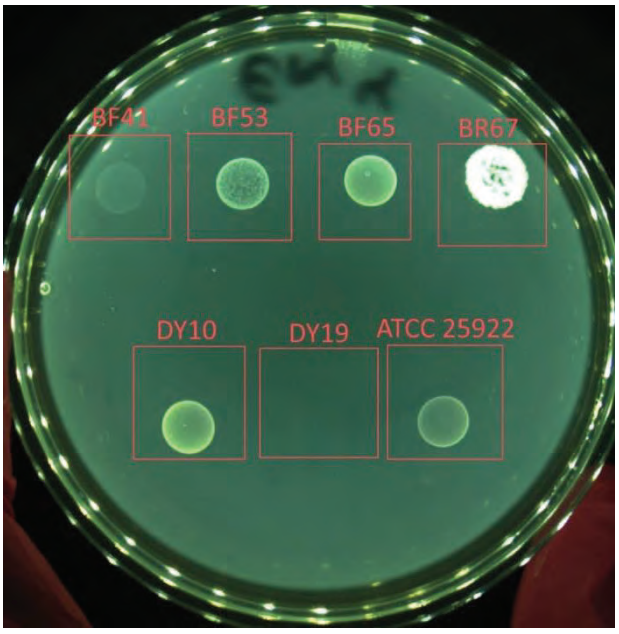
0.5 µg/mL Erythromycin



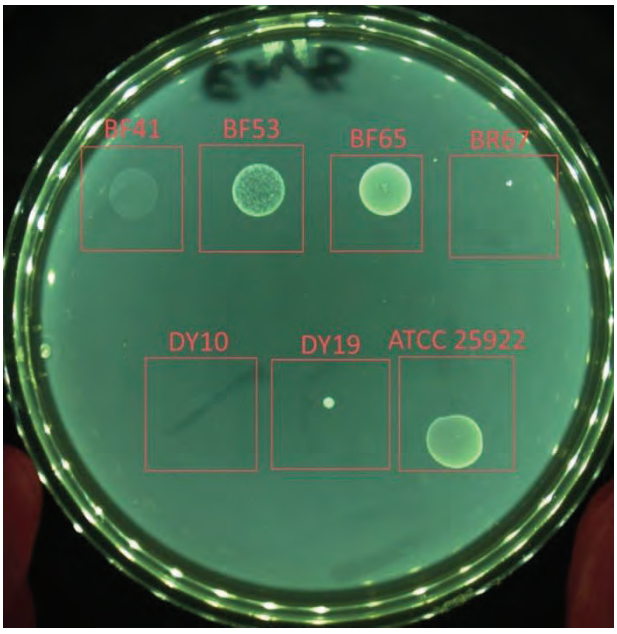
1 µg/mL Erythromycin



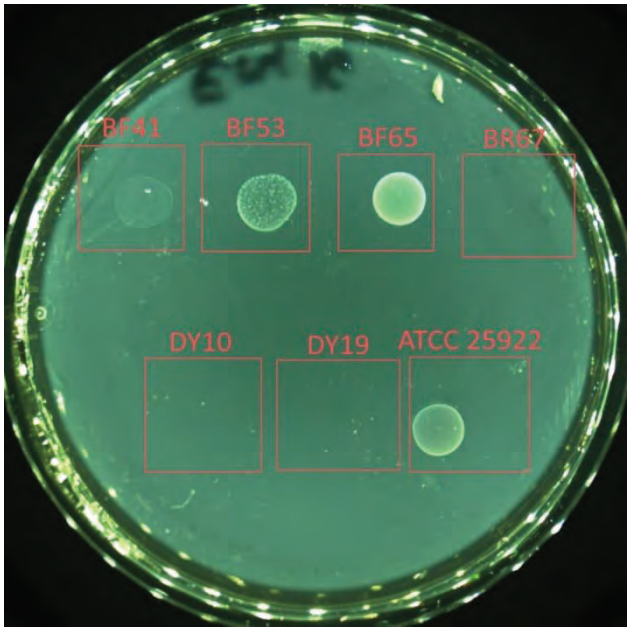
2 µg/mL Erythromycin



4 µg/mL Erythromycin



8 µg/mL Erythromycin



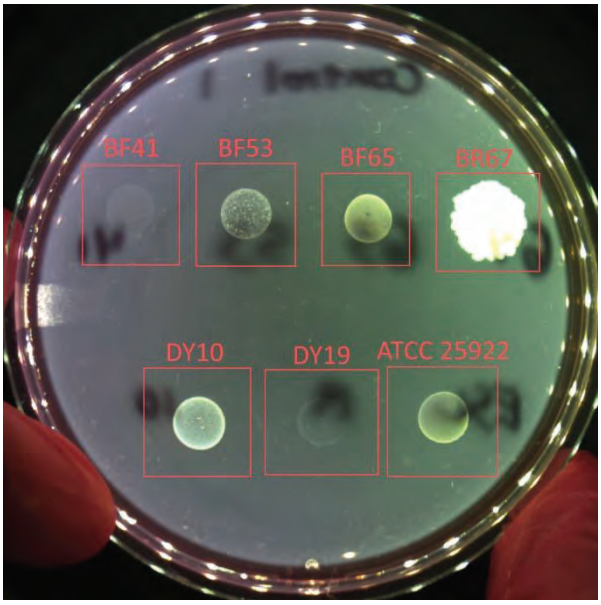
16 µg/ml Erythromycin

Table C-5. Agar Dilution Antibiotic Results and Susceptibility Photos: Gentamicin

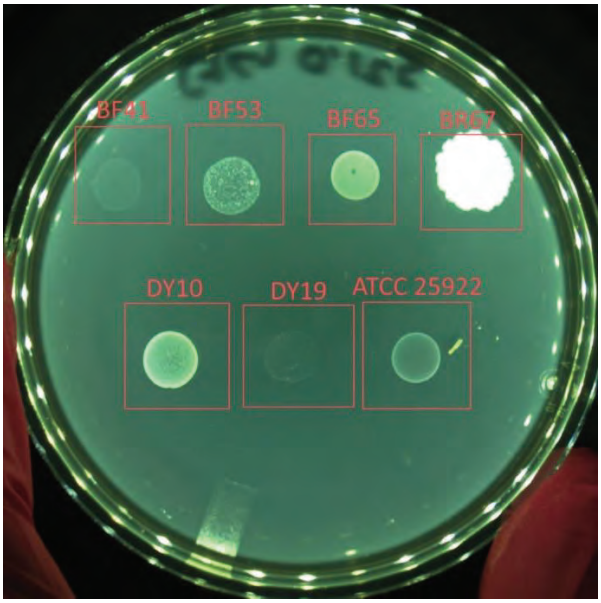
	Gentamicin Concentration (µg/mL)									
Organism	**0 (Control)**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	G	G
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	G	G
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G	G

G = Growth
NG = No Growth

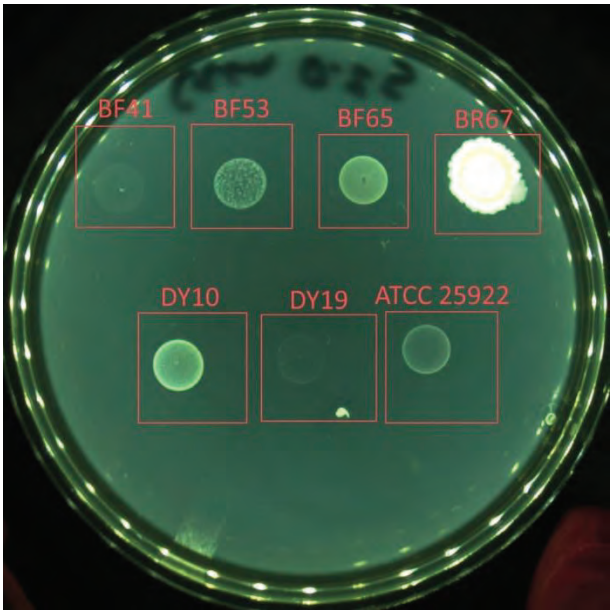
Agar Dilution Antibiotic Susceptibility Photos: Gentamicin



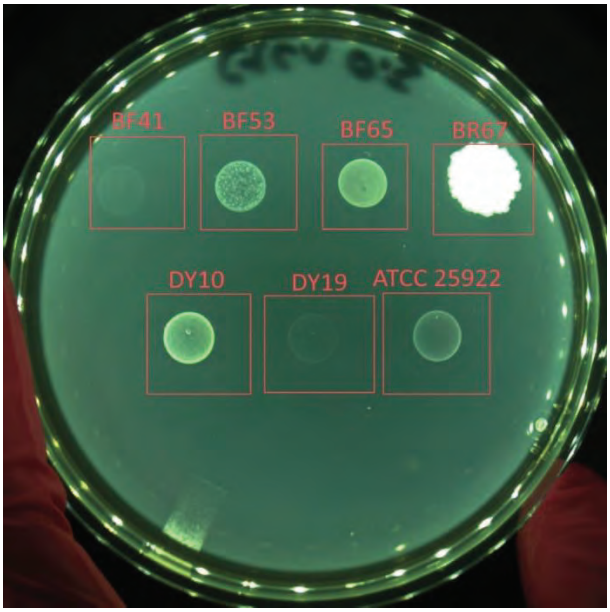
0 µg/mL Gentamicin



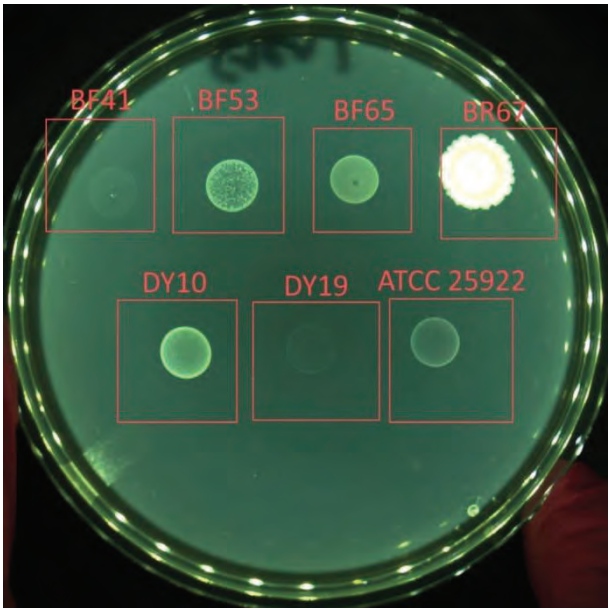
0.125 µg/mL Gentamicin



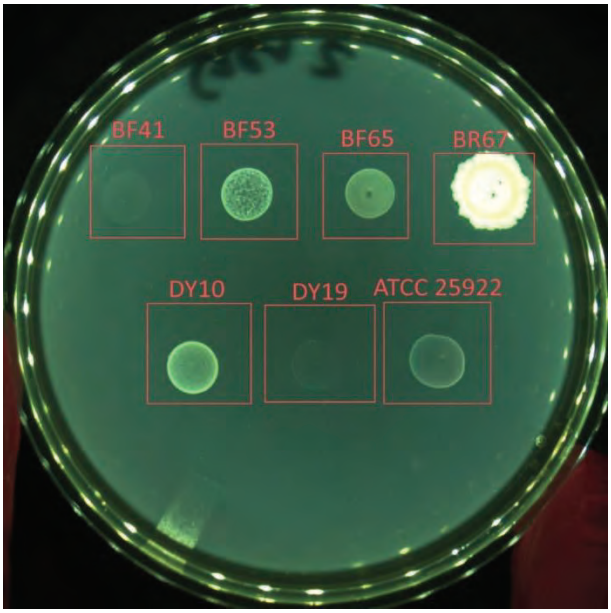
0.25 µg/mL Gentamicin



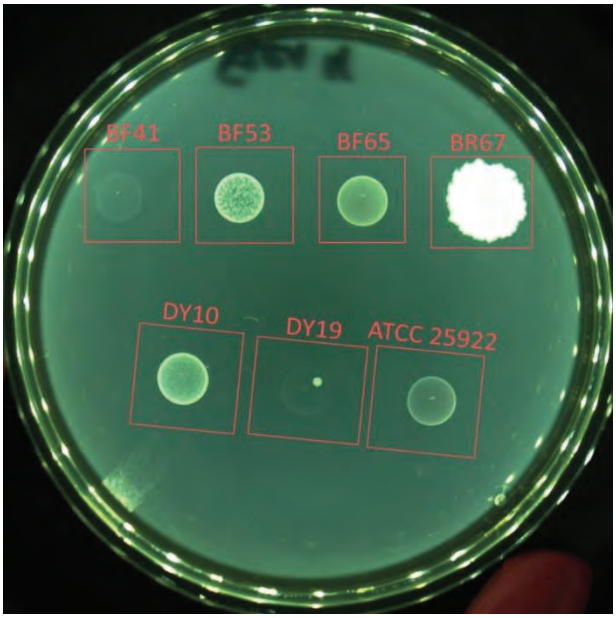
0.5 µg/mL Gentamicin



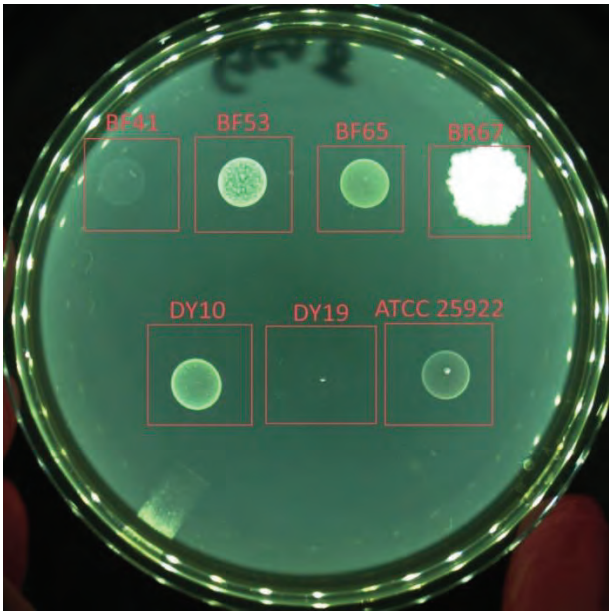
1 µg/mL Gentamicin



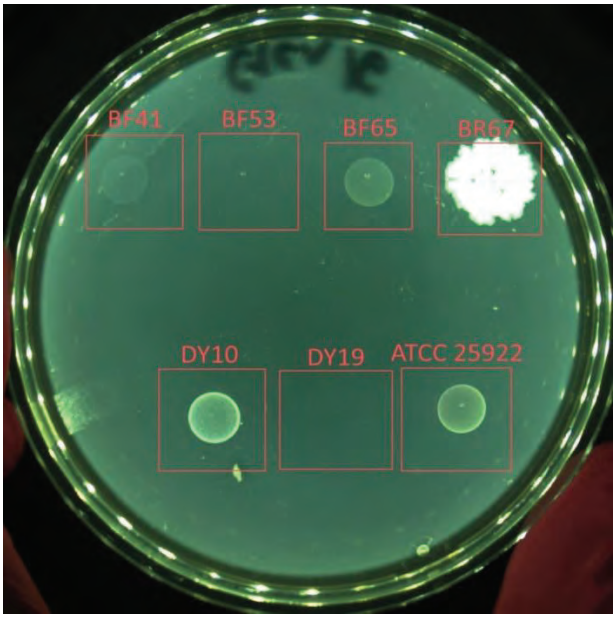
2 µg/mL Gentamicin



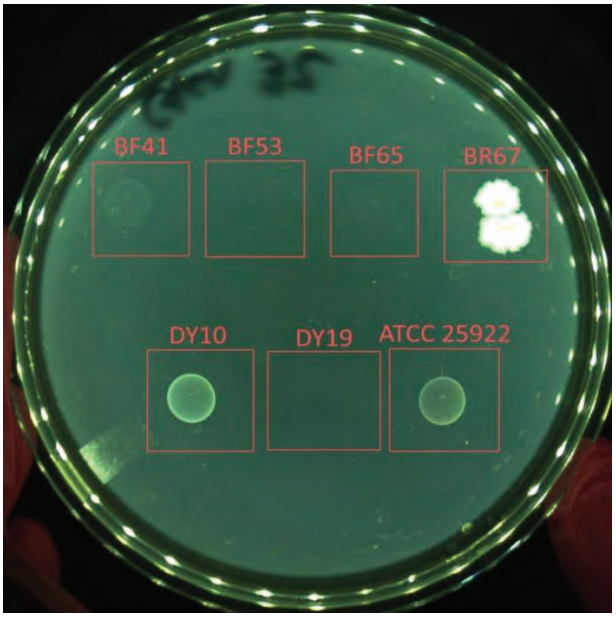
4 µg/mL Gentamicin



8 µg/mL Gentamicin



16 µg/mL Gentamicin



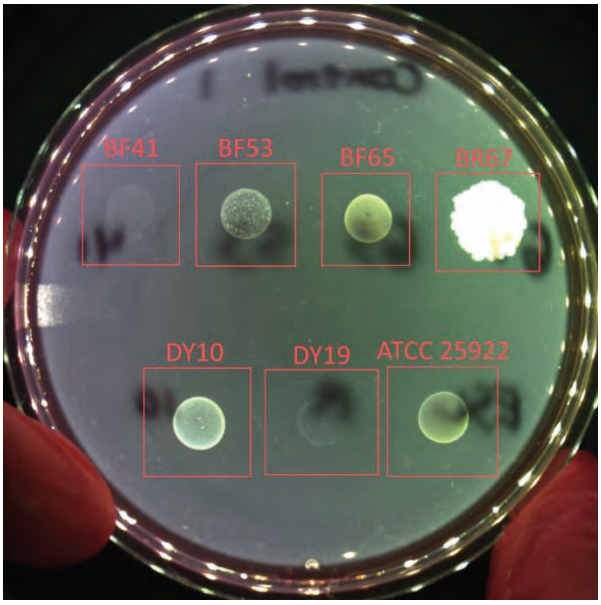
32 µg/mL Gentamicin

Table C-6. Agar Dilution Antibiotic Results and Susceptibility Photos: Kanamycin

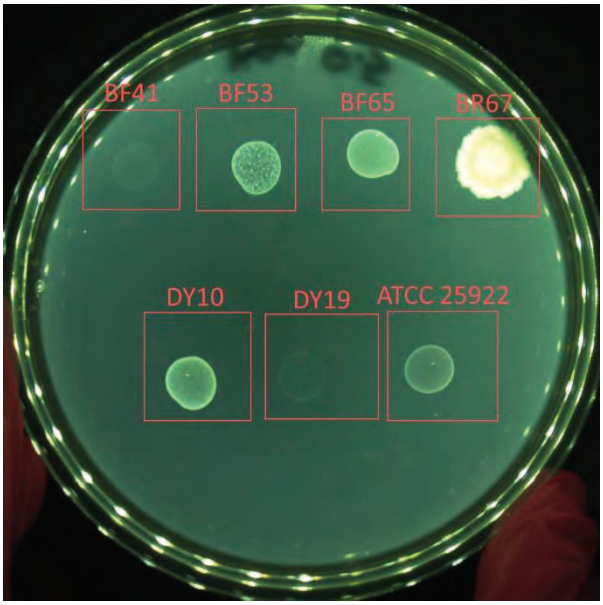
	Kanamycin Concentration (µg/mL)								
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	G
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	G	G
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G

G = Growth
NG = No Growth

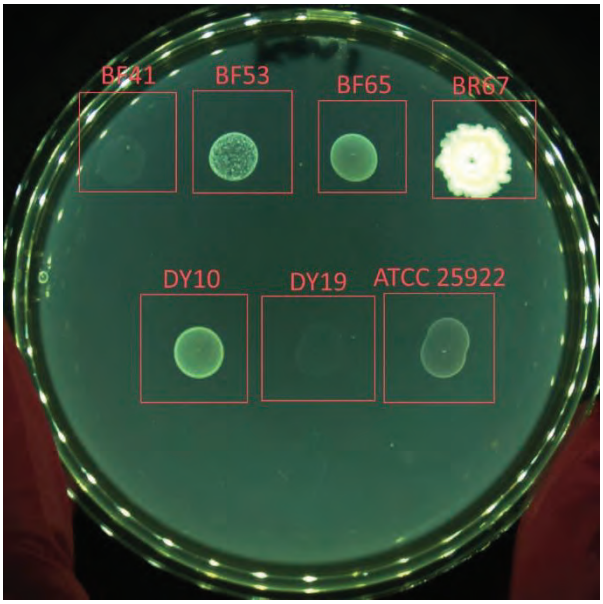
Agar Dilution Antibiotic Susceptibility Photos: Kanamycin



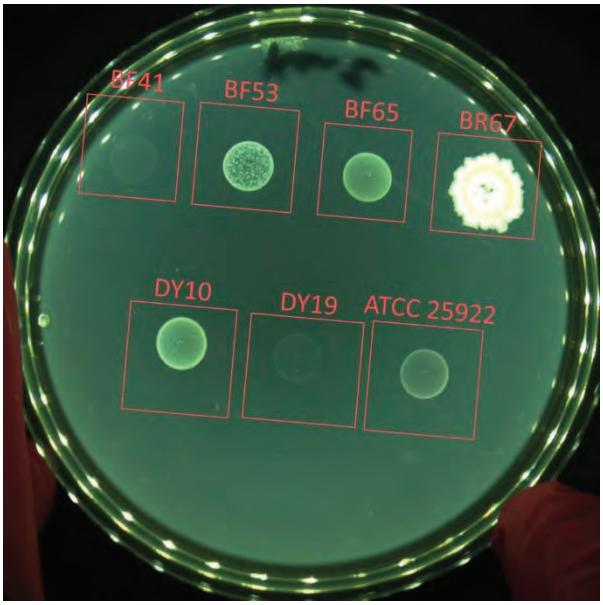
0 µg/mL Kanamycin



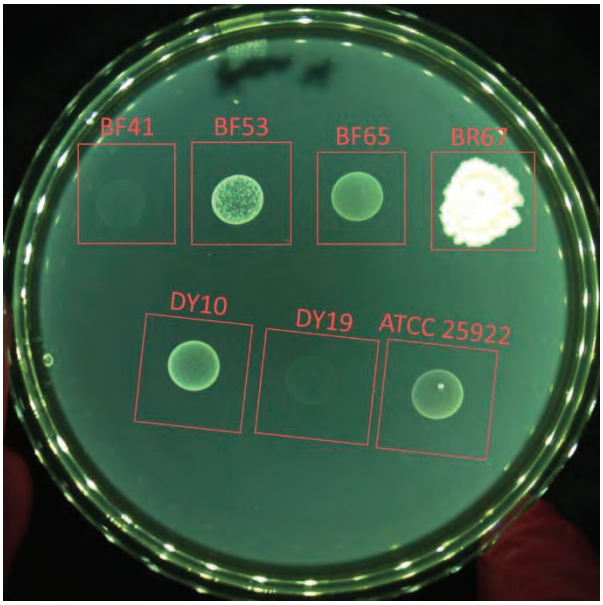
0.5 µg/mL Kanamycin



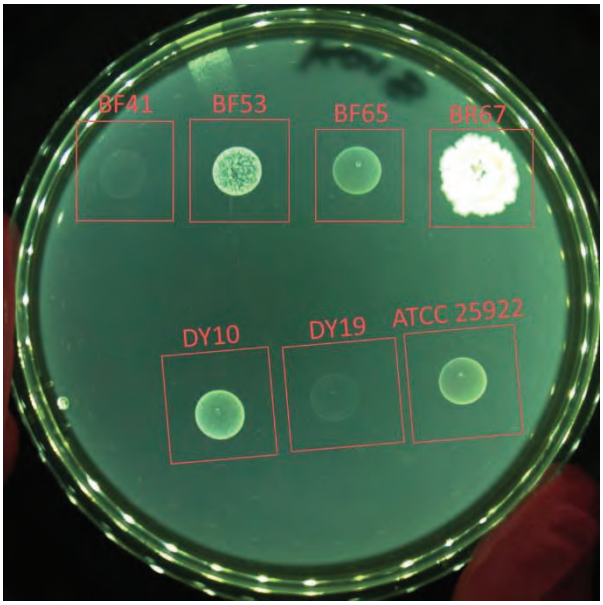
1 µg/mL Kanamycin



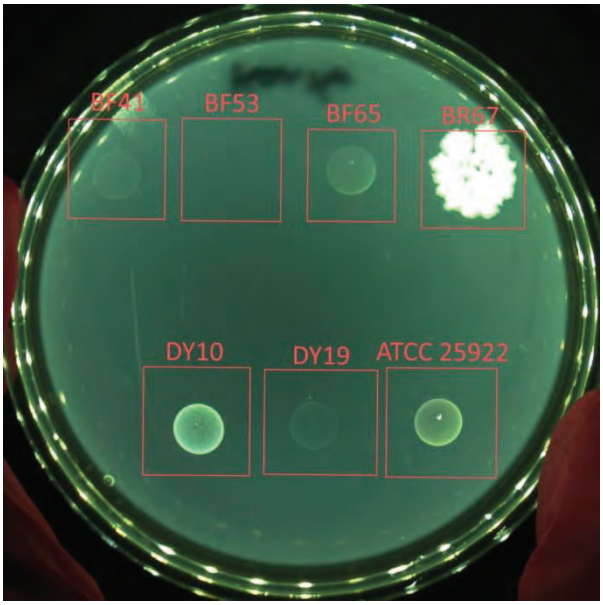
2 µg/mL Kanamycin



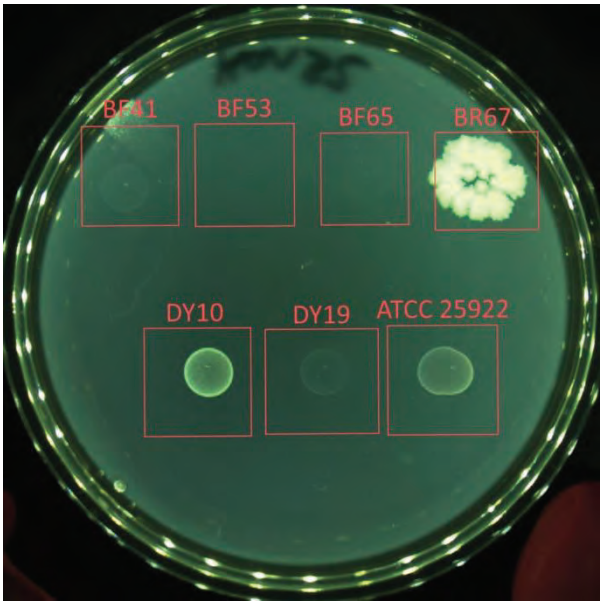
4 µg/mL Kanamycin



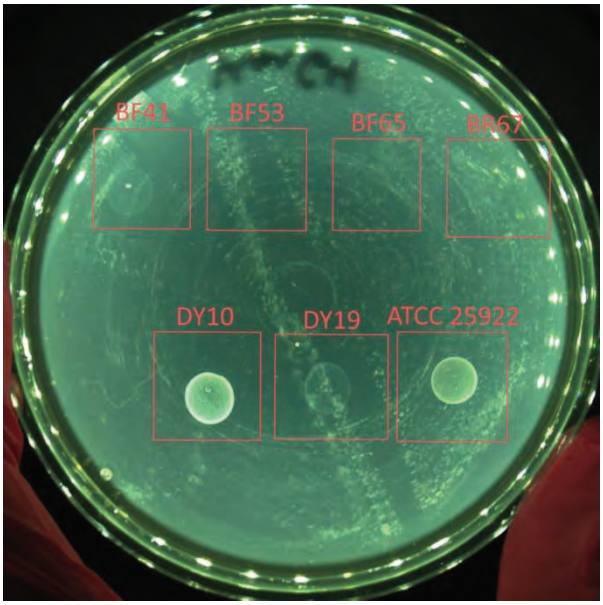
8 µg/mL Kanamycin



16 µg/mL Kanamycin



32 µg/mL Kanamycin



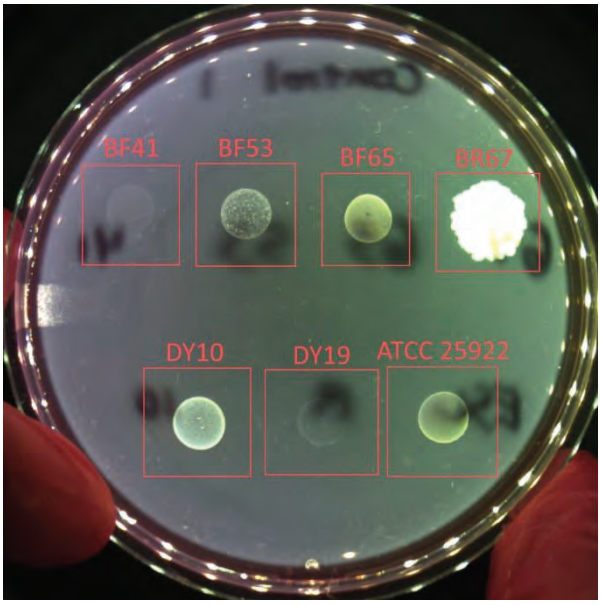
64 µg/mL Kanamycin

Table C-7. Agar Dilution Antibiotic Results and Susceptibility Photos: Streptomycin

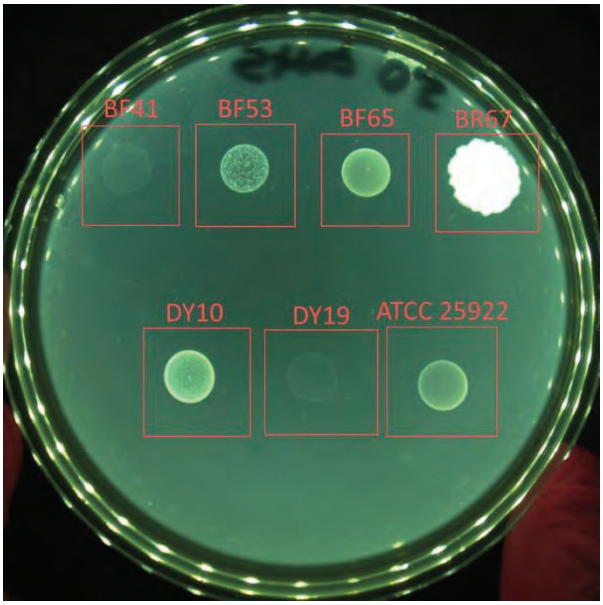
	Streptomycin Concentration (µg/mL)								
Organism	**0 (Control)**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	G	G	G	G
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	G
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G

G = Growth
NG = No Growth

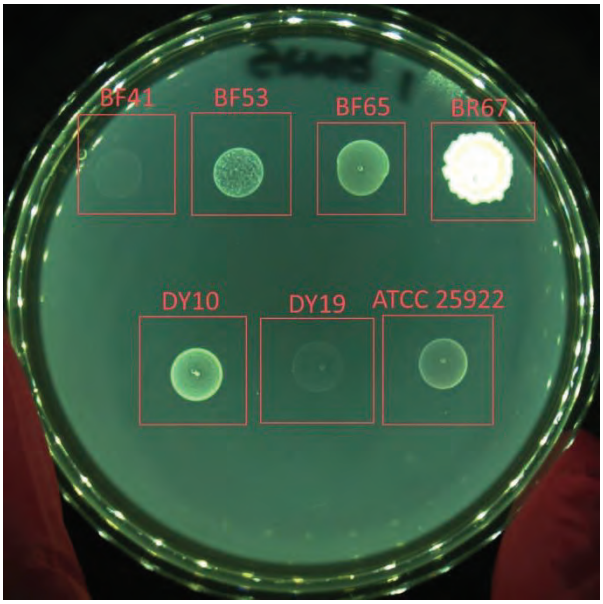
Agar Dilution Antibiotic Susceptibility Photos: Streptomycin



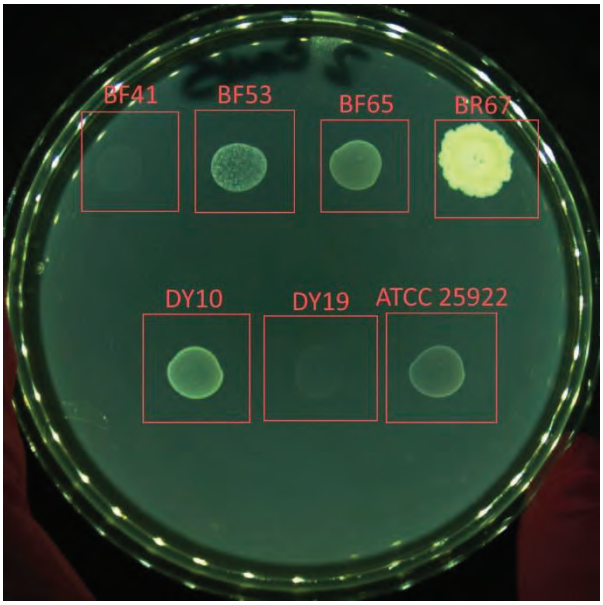
0 µg/mL Streptomycin



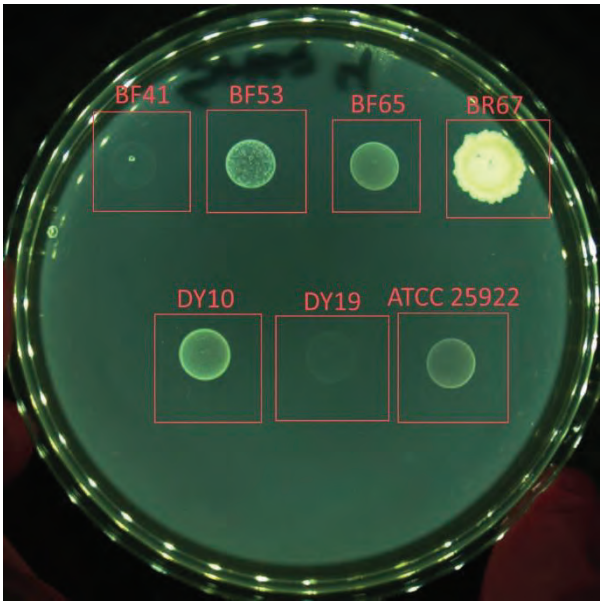
0.5 µg/mL Streptomycin



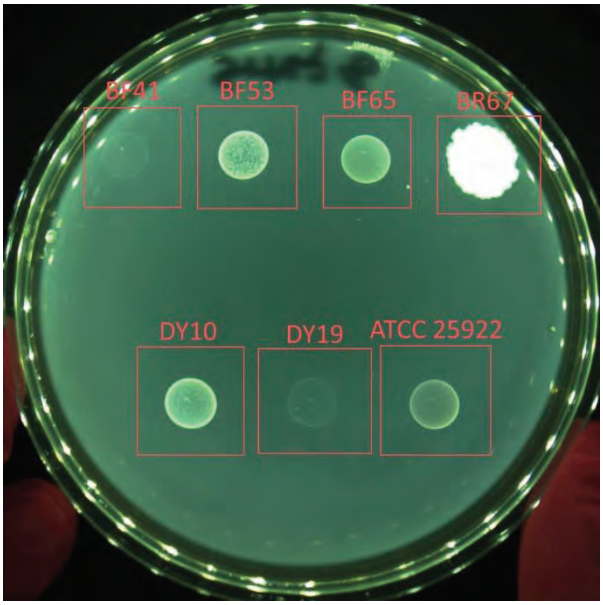
1 µg/mL Streptomycin



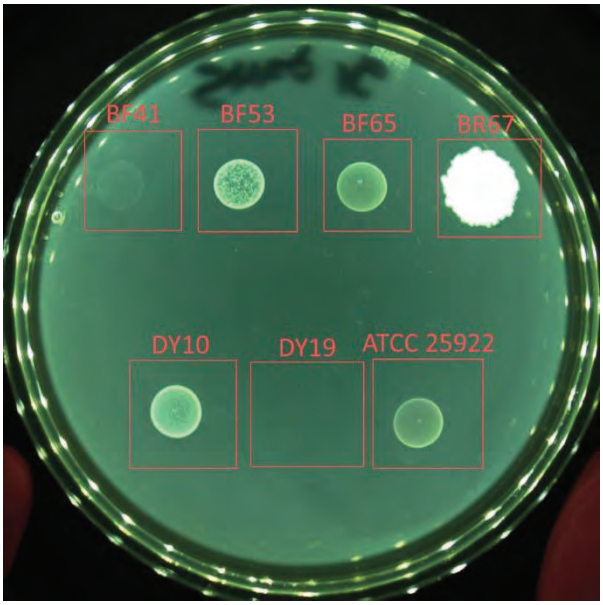
2 µg/mL Streptomycin



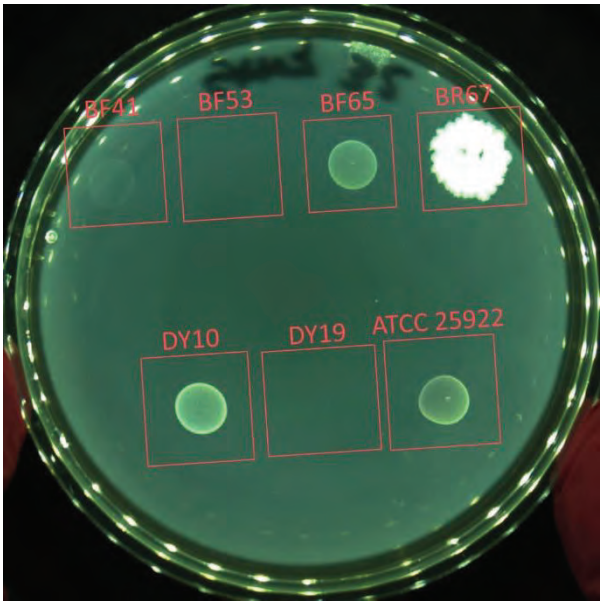
4 µg/mL Streptomycin



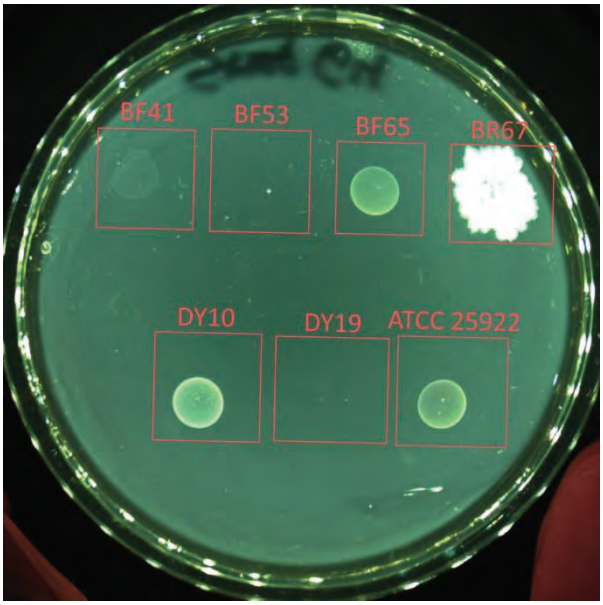
8 µg/mL Streptomycin



16 µg/mL Streptomycin



32 µg/mL Streptomycin



64 µg/mL Streptomycin

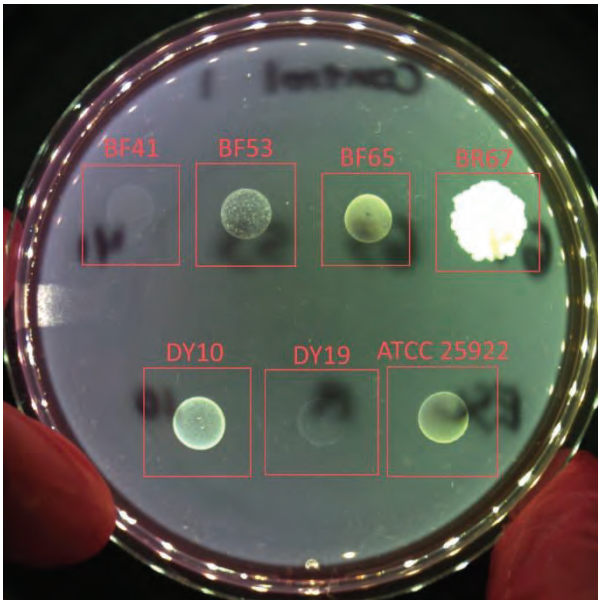
Table C-8. Agar Dilution Antibiotic Results and Susceptibility Photos: Tetracycline

	Tetracycline Concentration (µg/mL)											
Organism	**0 (Control)**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	G	G	G	G
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	G	G	G	G	G	G	G	G	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	NG	NG	NG	NG	NG	NG	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	G	G	G	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	G	G	G	G	G	G	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	NG	NG	NG	NG	NG	NG

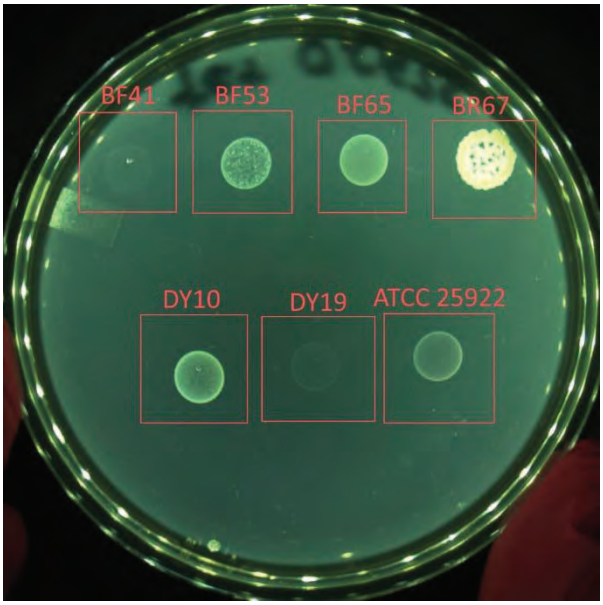
G = Growth

NG = No Growth

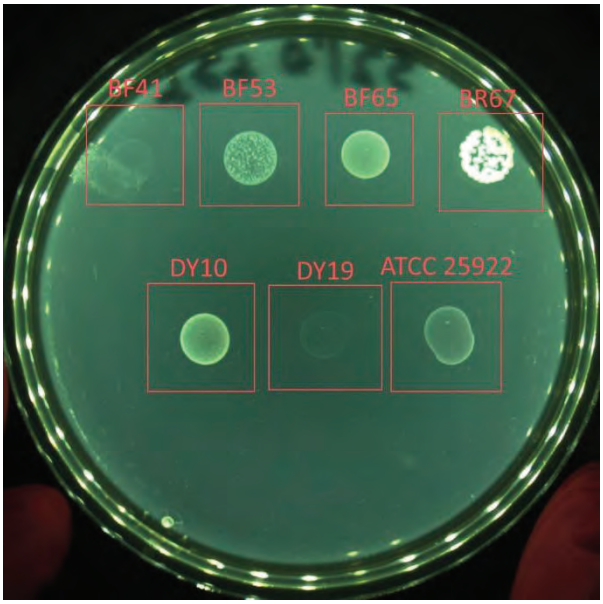
Agar Dilution Antibiotic Susceptibility Photos: Tetracycline



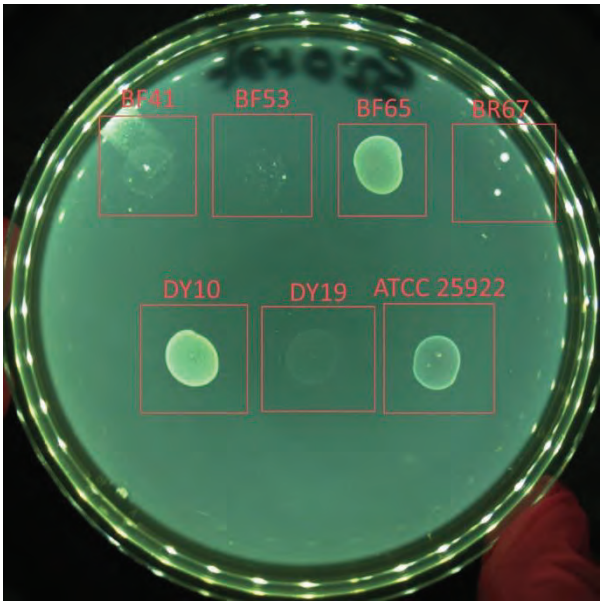
0 µg/mL Tetracycline



0.0625 µg/mL Tetracycline



0.125 µg/mL Tetracycline



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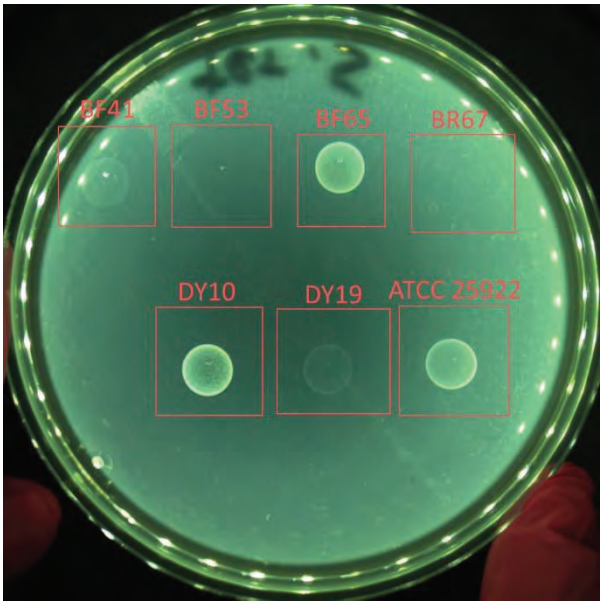

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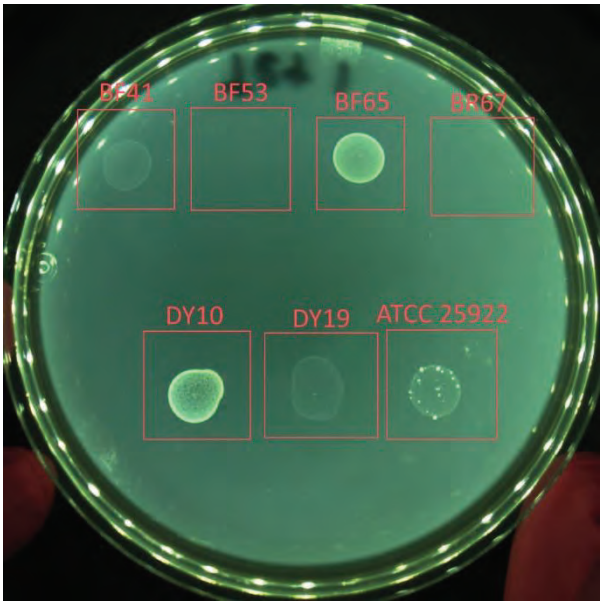
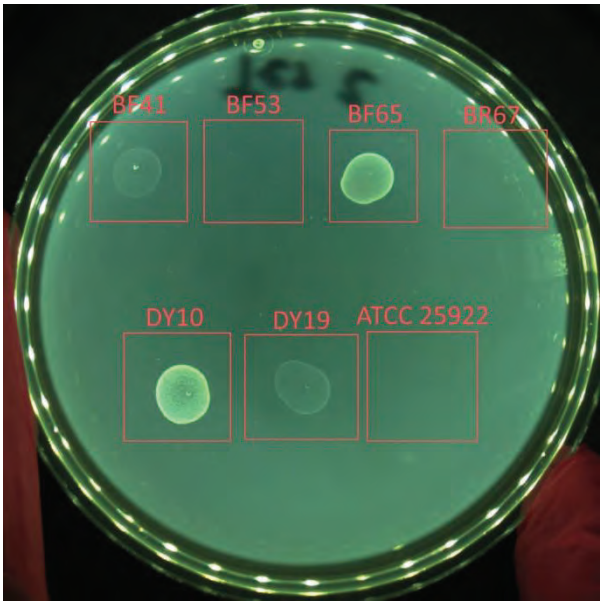
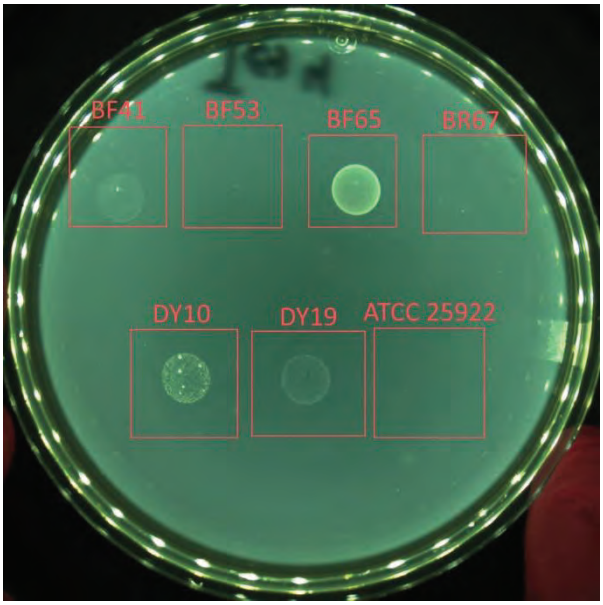


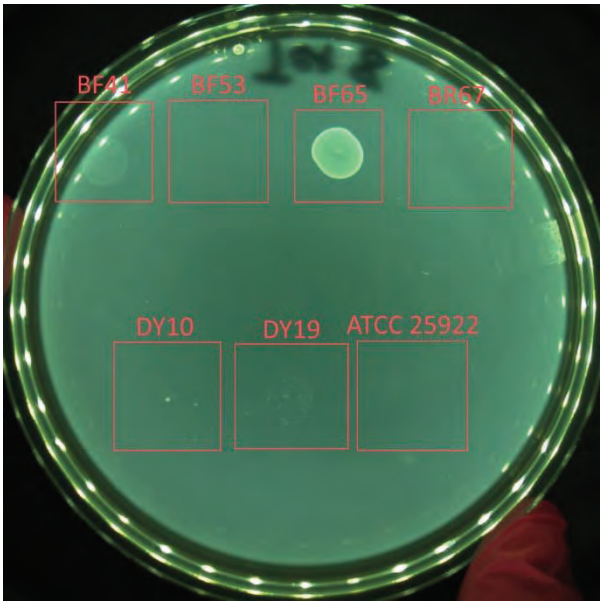
1 µg/mL Tetracycline



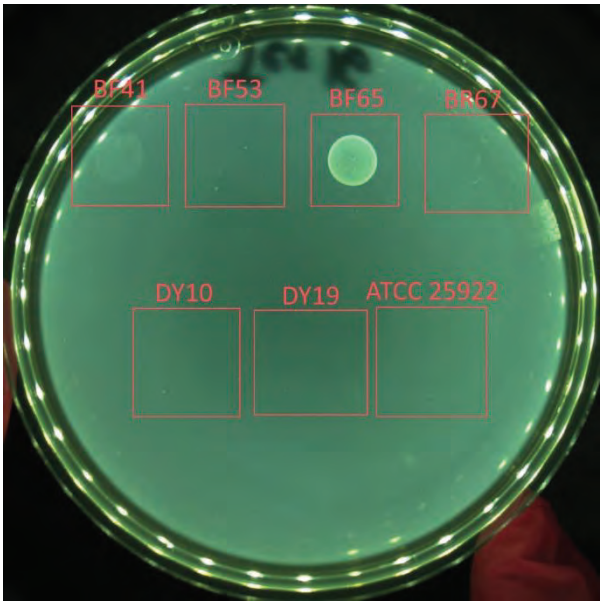
2 µg/mL Tetracycline



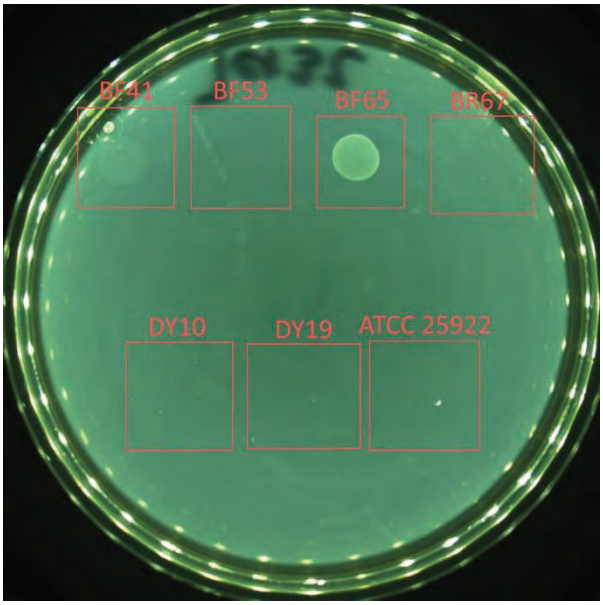
4 µg/mL Tetracycline



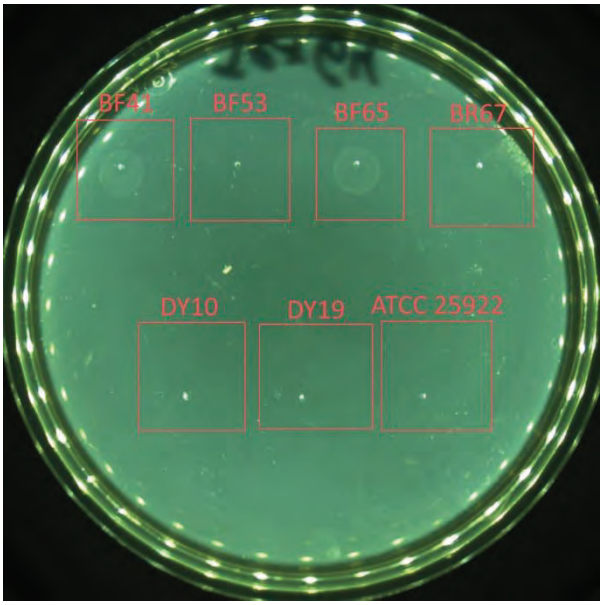
8 µg/mL Tetracycline



16 µg/mL Tetracycline



32 µg/mL Tetracycline



64 µg/mL Tetracycline

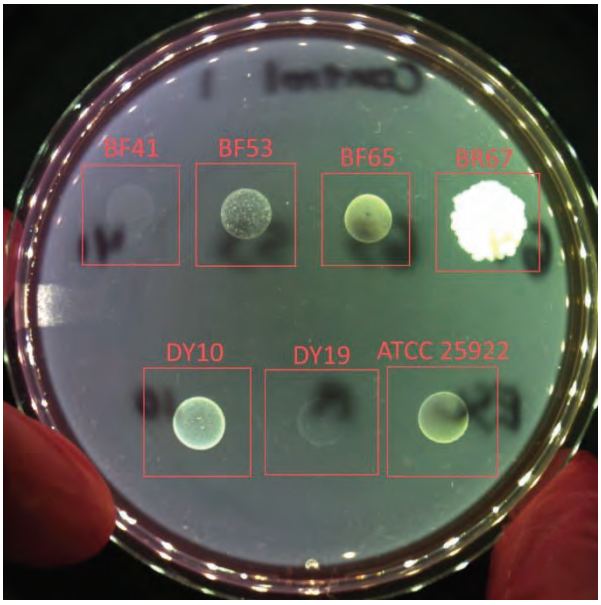
Table C-9. Agar Dilution Antibiotic Results and Susceptibility Photos: Vancomycin

	Vancomycin Concentration (µg/mL)									
Organism	**0 (Control)**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**
Prevotella albensis ASCUSBF41 (BF41)	G	G	G	G	G	G	G	G	NG	NG
Succinivibrio dextrinosolvens ASCUSBF53 (BF53)	G	G	G	G	G	G	G	G	G	G
Chordacoccus ruminofurens ASCUSBF65 (BF65)	G	G	G	NG	NG	NG	NG	NG	NG	NG
Clostridium beijerinckii ASCUSBR67 (BR67)	G	G	G	G	G	NG	NG	NG	NG	NG
Ruminococcus bovis ASCUSDY10 (DY10)	G	G	G	G	G	NG	NG	NG	NG	NG
Butyrivibrio fibrisolvens ASCUSDY19 (DY19)	G	G	NG	NG	NG	NG	NG	NG	NG	NG
Escherichia coli ATCC 25922	G	G	G	G	G	G	G	G	G	G

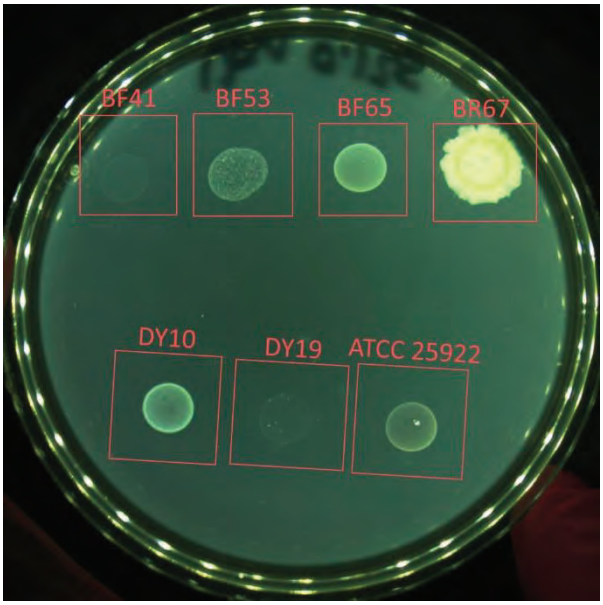
G = Growth

NG = No Growth

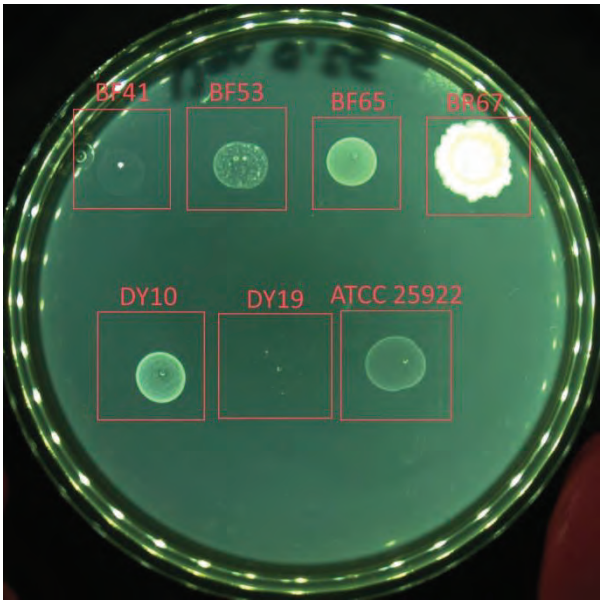
Agar Dilution Antibiotic Susceptibility Photos: Vancomycin



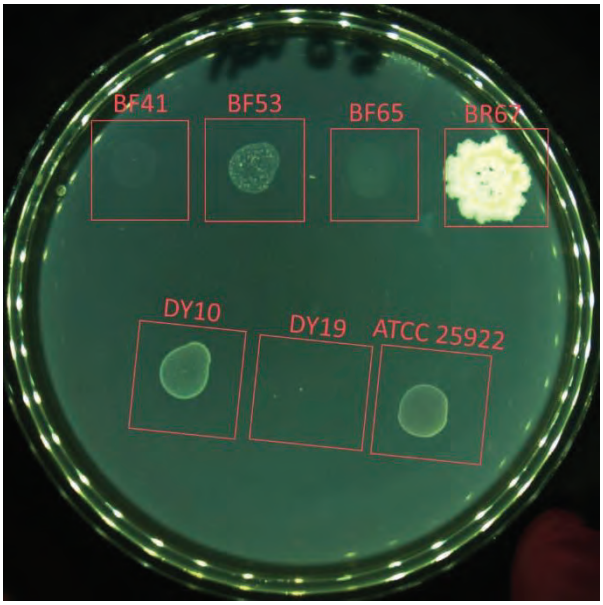
0 µg/mL Vancomycin



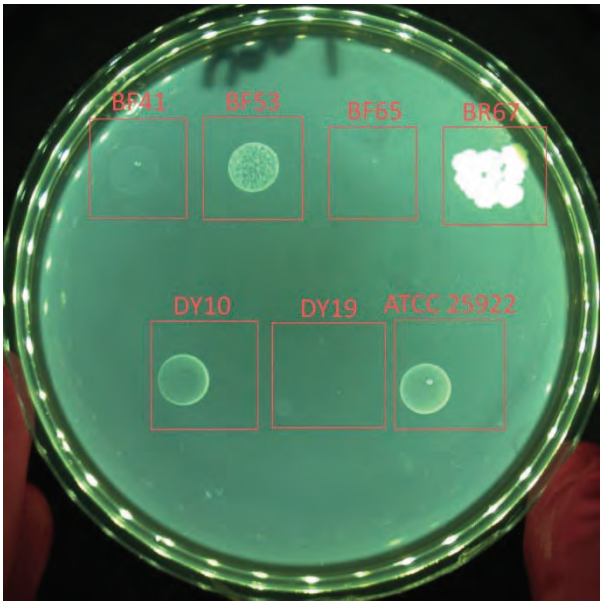
0.125 µg/mL Vancomycin



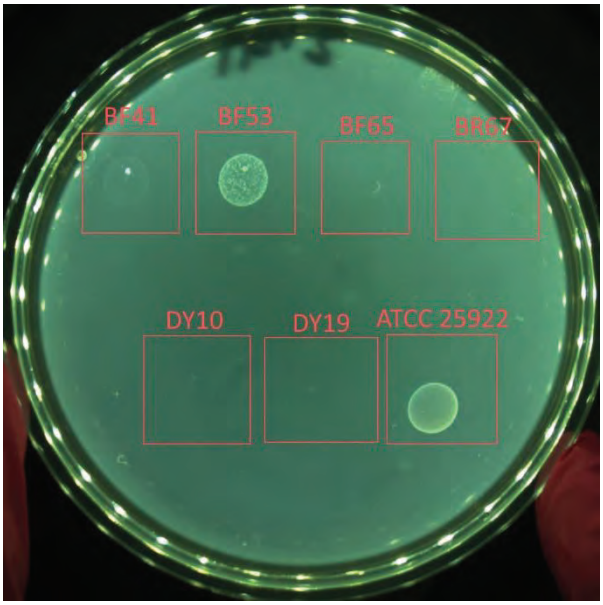
0.25 µg/mL Vancomycin



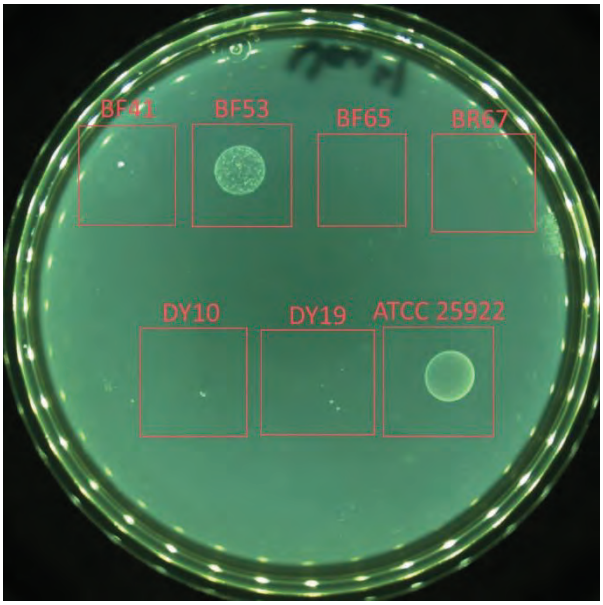
0.5 µg/mL Vancomycin



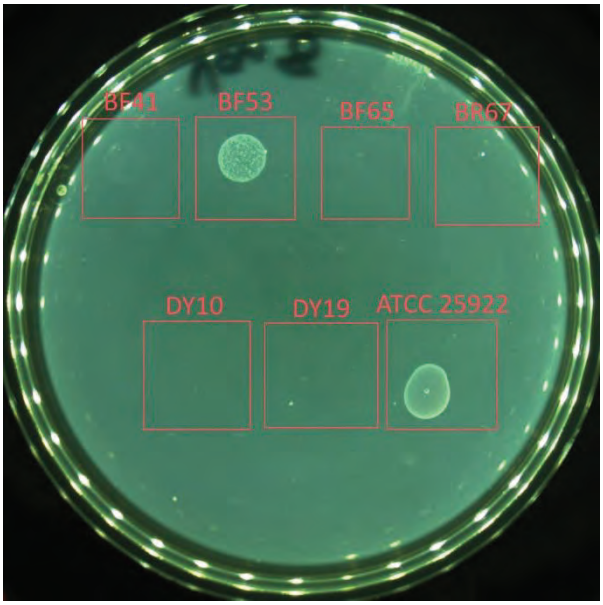
1 µg/mL Vancomycin



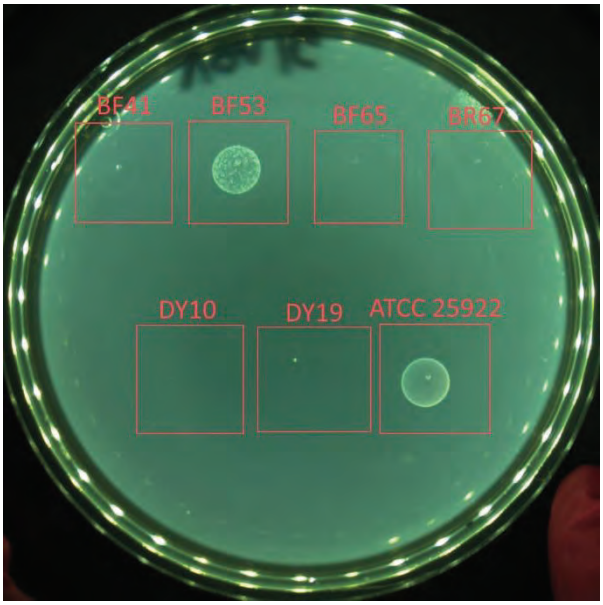
2 µg/mL Vancomycin



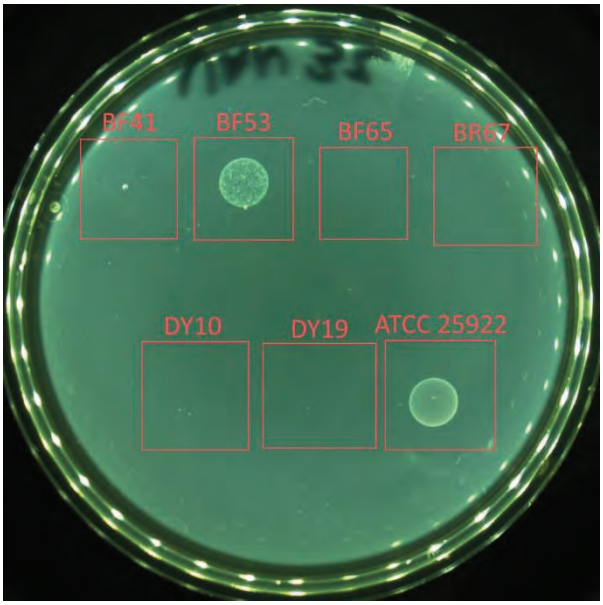
4 µg/mL Vancomycin



8 µg/mL Vancomycin



16 µg/mL Vancomycin



32 µg/mL Vancomycin

(b) (4)

FINAL REPORT

TITLE: **Characterization of Ascus Biosciences *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53) Production Strain: Absence of Antimicrobial Activity**

INVESTIGATOR'S STUDY NUMBER: (b) (4)

CONDUCT DATES: Receipt of supernatant: November 20, 2019
Testing of supernatant: November 27, 2019 – December 5, 2019

SPONSOR: Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121

INVESTIGATOR: (b) (4)

VERSION: FINAL

SIGNATURE: (b)(6) _____ 2/21/20
Principal Investigator Date

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LIST OF TABLES AND APPENDICES

OBJECTIVES

To determine the antimicrobial properties of the *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53) production strain supernatant.

STANDARDS OF COMPLIANCE

This study was conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs as detailed in the protocol.

STUDY SITE

Antimicrobial property testing of the product was performed by (b) (4) (b) (4)

MATERIALS

The sponsor provided Beef-53 supernatant (Lot number 20191031 V2) was prepared by centrifugation at (b) (4) The sample was received on November 20, 2019.

ANTIMICROBIAL PROPERTIES

A portion of the growth medium from a typical production batch of the *Succinivibrio dextrinosolvens* ASCUSBF53 (Beef-53), or a scaled down version, was kept refrigerated (2-8°C) and shipped to (b) (4) and used 13 days after receipt.

1.1. Preparation of Culture Plates

The following six organisms were tested against the supernatant:

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

(b) (4)

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D

The supernatant was discarded after autoclaving and issue of the final report. No retention sample was maintained.

RESULTS

No zones of inhibition were observed for the Beef-53 supernatant lot, or the sterile distilled water control. A zone of inhibition was observed for the enrofloxacin positive control for each organism as indicated in the table below:

Table 1. Zone Diameters from Beef-53 Supernatant and Controls

Organism	ATCC number	(b) (4) code	Zone Diameter for the indicated solution (mm)		
			Beef-53 Supernatant	Sterile Distilled water	Enrofloxacin
Staphylococcus aureus	6538	Sta 11	(b) (4)		
Escherichia coli	11229	EC 96			
Bacillus cereus	2	BC 5			
Bacillus circulans	4516	Bi 1			
Streptococcus pyogenes	12344	Str 59			
Serratia marcescens	14041	SM 4			

Following incubation, pictures were taken of each organism seeded into the agar onto which a saturated disk of supernatant and controls were placed according to the protocol. These pictures are included in Appendix B. No zones of inhibition are observed in these pictures.

CONCLUSION

The Beef-53 supernatant exhibited no antibacterial activity against the 6 strains representative of Gram positive and Gram negative bacteria.

APPENDIX A. Protocol

(b) (4)

STUDY PROTOCOL

TITLE:	**Characterization of Ascus Biosciences Various Production Strain: Absence of Antimicrobial Activity**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b)(6)
VERSION:	FINAL

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SIGNATURES

Sponsor Representative

Jordan Embree
Ascus Biosciences
6450 Lusk Blvd
Suites E109/209
San Diego, CA 92121
Email: jordan@ascusbiosciences.com
Tel. 877-696-8945 x709

(b)(6) 11/14/19

Signature Date

Investigator (b) (4), (b)(6)

(b)(6)

11/14/19

ate

1. OBJECTIVES

Determination of the antimicrobial properties of various production strain supernatants.

2. STUDY TIMELINE

Anticipated study dates are:
Antimicrobial Properties: November 2019

3. STANDARDS OF COMPLIANCE

This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs as detailed in this protocol.

4. STUDY SITE

Antimicrobial properties testing of the products will be performed by (b) (4) (b) (4)

5. MATERIALS AND METHODS

5.1. Supernatant

(b) (4)

6. ABSENCE OF ANTIMICROBIAL PRODUCTION[1]

(b) (4)

[1] FAO (2006) Determination of Antibacterial Activity of enzyme preparations from the Combined Compendium of Food Additive Specifications, Vol. 4 (FAO/JECFA), pg 122

6.1. Preparation of Culture Plates

The following six organisms will be tested against each supernatant: Volumes of media and numbers of plates should be adjusted as required, based upon the number of supernatants tested.

Organism	ATCC number	(b) (4) code	Dilution tested
Staphylococcus aureus	6538	Sta 11	1:10
Escherichia coli	11229	EC 96	1:10
Bacillus cereus	2	BC 5	1:10
Bacillus circulans	4516	Bi 1	1:10
Streptococcus pyogenes	12344	Str 59	1:20
Serratia marcescens	14041	SM 4	1:10

(b) (4)

(b) (4)

7. RAW DATA, RECORDS, AND REPORTS

7.1. Data

All raw data will be recorded, handled, and stored according to facility SOPs, this protocol, and applicable regulatory requirements. All original data collected and records generated in connection with the study will be archived at the study site. The following records will be maintained:

- Quality control records generated concurrent with all media and materials preparation, and lab testing.
- Protocols, protocol amendments, correspondence, reports and other documentation, including drafts of the final report
- Raw data and logs
- Documents related to any occurrence or situation that develops during the course of the trial that may affect the test results

All records will be maintained appropriately in labs and files as the project is ongoing, and thereafter in archives storage at (b) (4)

7.2. Reporting of Results

A separate report will be issued for the production strain for each of the tests performed. If additional production strains are tested, reports will be issued in a similar manner, depending upon the tests required.

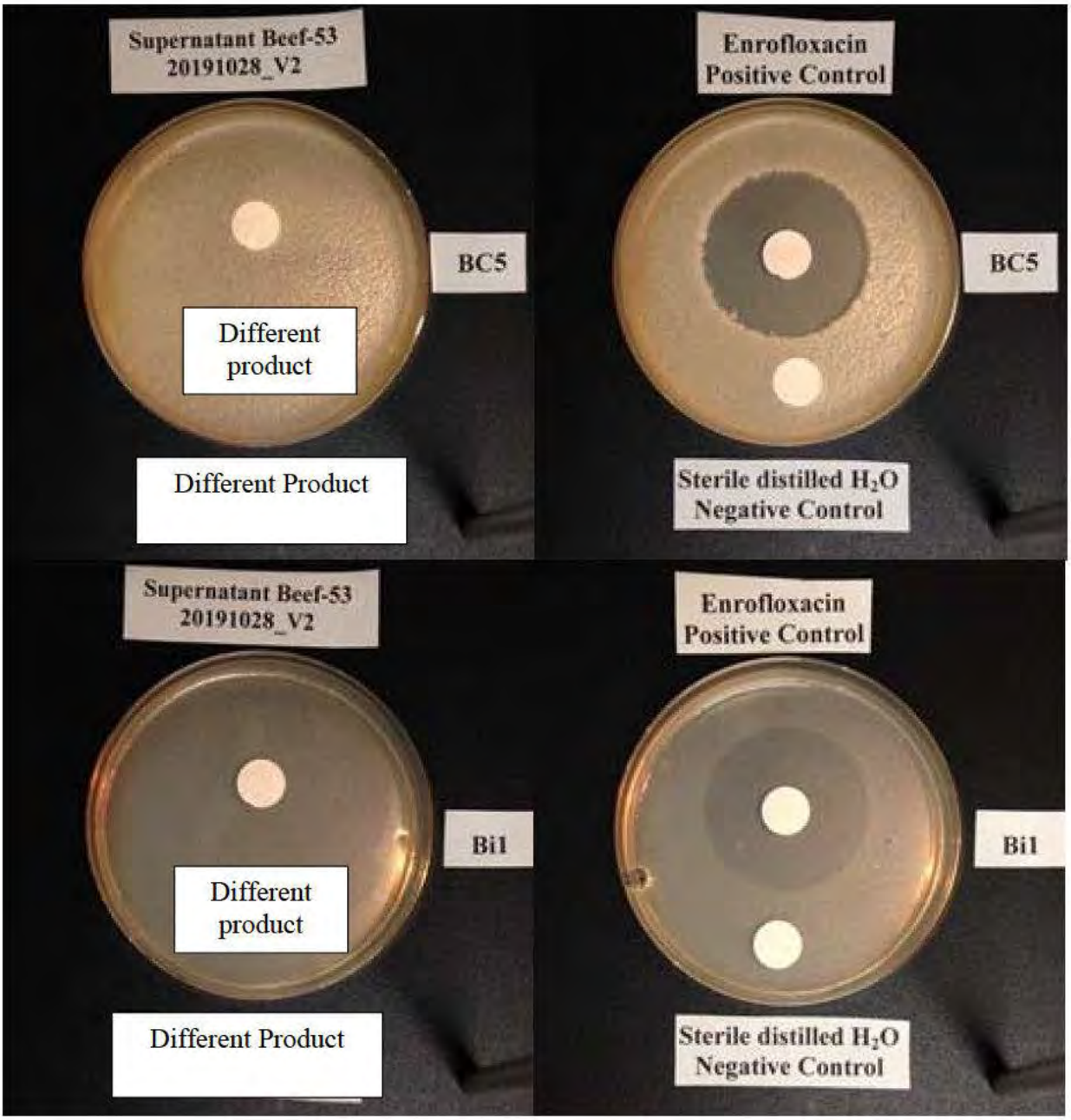
8. DISPOSITIONS

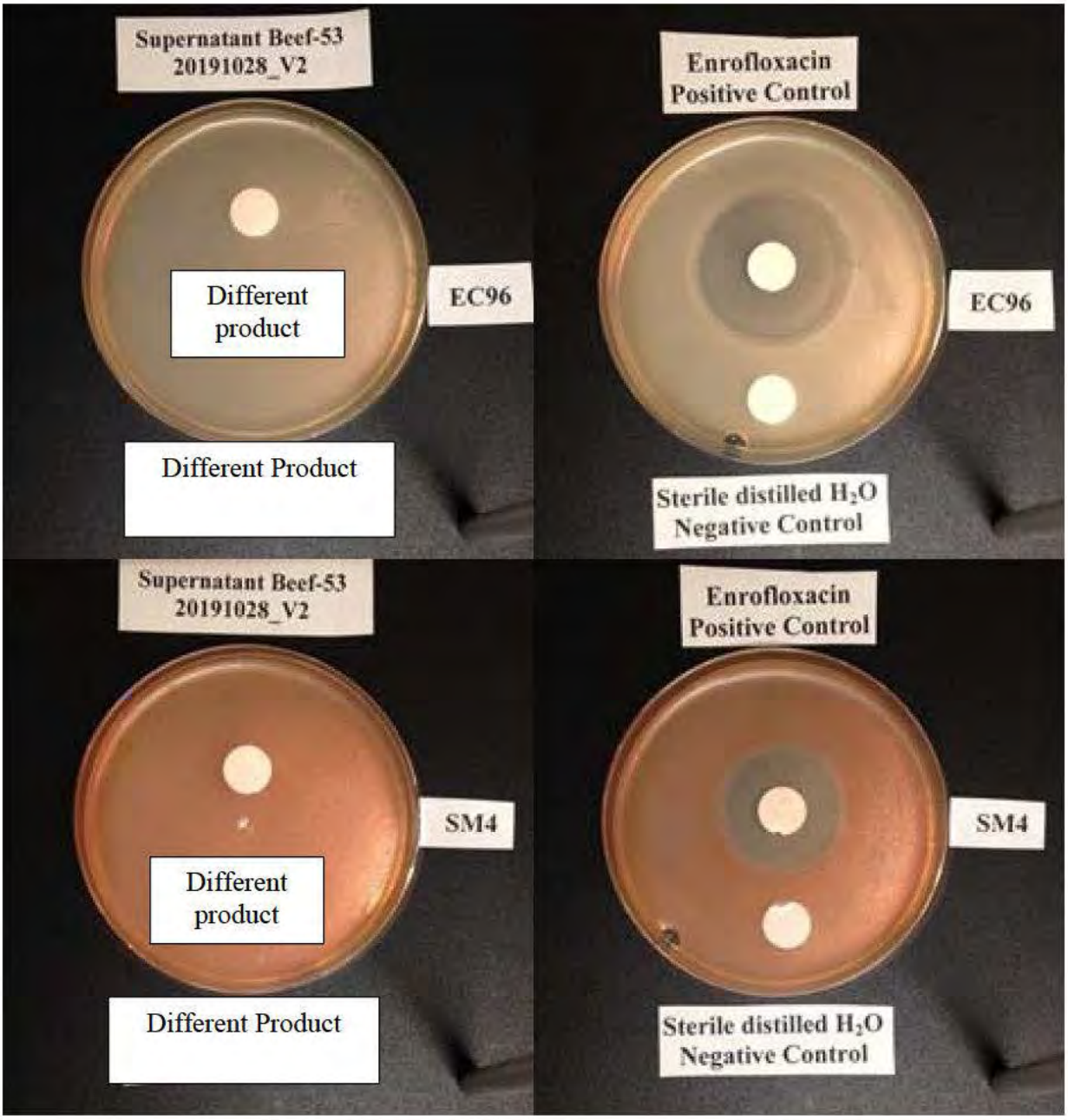
8.1. Supernatants
All surplus quantities of the provided supernatants will be discarded after autoclaving following report issue. No reserve samples will be maintained.

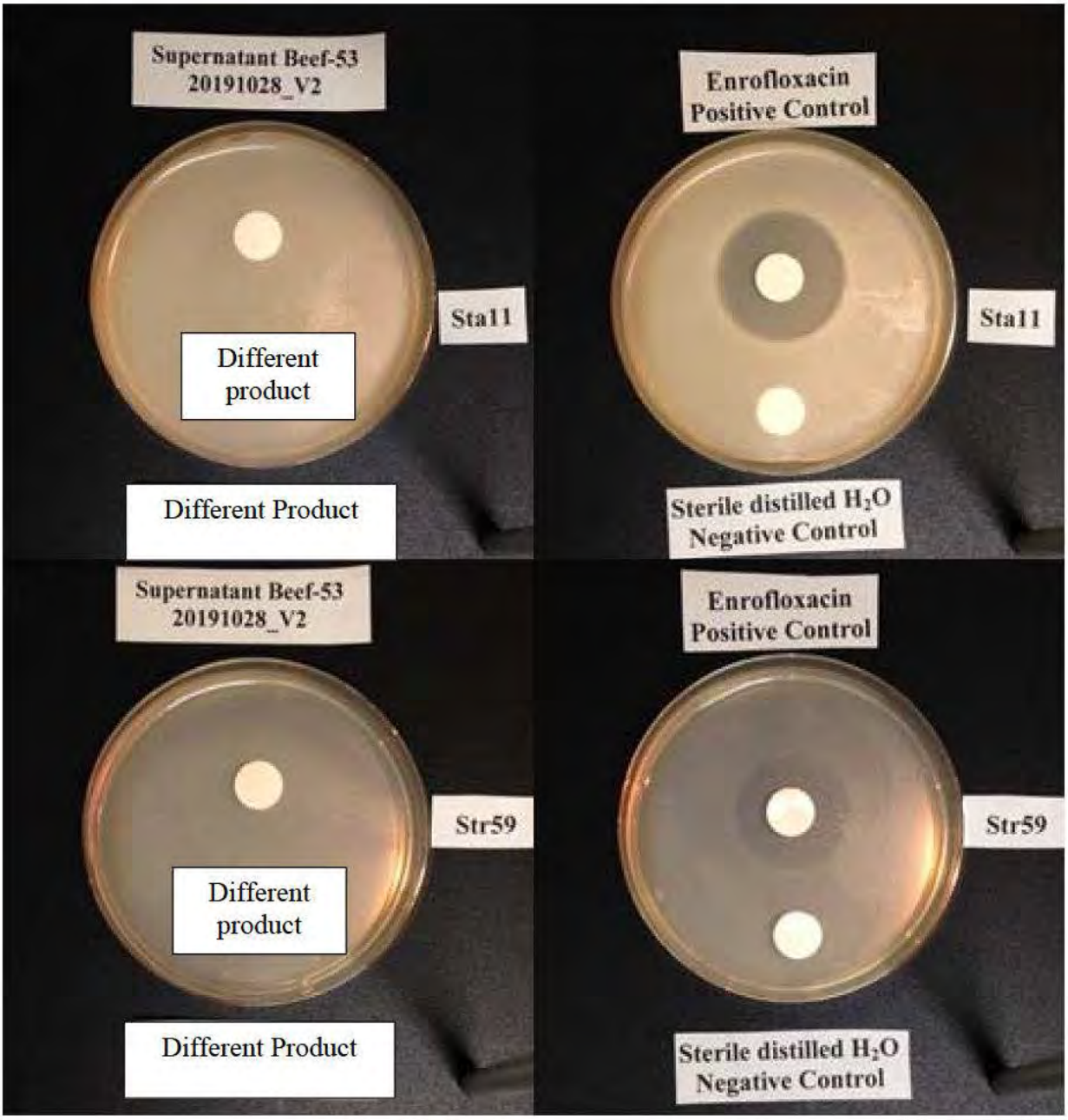
9. CHANGES TO PROTOCOL

Any change or revision to the approved protocol will be documented by written amendment that will be maintained with the protocol. As a minimum, the amendment will indicate the changes or revisions made, indicate the effective date, identify the protocol sections affected, explain the reasons for change and describe the impact on the study. The amendment will be signed and dated by those who signed the protocol. Signatures will be obtained before implementation of the change if possible. If such is not possible, the investigator will attempt to obtain verbal prior authorization from the sponsor and follow with written documentation at the earliest opportunity. Protocol deviations are defined as unintended or unforeseeable necessary changes to the protocol. Protocol deviation reports list any action that is not/was not in accordance with the protocol. They must contain a detailed description of the deviation, its reason, and a description of its effect on the study.

APPENDIX B: Photos




Supernatant Beef-53
20191028_V2
Different product
EC96
Different Product
Enrofloxacin
Positive Control
EC96
Sterile distilled H_2O
Negative Control
Supernatant Beef-53
20191028_V2
Different product
SM4
Different Product
Enrofloxacin
Positive Control
SM4
Sterile distilled H_2O
Negative Control


Supernatant Beef-53
20191028_V2
Sta11
Different product
Different Product
Enrofloxacin
Positive Control
Sta11
Sterile distilled H_2O
Negative Control
Supernatant Beef-53
20191028_V2
Str59
Different product
Different Product
Enrofloxacin
Positive Control
Str59
Sterile distilled H_2O
Negative Control

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

Reference: **AOAC Method 2015.01**

Approved: (b)(6)

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

- (b) (4)

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 – (b) (4)
(d) ICP-MS – (b) (4)

7.2 Reagents and Standards

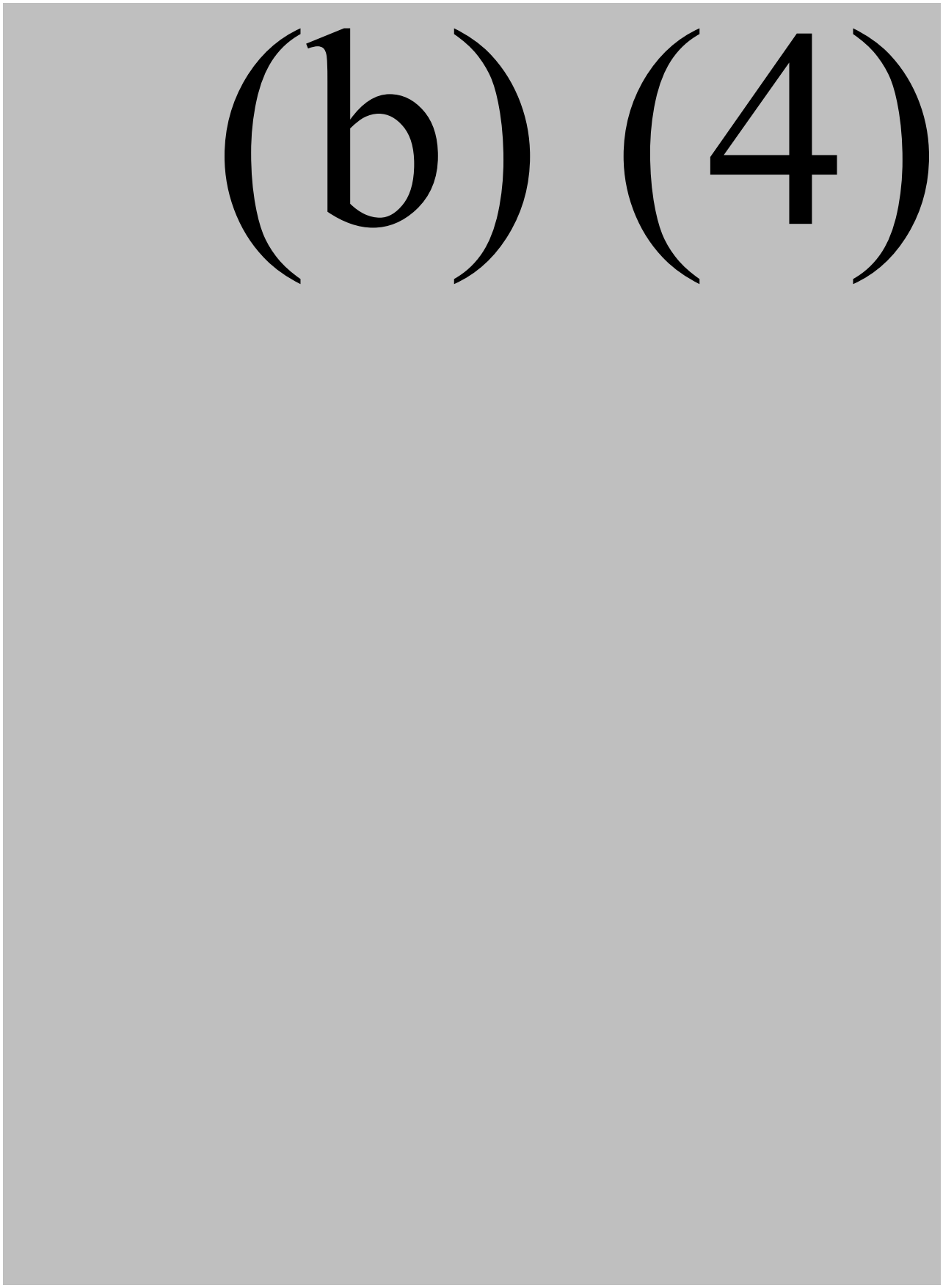
All reagents may contain impurities that may affect the integrity of the analytical results. Due to the high sensitivity of the ICP-MS, high-purity reagents, water, acids, glassware and sample tubes that are suitable for trace metal analysis must be used at all time.

(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) – (b) (4) trace metal grade
(g) Hydrochloric Acid (HCl) – Concentrated, trace element grade
(h) Internal Standard Solution – (b) (4)
(i) Deionized water (DI H_2O)

7.2.1 Working solutions

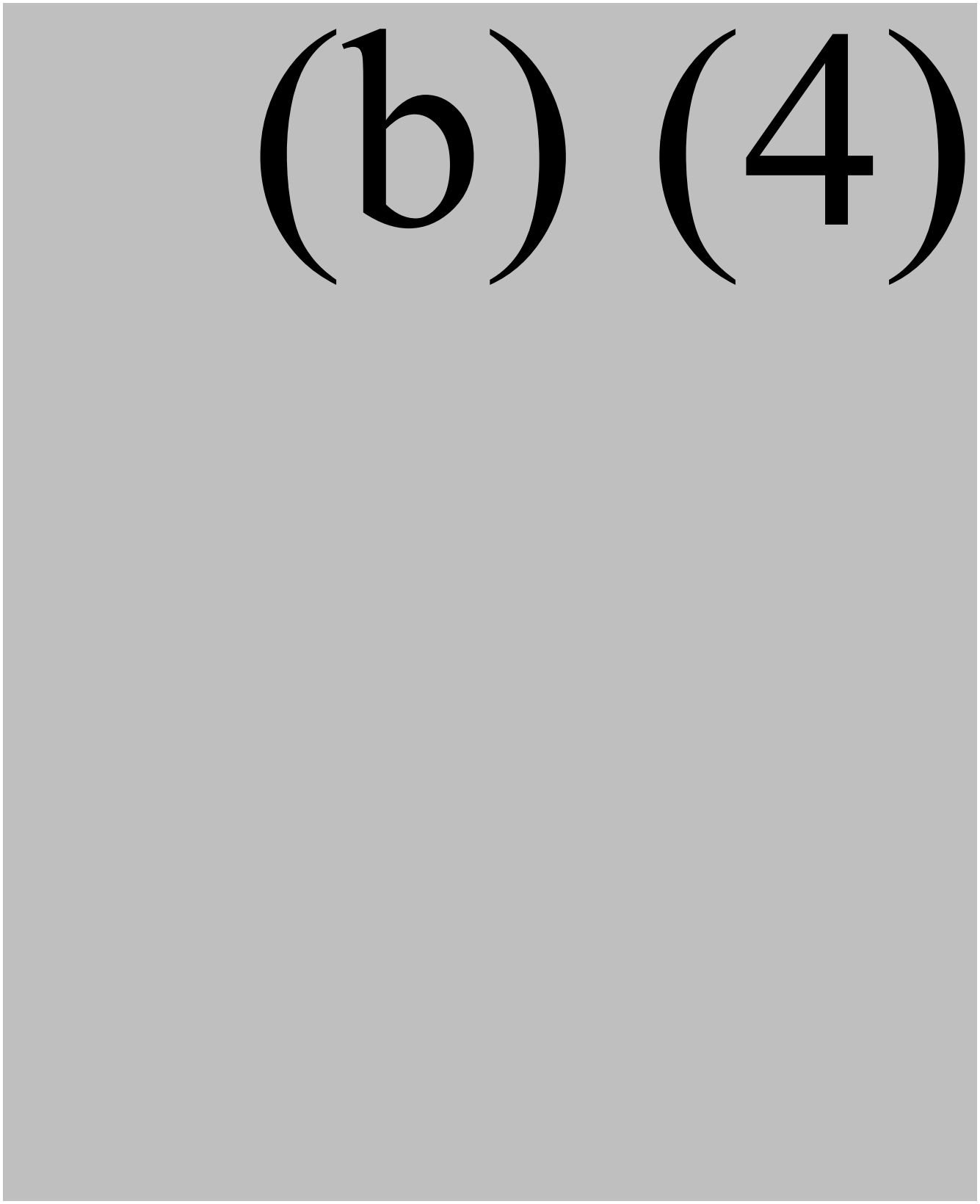
Please always use safety precautions when preparing solutions. Always add acid to water! Shake each solution after all the reagents are combined.

(b) (4)


(b) (4)

7.3 Test Sample Treatment

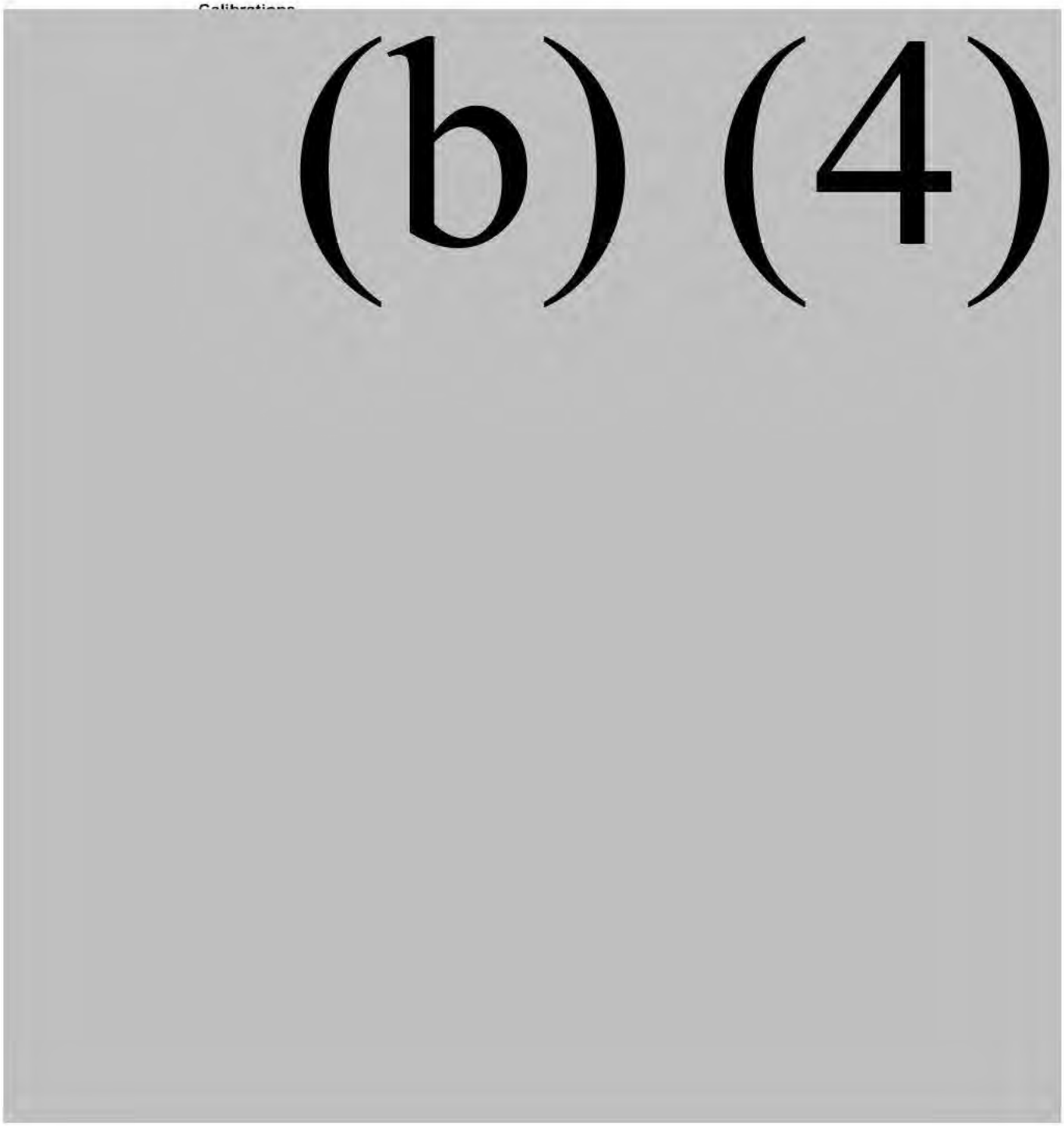
(b) (4)


(b) (4)

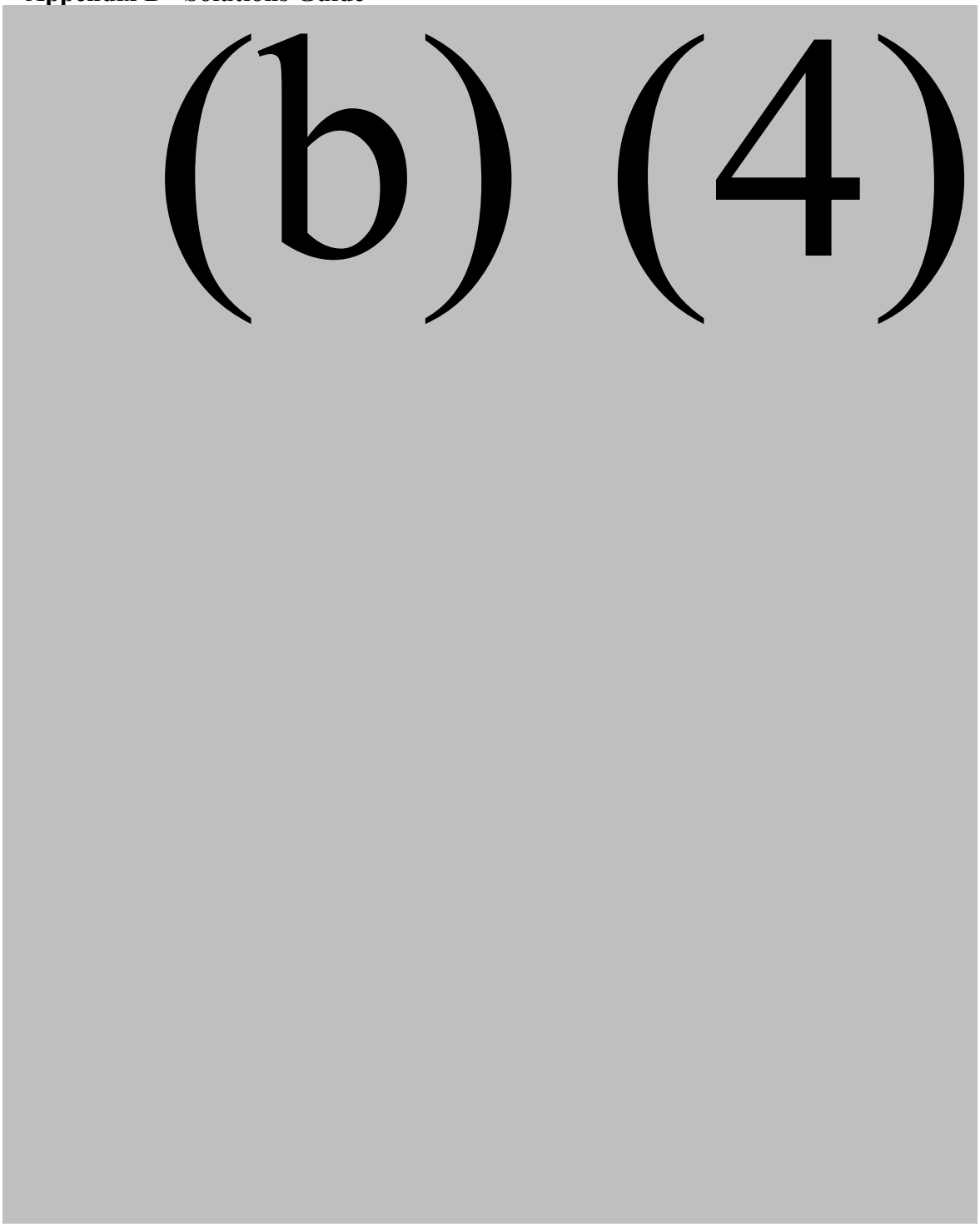
(b) (4)

(b) (4)

Appendix A - Calibration Concentrations



Appendix B - Solutions Guide



(b) (4)

(b) (4)

Name: Native Microbials, Inc.
Customer: (b)(6)
Address: 10255 Science Center Dr., Suite C2
San Diego, CA
92121
USA
877-696-8945

Order ID (b) (4)
Report ID
Date Received: 1/8/2021 10:00:51
Reported: 1/13/2021 15:28:35
P.O. #: N/A
Page: **1 of 1**

Report of Results

(b) (4) **Analysis Date:** 2021/01/08 **Receiving Temperature:** 2.0C **Sample Condition:** Okay

Description: Beef-53 Lot: 1801.2041 (says 2040)

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

(b) (4) **Analysis Date:** 2021/01/08 **Receiving Temperature:** 2.0C **Sample Condition:** Okay

Description: Beef-53 Lot: 1801.2042

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

(b) (4) **nalysis Date:** 2021/01/08 **Receiving Temperature:** 2.0C **Sample Condition:** Okay

Description: Beef-53 Lot: 1801.2044

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

(b) (4) **nalysis Date:** 2021/01/08 **Receiving Temperature:** 2.0C **Sample Condition:** Okay

Description: Beef-65 Lot: 1801.2039

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

(b) (4) **nalysis Date:** 2021/01/08 **Receiving Temperature:** 2.0C **Sample Condition:** Okay

Description: Beef-65 Lot: 1801.2043

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

(b) (4) **nalysis Date:** 2021/01/08 **Receiving Temperature:** 2.0C **Sample Condition:** Okay

Description: Beef-65 Lot: 1801.2045

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

(b) (4), (b)(6)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Product Data Sheet

Product Description:

(b) (4) 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:

(b) (4)

Typical Data:

(b) (4)

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

Packaging:

(b) (4) is available in 50 lb. beaded poly-lined cartons and in bulk liquid.

Storage and Handling:

(b) (4) 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. *

(b) (4)

(b) (4)

* Actual shelf-life may vary and is dependent 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 performance of (b) (4) in their finished product.

(b) (4)

Rev. date 06/09

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

(b) (4)

(b) (4)

Product Type	**FOOD GRADE – GENERAL PURPOSE PROCESS AID DEFOAMER**
Product Description	(b) (4) is a defoamer designated to control foam in many processes. (b) (4) 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 (b) (4) 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. (b) (4) 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 (b) (4) include

- Fermentation
- Egg washing

Incorporation

(b) (4) 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)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	2JI0075
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

Test	Specification		Result
	min	max	
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			< 3000 ppm

(b) (4), (b)(6)

(b) (4)

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

(b) (4)

Dear (b) (4)

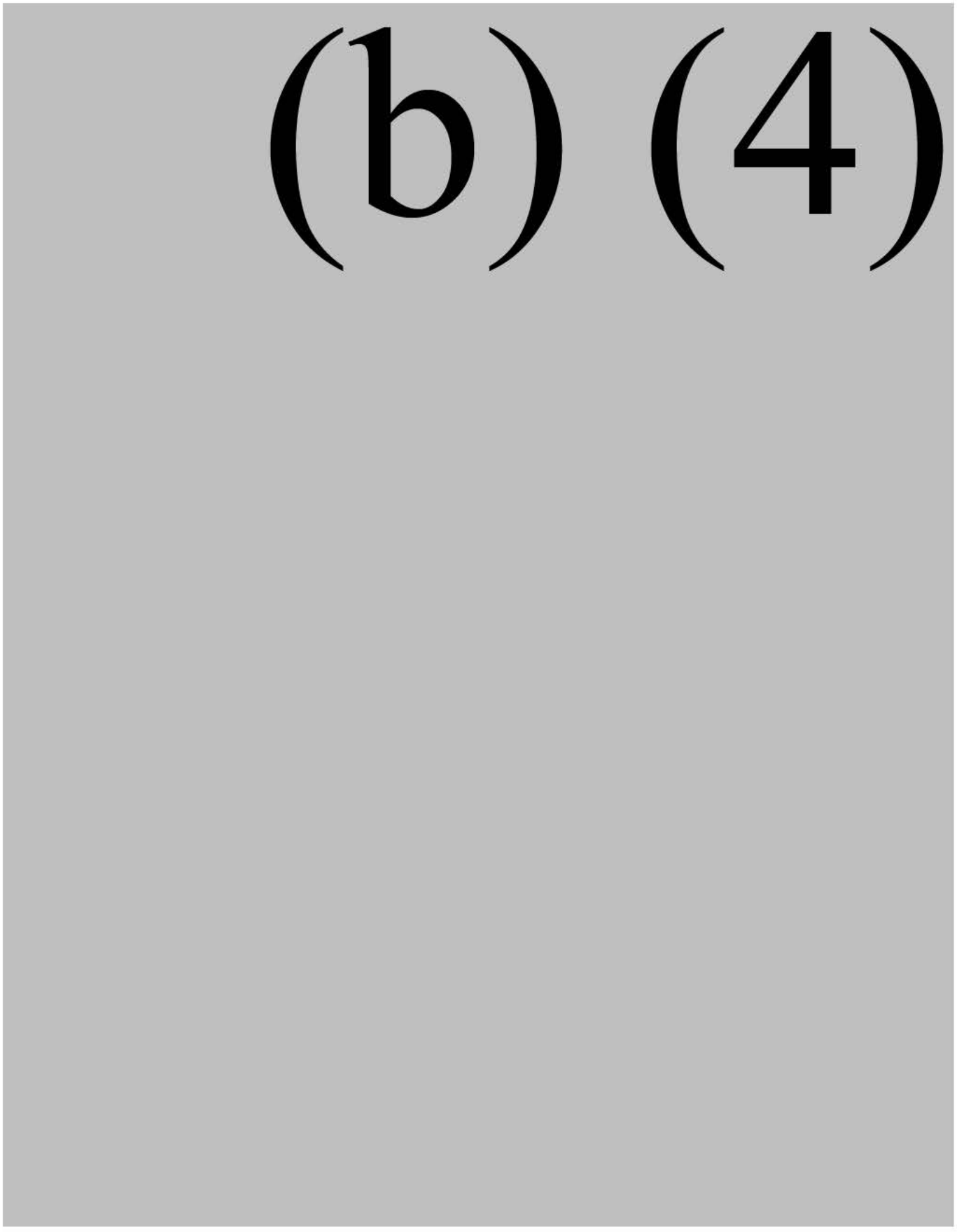
You requested, on behalf of the (b) (4) that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer),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

(b)(6)

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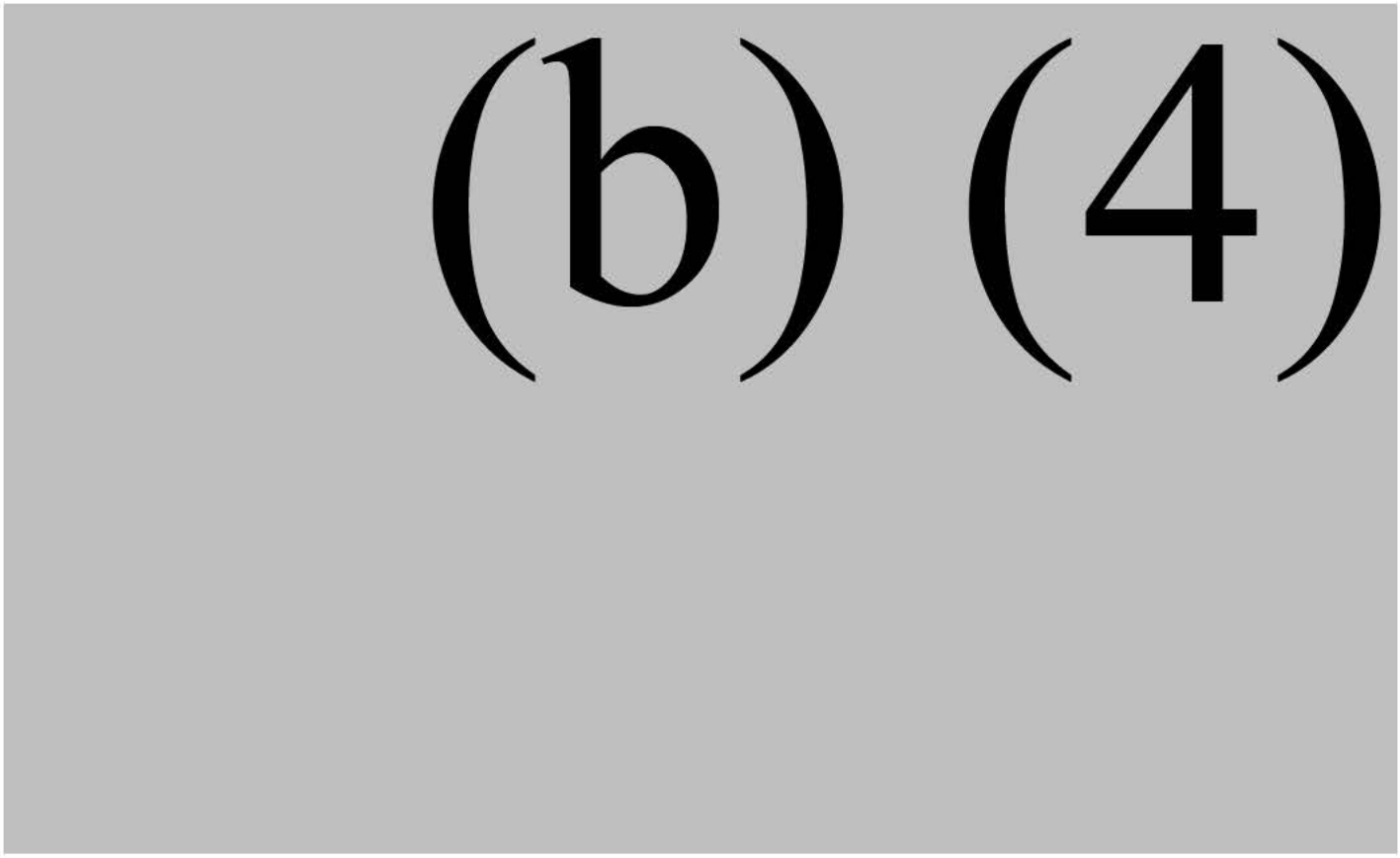
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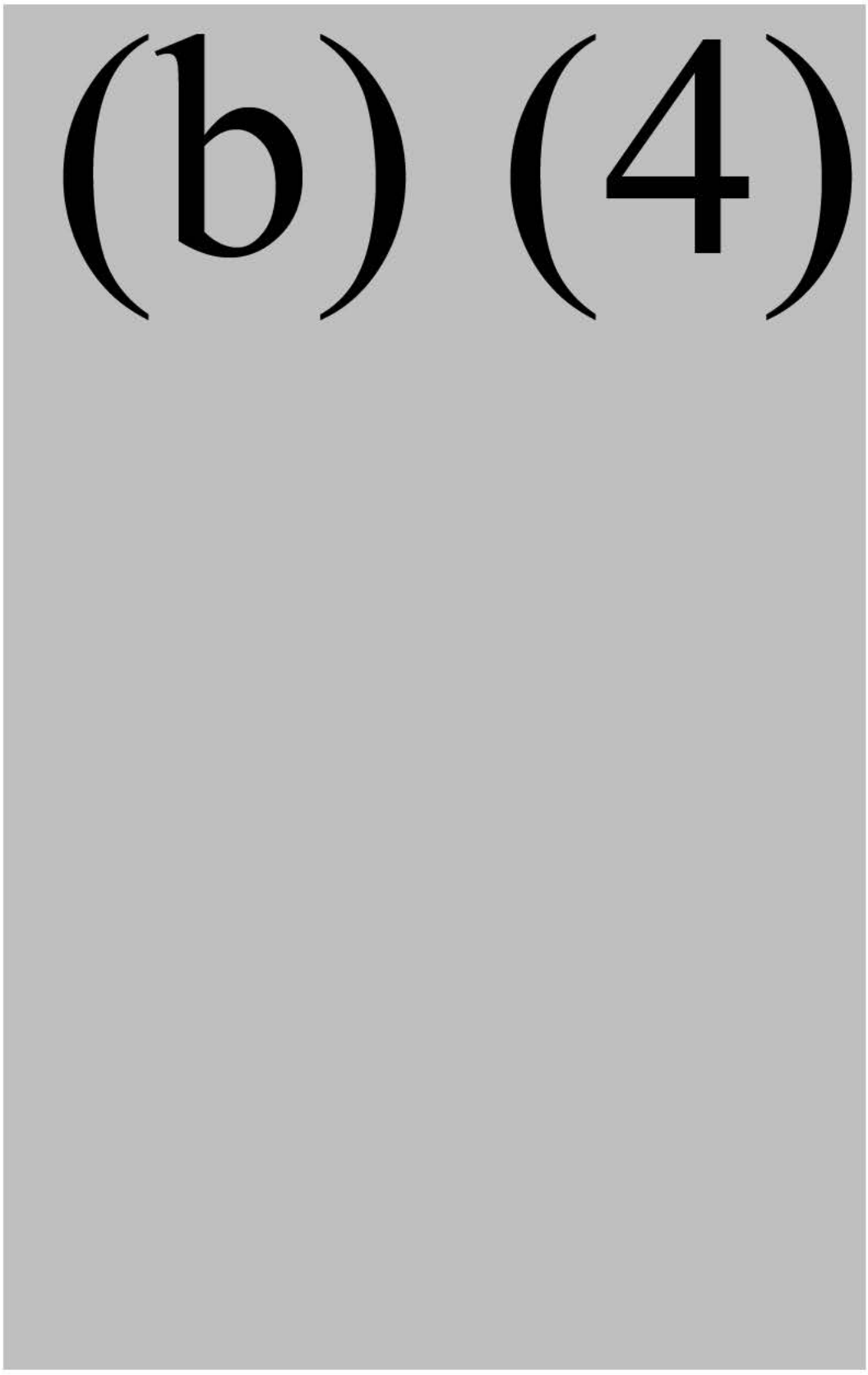
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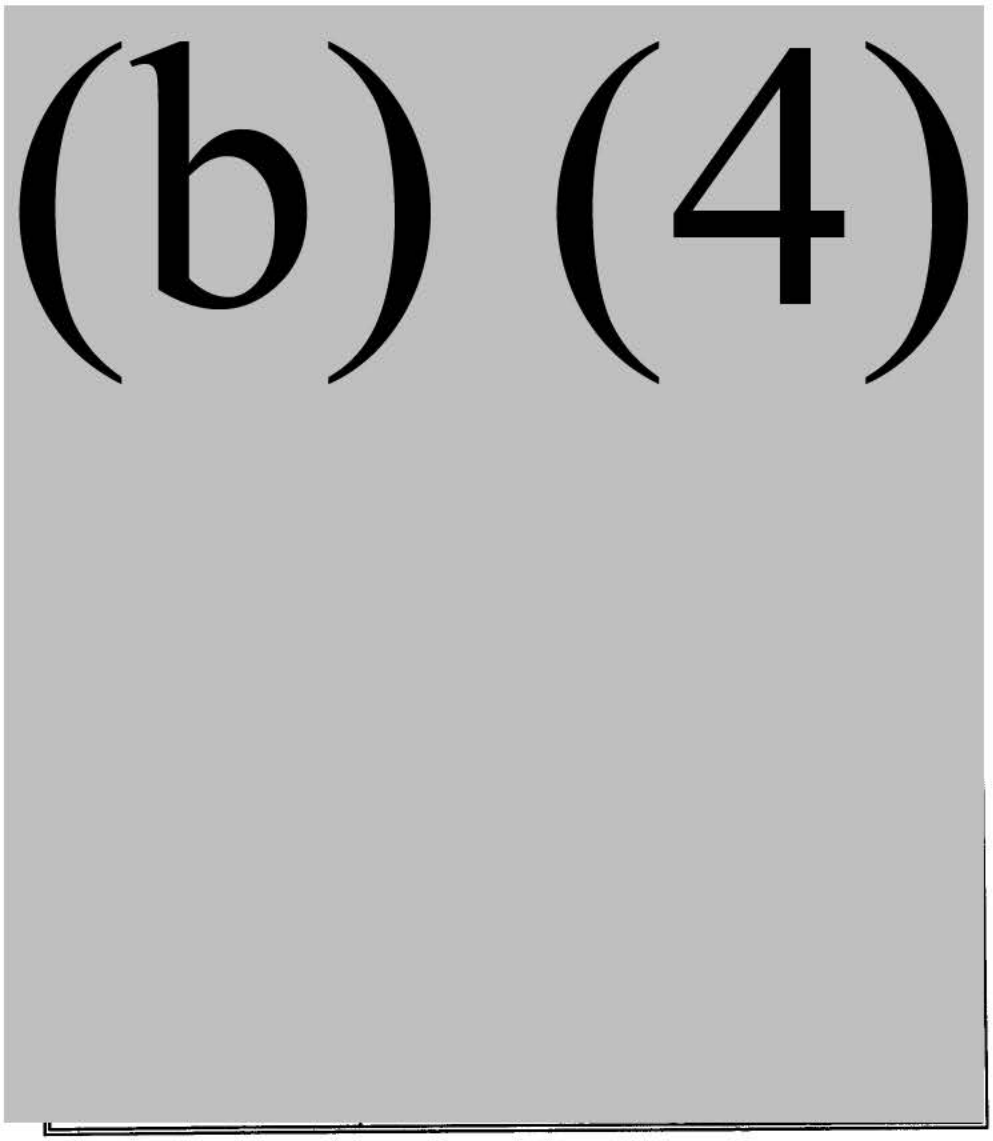

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	2JI0075
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

Test	Specification		Result
	min	max	
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			< 3000 ppm

(b) (4), (b)(6)

(b) (4)

(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 %	(b) (4)
SPECIFIC ROTATION $[a]_D$	+5.7° to +6.8°		
LOSS ON DRYING	8.0	12.0 %	
RELATED COMPOUNDS:			
INDIVIDUAL IMPURITY		0.5 %	
TOTAL IMPURITIES		2.0 %	
RESIDUE ON IGNITION		0.4 %	
SULFATE		0.03 %	
IRON		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), (b)(6)

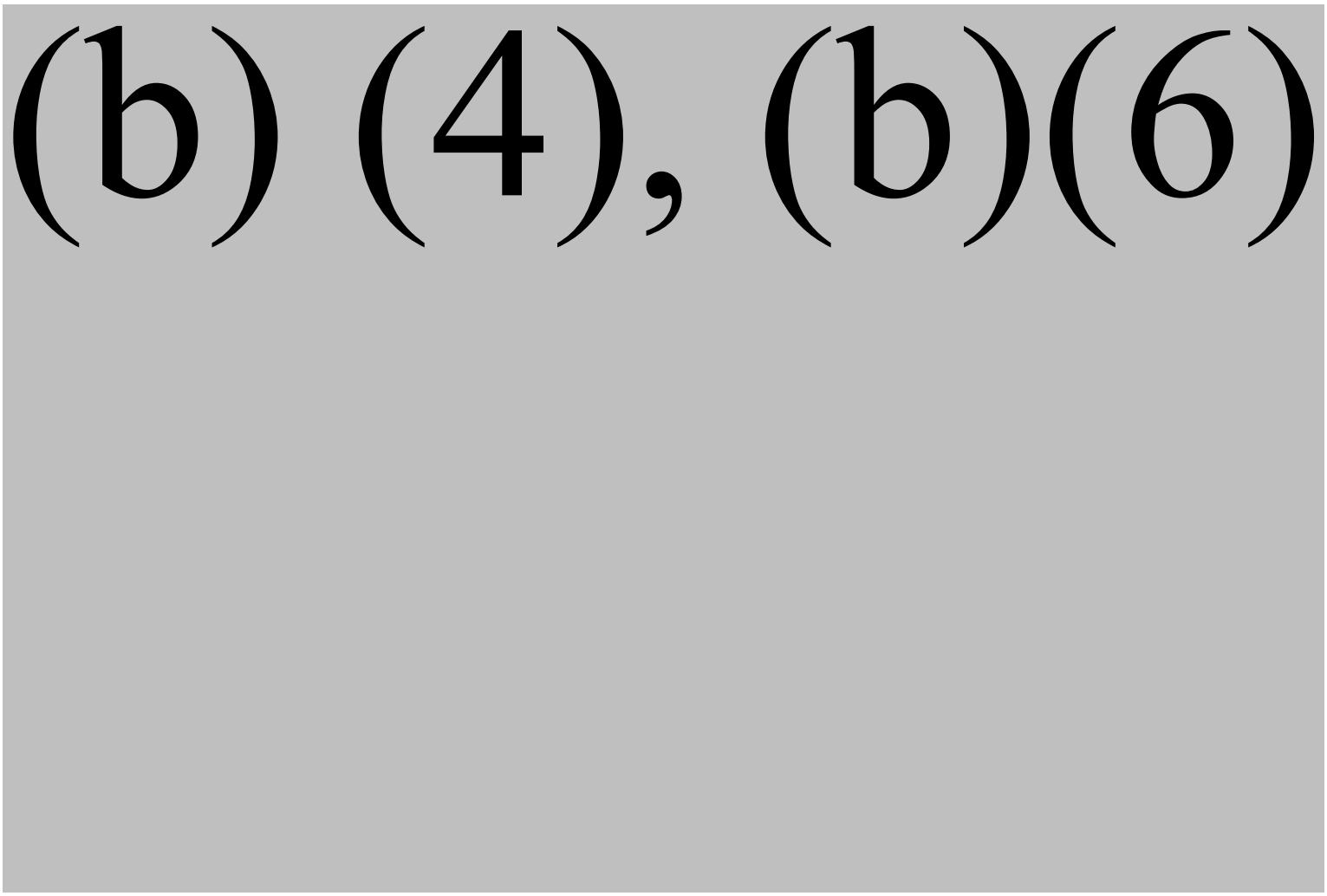
(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	PO204	Lot Number	1JH0518
Item	Dibasic Potassium Phosphate, Anhydrous, USP, EP, BP	Manufacturer Lot	26660003
CAS Number	7758-11-4	Manufacturer Code	283
Molecular Formula	K_2HPO_4	Molecular Weight	174.18

Test	Specification		Result
	min	max	
ASSAY (K_2HPO_4; DRIED BASIS)	98.0	100.5 %	(b) (4)
pH OF A 1 IN 20 SOLUTION	8.5	9.6	
LOSS ON DRYING		1.0 %	
INSOLUBLE SUBSTANCES		0.2 %	
CARBONATE	NOT MORE THAN A FEW BUBBLES ARE EVOLVED		NOT MORE THAN A FEW BUBBLES ARE EVOLVED
CHLORIDE (Cl)		0.02 %	(b) (4)
SULFATE		0.1 %	
ARSENIC (As)		2 ppm	
IRON (Fe)		0.001 %	
SODIUM (Na)	NO YELLOW COLOR		
SODIUM (EP)		0.1 %	
ELEMENTAL IMPURITIES	AS REPORTED		COMPLIES WITH STANDARD
FLUORIDE		0.001 %	(b) (4)
MONOBASIC OR TRIBASIC SALT		0.4 ml	
MONOPOTASSIUM PHOSPHATE		2.5 %	
APPEARANCE OF SOLUTION	CLEAR AND COLORLESS		CLEAR AND COLORLESS
REDUCING SUBSTANCES	SOLUTION REMAINS PINK		SOLUTION REMAINS PINK
IDENTIFICATION (A)	POSITIVE FOR POTASSIUM		POSITIVE FOR POTASSIUM
IDENTIFICATION (B)	POSITIVE FOR PHOSPHATE		POSITIVE FOR PHOSPHATE
IDENTIFICATION (C)	SLIIGTHLY ALKALINE		SLIIGTHLY ALKALINE
CERTIFIED KOSHER			CERTIFIED KOSHER
APPEARANCE			WHITE POWDER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			04-MAR-2023
DATE OF MANUFACTURE			04-MAR-2020
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION (USP)			(USP) 42
MONOGRAPH EDITION (EP)			(EP) 10
MONOGRAPH EDITION (BP)			(BP) 2020


(b) (4), (b)(6)

(b) (4)

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 %		(b) (4)
ARSENIC (As)		3 mg/kg	
FLUORIDE		10 mg/kg	
INSOLUBLE SUBSTANCES		0.2 %	
LEAD (Pb)		2 mg/kg	
LOSS ON DRYING		1 %	
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), (b)(6)

(b) (4)

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 %	(b) (4)
pH OF A 3% SOLUTION @ 25°C	7.5	9.2	
LOSS ON DRYING		1.0 %	
INSOLUBLE MATTER		0.05 %	
CHLORIDE (Cl)		350 ppm	
SULFATES (SO_4)		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), (b)(6)

(b) (4)

(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 %		(b) (4)
ARSENIC (As)		3 mg/kg	
CARBONATE (as Na_2CO_3)		3.0 %	
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), (b)(6)

(b) (4)

Specifications for Sodium Sulfate

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

(b) (4)

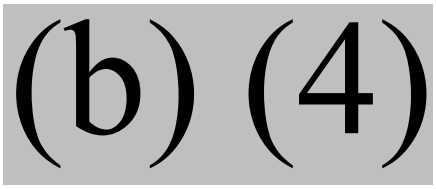
Certificate Of Analysis

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°		(b) (4)
CONDUCTIVITY @ 20 C		35 µS/cm	
COLOR VALUE		75	
LOSS ON DRYING		0.1 %	
SULFITE		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), (b)(6)

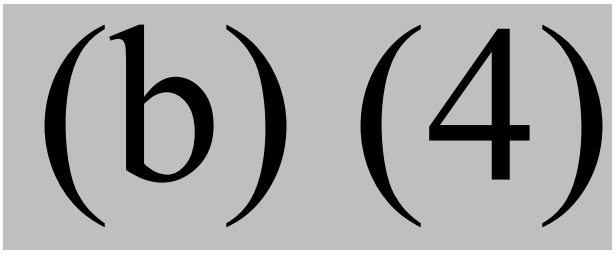
(b) (4)



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		



Biotin

(b) (4)

Specification for Biotin, Powder, FCC (BI115)

Item Number	BI115
Item	Biotin, Powder, FCC
CAS Number	58-85-5
Molecular Formula	$C_{10}H_{16}N_2O_3S$
Molecular Weight	244.31
MDL Number	
Synonyms	Vitamin H

Test	Specification	
	Min	Max
ASSAY ($C_{10}H_{16}N_2O_3S$)	97.5-100.5 %	
MELTING RANGE	229 - 232 C (dec	
OPTICAL ROTATION	+89 to+93	
LEAD (Pb)		2 mg/kg
IDENTIFICATION		TO PASS TEST
RETEST DATE		

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	CA159	Lot Number	1IK0060
Item	Calcium Pantothenate, Powder, USP		
CAS Number	137-08-6		
Molecular Formula	$C_{18}H_{32}CaN_2O_{10}$	Molecular Weight	476.53

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.0	102.0 %	(b) (4)
CALCIUM CONTENT (Ca; DRIED BASIS)	8.2	8.6 %	
OPTICAL ROTATION	+25.0° to +27.5°		
LOSS ON DRYING		5.0 %	
ALKALINITY	NO PINK COLOR		
ELEMENTAL IMPURITIES:			
CADMIUM (Cd)	AS REPORTED		
LEAD (Pb)	AS REPORTED		
ARSENIC (As)	AS REPORTED		
MERCURY (Hg)	AS REPORTED		
CHROMIUM (Cr)	AS REPORTED		
IDENTIFICATION (A)	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
IDENTIFICATION (B)	POSITIVE FOR CALCIUM		POSITIVE FOR CALCIUM
IDENTIFICATION (C)	+25.0°to+27.5°		+26.0°
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			31-MAY-2022
DATE OF MANUFACTURE			01-JUN-2019
APPEARANCE			WHITE POWDER
RESIDUAL SOLVENTS	AS REPORTED		.
CLASS 2 (SOLVENT) / METHANOL			<3000 ppm

(b) (4), (b)(6)

(b) (4)

(b) (4) **Certificate Of Analysis**

Item Number	C1454	Lot Number	1HE1065
Item	Vitamin B12, FCC		
CAS Number	68-19-9		
Molecular Formula	$C_{63}H_{88}CoN_{14}O_{14}P$	Molecular Weight	1355.37

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	96.0	100.5 %	(b) (4)
LOSS ON DRYING		12.0 %	
PSEUDO CYANOCOBALAMIN		TO PASS TEST	PASSES TEST
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			09-MAY-2022
DATE OF MANUFACTURE			10-MAY-2017
APPEARANCE			DARK RED POWDER

(b) (4), (b)(6)

(b) (4) **Certificate Of Analysis**

Item Number	C1454	Lot Number	1HE1065
Item	Vitamin B12, FCC		
CAS Number	68-19-9		
Molecular Formula	$C_{63}H_{88}CoN_{14}O_{14}P$	Molecular Weight	1355.37

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	96.0	100.5 %	(b) (4)
LOSS ON DRYING		12.0 %	
PSEUDO CYANOCOBALAMIN		TO PASS TEST	PASSES TEST
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			09-MAY-2022
DATE OF MANUFACTURE			10-MAY-2017
APPEARANCE			DARK RED POWDER

(b) (4), (b)(6)

(b) (4)

SPECIFICATIONS

Ref: B31-003A40

(b) (4)

DEFINITION :

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

SPECIFICATIONS :

(b) (4)

LOSS ON DRYING (%)
REDUCING SUGARS (% d.b. Bertrand)
pH
ASH (% d.b.)
PROTEIN (% d.b.)
NITROGEN (% d.b.)
AMINO NITROGEN (% d.b.)
ACIDITY as LACTIC ACID (% d.b.)
PHOSPHOROUS (total, % d.b.)

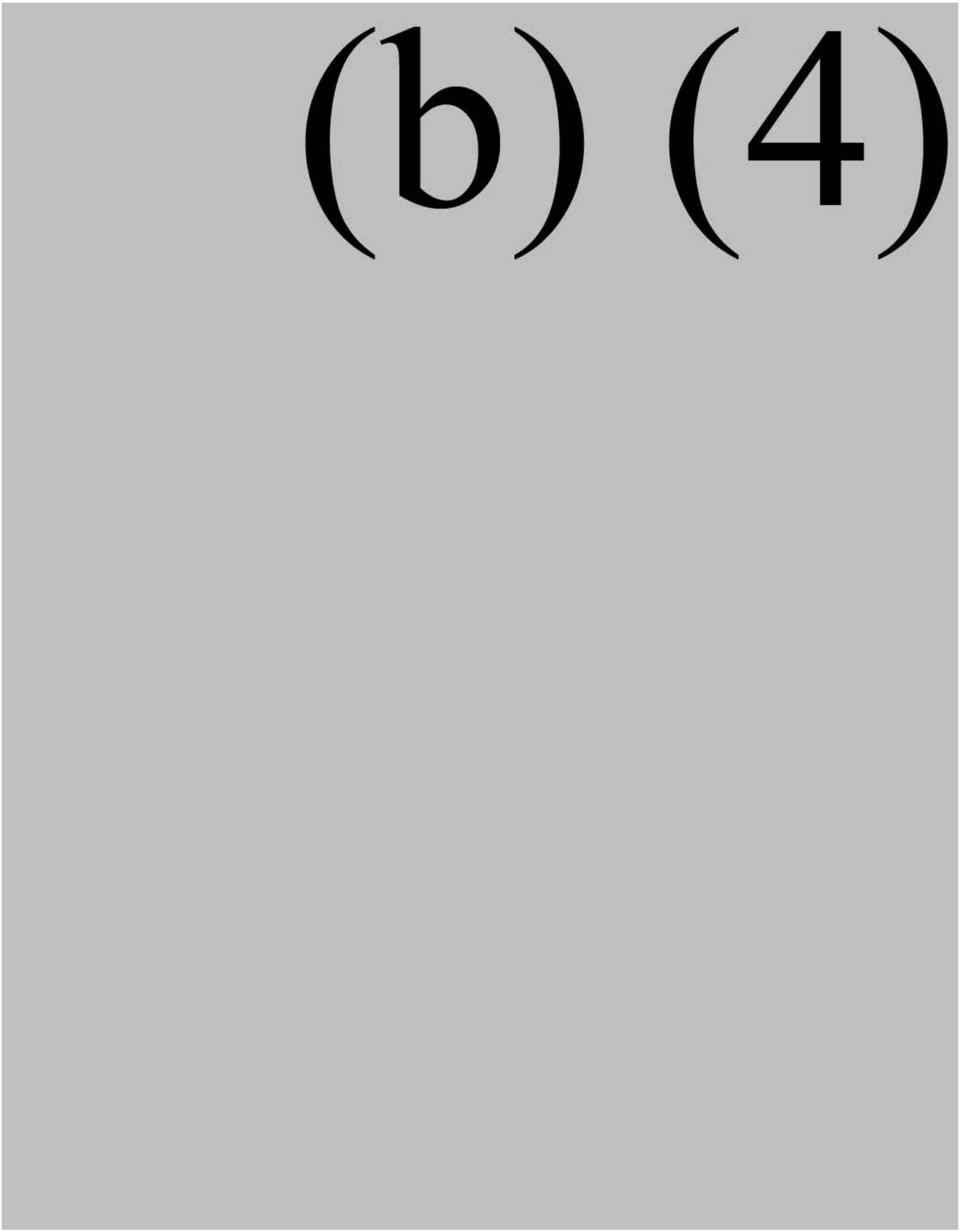
COMMENTS :

(b) (4) is a spray-dried version of the Roquette (b) (4) corn steep liquor. (b) (4) 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) (4), (b)(6)

February 10, 2016

(b) (4)


(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	F1000	Lot Number	1IG0330
Item	Ferric Ammonium Citrate, Brown, Powder, FCC		
CAS Number	1185-57-5		
Molecular Formula		Molecular Weight	

Test	Specification		Result
	min	max	
ASSAY (Fe)	16.5 - 18.5 %		(b) (4)
FERRIC CITRATE		TO PASS TEST	PASSES TEST
OXALATE (C_2O_4)		TO PASS TEST	PASSES TEST
LEAD (Pb)		2 mg/kg	0.01 mg/kg
MERCURY		1 mg/kg	<1 mg/kg
SULFATE		0.3 %	<0.3 %
IDENTIFICATION		TO PASS TEST	PASSES TEST
RETEST DATE			04-JUN-2022
DATE OF MANUFACTURE			04-JUN-2019
APPEARANCE			BROWN POWDER

(b) (4), (b)(6)

(b) (4)

Certificate Of Analysis

Item Number	FO105	Lot Number	2JJ0366
Item	Folic Acid, Powder, USP		
CAS Number	59-30-3		
Molecular Formula	$C_{19}H_{19}N_7O_6$	Molecular Weight	441.40

Test	Specification		Result
	min	max	
ASSAY (ANHYDROUS BASIS)	97.0	102.0 %	(b) (4)
WATER DETERMINATION		8.5 %	
RESIDUE ON IGNITION		0.3 %	
RELATED COMPOUNDS		2.0 %	
ELEMENTAL IMPURITIES:			
CADMIUM (Cd)	AS REPORTED		
LEAD (Pb)	AS REPORTED		
ARSENIC (As)	AS REPORTED		
MERCURY (Hg)	AS REPORTED		
IDENTIFICATION A . ULTRAVIOLET ABSORPTION	The ratio A256 / A365 is 2.80 - 3.00		
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			26-SEP-2021
DATE OF MANUFACTURE			27-SEP-2019
APPEARANCE			ORANGE POWDER
RESIDUAL SOLVENTS:	AS REPORTED		NO RESIDUAL SOLVENTS USED

(b) (4), (b)(6)

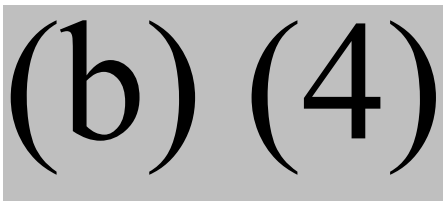
(b) (4)

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 %		(b) (4)
COLOR		TO PASS TEST	
SPECIFIC GRAVITY		TO PASS TEST	
IRON (Fe)		5 mg/kg	
LEAD (Pb)		1 mg/kg	
MERCURY		0.10 mg/kg	
NONVOLATILE RESIDUE		0.5 %	
ORGANIC COMPOUNDS		TO PASS TEST	
OXIDIZING SUBSTANCES (as Cl2)		0.003 %	
REDUCING SUBSTANCES (as SO_3)		0.007 %	
SULFATE		0.5 %	
IDENTIFICATION		TO PASS TEST	PASSES TEST
EXPIRATION DATE			28-FEB-2021
DATE OF MANUFACTURE			28-FEB-2019
APPEARANCE			CLEAR COLORLESS LIQUID

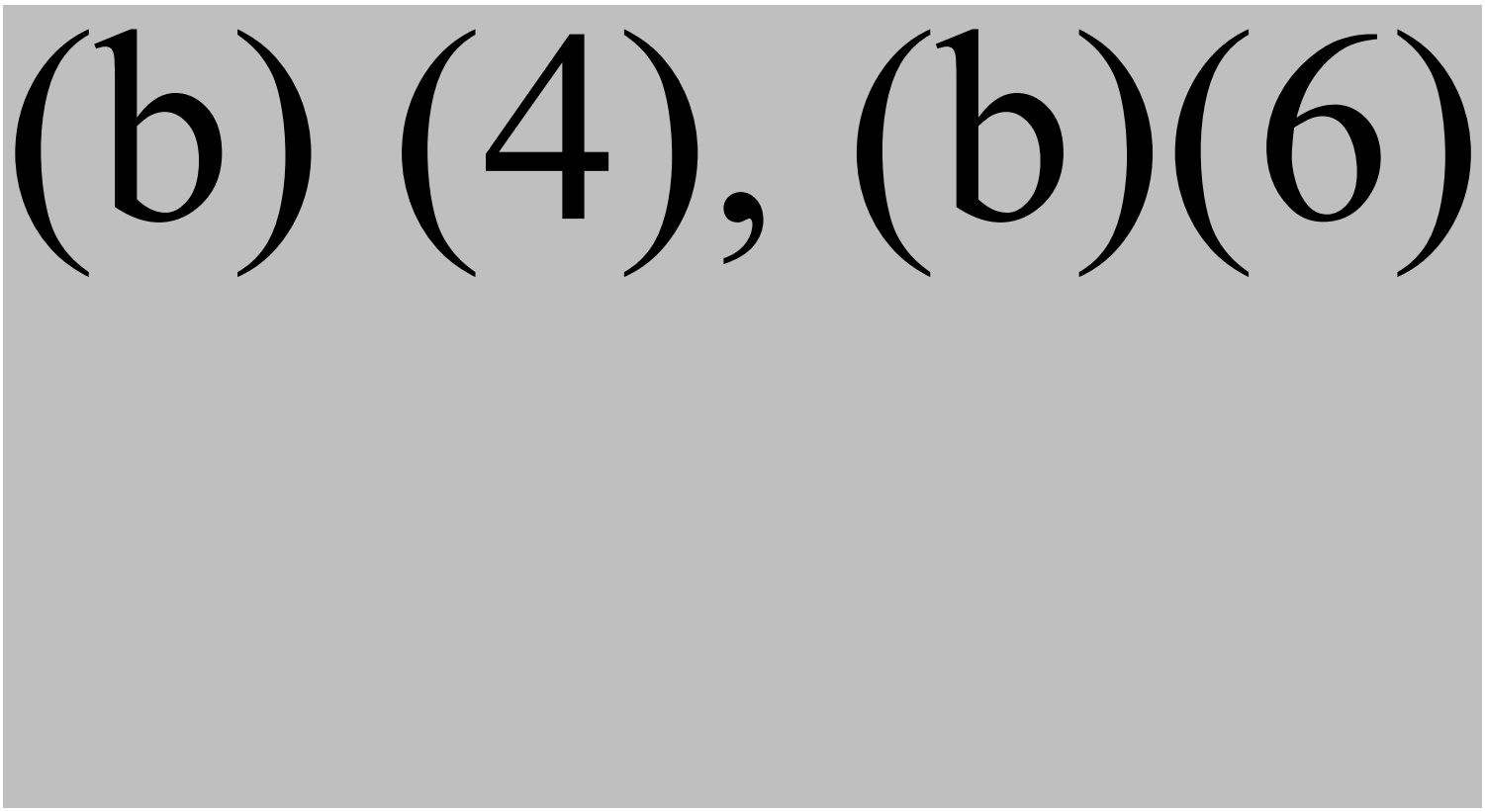
(b) (4), (b)(6)



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 %	(b) (4)
ARSENIC		3 ppm	
LEAD		10 ppm	
MERCURY		3 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

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 %	(b) (4)
pH OF A 5% SOLUTION @ 25°C	5.0	9.2	
LOSS ON IGNITION	48.0	52.0 %	
APPEARANCE OF SOLUTION	TO PASS TEST		PASSES TEST
IDENTIFICATION	TO PASS TEST		PASSES TEST
CHLORIDE		0.014 %	(b) (4)
IRON (Fe)		20 µg/g	
ELEMENTAL IMPURITIES	AS REPORTED		
SELENIUM		30 µg/g	
ALKALINITY OR ACIDITY	TO PASS TEST		
ARSENIC		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), (b)(6)

(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%	(b) (4)
ASSAY (IGNITED)	99.0	101.0%	
LOSS ON HEATING	10.0	12.0%	
APPEARANCE OF SOLUTION	TO PASS TEST		
ARSENIC (As)		3 mg/kg	
LEAD (Pb)		4 mg/kg	
SELENIUM (Se)		0.003%	
HEAVY METALS		20 ppm	
ELEMENTAL IMPURITIES		AS REPORTED	COMPLIES WITH STANDARD
IRON		10 ppm	(b) (4)
ZINC (Zn)		50 ppm	
CHLORIDE (Cl)		100 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), (b)(6)

(b) (4)

Certificate Of Analysis

Item Number	NI100	Lot Number	1JD0426
Item	Niacin, Powder, USP		
CAS Number	59-67-6		
Molecular Formula	$C_6H_5NO_2$	Molecular Weight	123.11

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.0%	102.0%	(b) (4)
LOSS ON DRYING		1.0 %	
RESIDUE ON IGNITION		0.1 %	
CHLORIDE (Cl)		0.02 %	
SULFATES (SO_4)		0.02 %	
ELEMENTAL IMPURITIES:			
CADMIUM (Cd)		AS REPORTED	
LEAD (Pb)		AS REPORTED	
ARSENIC (As)		AS REPORTED	
MERCURY (Hg)		AS REPORTED	
RELATED COMPOUNDS		TO PASS TEST	PASSES TEST
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			WHITE PWDER
EXPIRATION DATE			20-JUN-2022
DATE OF MANUFACTURE			21-JUN-2019
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (4), (b)(6)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	AM150	Lot Number	1HL0262
Item	Aminobenzoic Acid, USP		
CAS Number	150-13-0		
Molecular Formula	$C_7H_7NO_2$	Molecular Weight	137.14

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	98.0	102.0 %	(b) (4)
LOSS ON DRYING		0.2 %	
RESIDUE ON IGNITION		0.1 %	
ELEMENTAL IMPURITIES:			
LEAD (Pb)	AS REPORTED		
ARSENIC (As)	AS REPORTED		
ANILINE		10 ppm	
p-TOLUIDINE		10 ppm	
ORGANIC IMPURITIES:			
4-NITROBENZOIC ACID		0.2 %	
BENZOCAINE		0.2 %	
ANY INDIVIDUAL, UNSPECIFIED IMPURITY		0.1 %	
TOTAL IMPURITIES		0.5 %	
IDENTIFICATION A) FTIR	(b) (4) MATCHES REFERENCE		(b) (4) MATCHES REFERENCE
IDENTIFICATION B (HPLC)	RETENTION TIME MATCHES STANDARD		RETENTION TIME MATCHES STANDARD
EXPIRATION DATE			01-DEC-2020
DATE OF MANUFACTURE			01-DEC-2018
APPEARANCE			WHITE POWDER
RESIDUAL SOLVENTS		AS REPORTED	NO RESIDUAL SOLVENTS USED

(b) (4), (b)(6)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	PH195	Lot Number	1JD0740
Item	Phytonadione, USP		
CAS Number	81818-54-4		
Molecular Formula	$C_{31}H_{46}O_2$	Molecular Weight	450.70

Test	Specification		Result
	min	max	
ASSAY	97.0	103.0 %	(b) (4)
REFRACTIVE INDEX @ 25°C	1.523	1.526	
REACTION	NEUTRAL TO LITMUS		NEUTRAL TO LITMUS
MENADIONE	NO PURPLE OR BLUE COLOR		NO PURPLE OR BLUE COLOR
Z-ISOMER CONTENT		21.0 %	(b) (4)
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 (UV)		3.0 %	<3.0 %
EXPIRATION DATE			29-NOV-2021
DATE OF MANUFACTURE			30-NOV-2019
APPEARANCE			CLEAR YELLOW LIQUID
RESIDUAL SOLVENTS	AS REPORTED		.
CLASS 2 (SOLVENT) / METHANOL			(b) (4)
CLASS 3 (solvent) / ACETONE			
CLASS 3 (solvent) / ETHANOL			
MONOGRAPH EDITION			(USP) 42

(b) (4), (b)(6)

(b) (4)

Safety Evaluation of Phytonadione (Natural Vitamin K1) for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

Native Microbials, Inc.

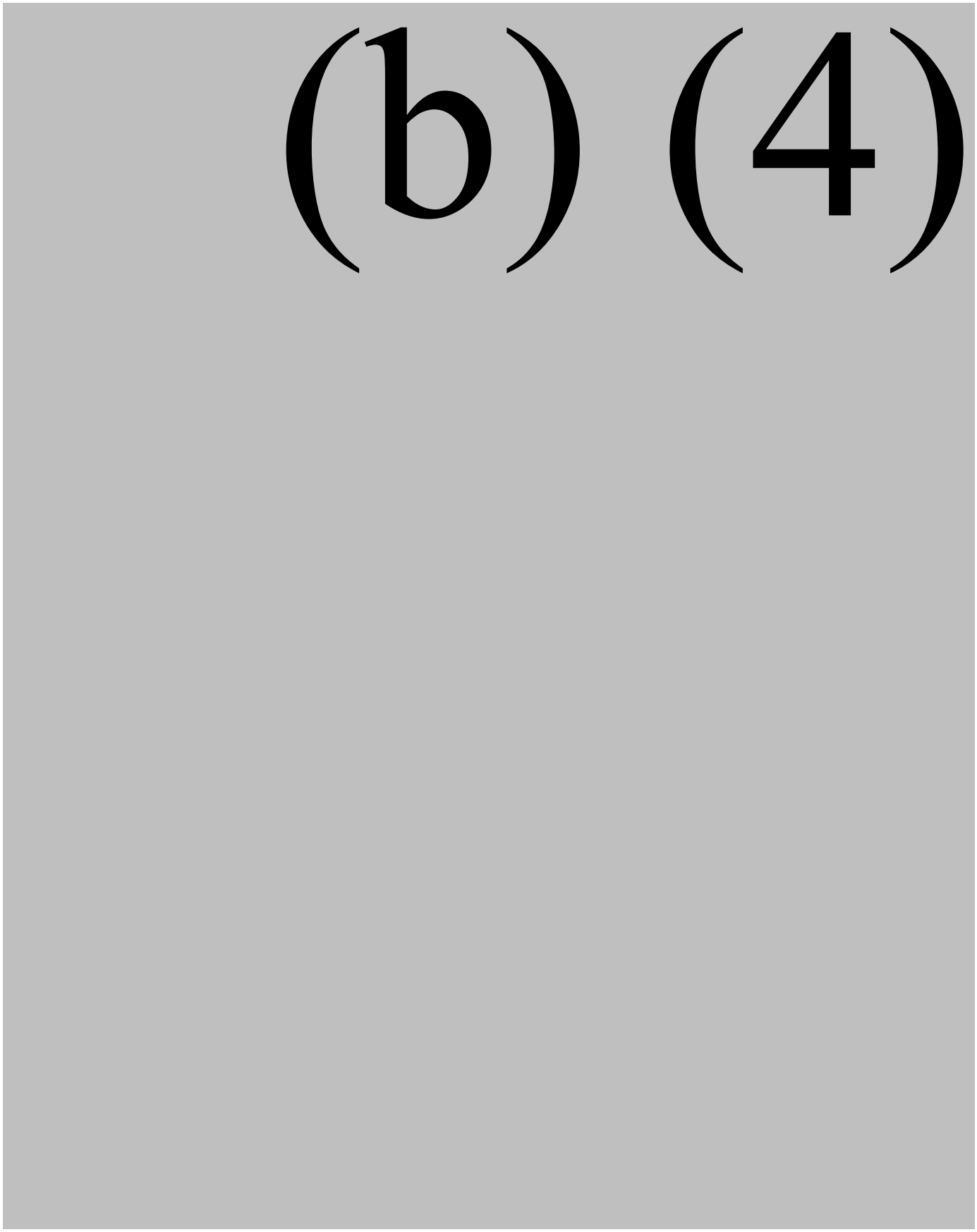
January, 2021

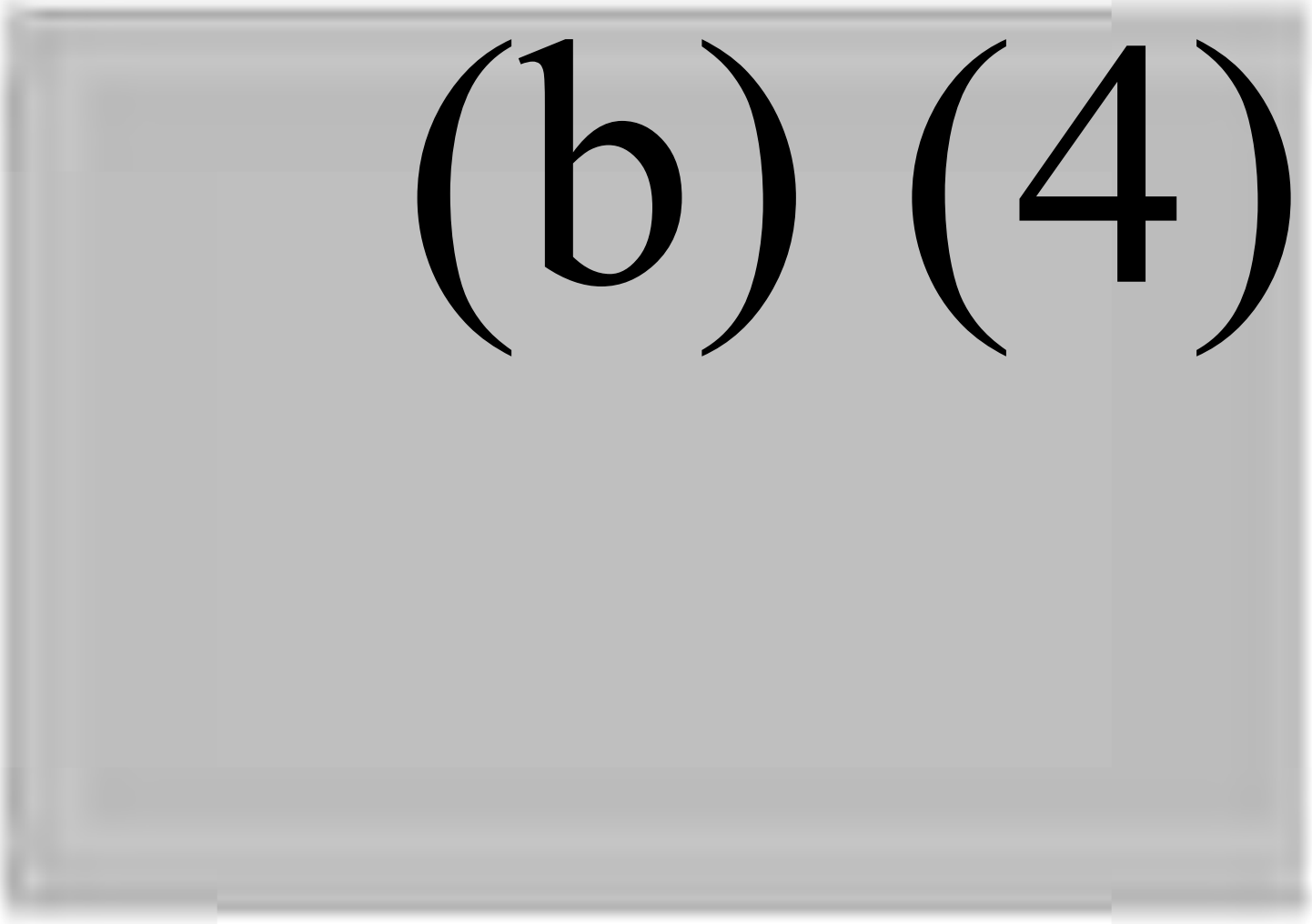
Safety Evaluation of Phytonadione (Natural Vitamin K1) for Use in the Production of Direct-Fed Microbials for Use in Animal Feed

1. INTRODUCTION

Native Microbials, Inc. develops direct-fed microbial (DFM) products for use as supplementary feeds for poultry and cattle in the United States (U.S.). (b) (4)

(b) (4)


(b) (4)


(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	RI103	Lot Number	1JE0551
Item	Riboflavin, USP		
CAS Number	83-88-5		
Molecular Formula	$C_{17}H_{20}N_4O_6$	Molecular Weight	376.36

Test	Specification		Result
	min	max	
ASSAY ($C_{17}H_{20}N_4O_6$)	98.0%	102.0 %	(b) (4)
SPECIFIC ROTATION $[a]_D$	-115° to -135°		
LOSS ON DRYING		1.5 %	
RESIDUE ON IGNITION		0.3 %	
LUMIFLAVIN		0.025	
ELEMENTAL IMPURITIES:			
CADMIUM (Cd)	AS REPORTED		
LEAD (Pb)	AS REPORTED		
ARSENIC (As)	AS REPORTED		
MERCURY (Hg)	AS REPORTED		
IDENTIFICATION	PALE GREENISH YELLOW WITH YELLOWISH-GREEN FLUORESCENCE		PALE GREENISH YELLOW WITH YELLOWISH-GREEN FLUORESCENCE
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
APPEARANCE			ORANGE POWDER
EXPIRATION DATE			01-MAR-2022
DATE OF MANUFACTURE			02-MAR-2019
RESIDUAL SOLVENTS	AS REPORTED		NO RESIDUAL SOLVENTS USED
MONOGRAPH EDITION			(USP) 42

(b) (4), (b)(6)

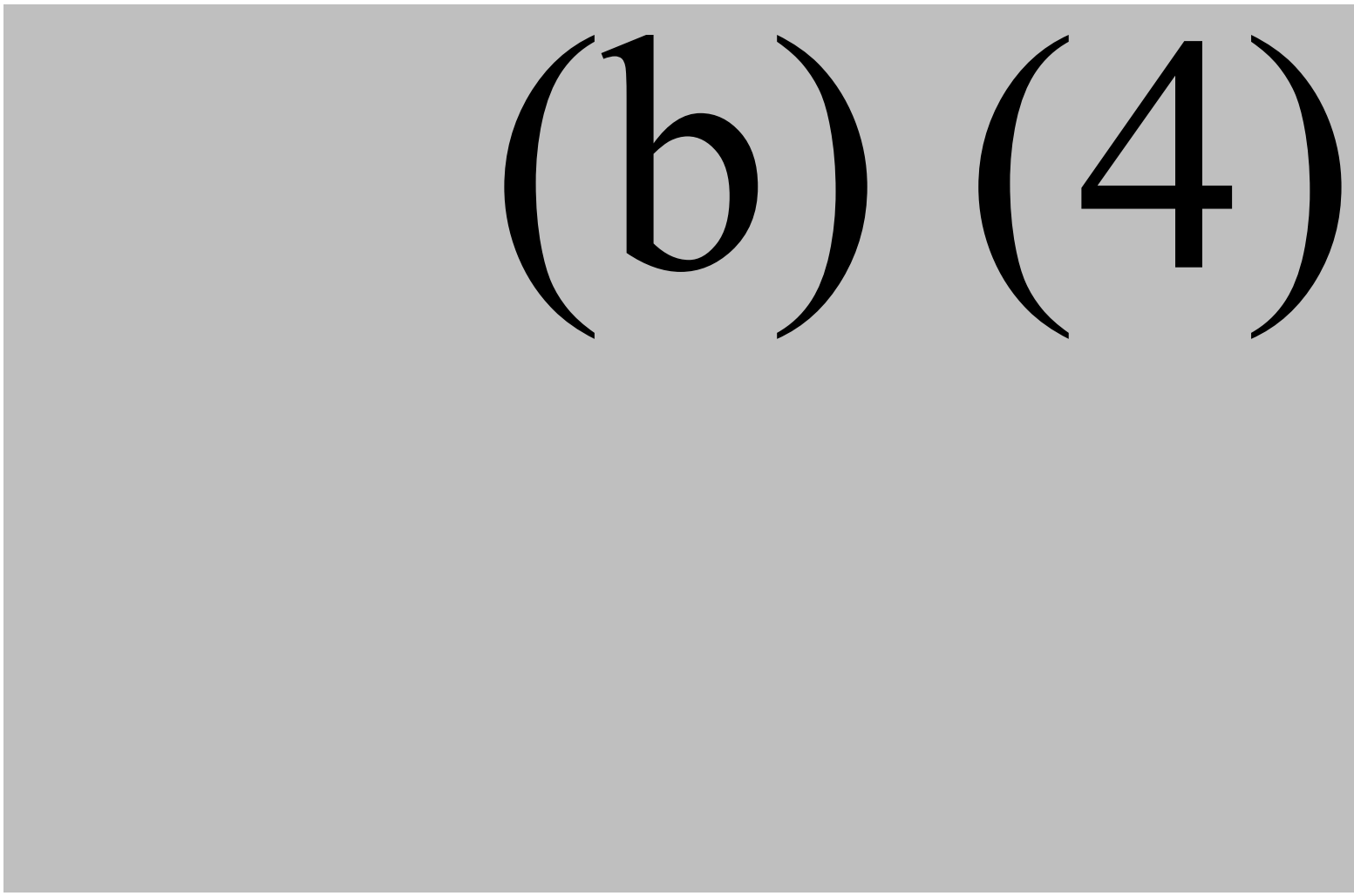
(b) (4)

(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 %	(b) (4)
APPEARANCE OF SOLUTION	CLEAR COLORLESS		
ACIDITY OR ALKALINITY		0.5 ml	
LOSS ON DRYING		0.5%	
ALUMINUM		0.2 ppm	
BROMIDES		100 ppm	
PHOSPHATES		25 ppm	
POTASSIUM		500 ppm	
IODIDES	NO BLUE COLOR		
MAGNESIUM AND ALKALINE-EARTH METALS (as Ca)		100 ppm	
ARSENIC (As)		1 ppm	
IRON (Fe)		2 ppm	
BARIUM (Ba)	OPALESCENCE LESS THAN REFERENCE		OPALESCENCE LESS THAN REFERENCE
FERROCYANIDES	NO BLUE COLOR		NO BLUE COLOR
SULFATE (SO_4)		200 ppm	(b) (4)
NITRITES		0.01	
BACTERIAL ENDOTOXINS		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


(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	1JA0625
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

Test	Specification		Result
	min	max	
ASSAY (ANHYDROUS BASIS)	91.0	100.5%	(b) (4)
pH OF A 10% (w/w) SOLUTION	3.5	7.0	
LEAD (Pb)		1 mg/kg	
NICKEL (Ni)		1 mg/kg	
REDUCING SUGARS		0.3%	
RESIDUE ON IGNITION		0.1 %	
WATER		1.5%	
IDENTIFICATION	TO PASS TEST		PASSES TEST
RETEST DATE			12-OCT-2021
DATE OF MANUFACTURE			01-OCT-2019
APPEARANCE			WHITE POWDER

(b) (4)

Appendix 009ZD Intentionally Left Blank

(b) (4)

Certificate Of Analysis

Item Number	T1053	Lot Number	2HG0513
Item	Thiamine Hydrochloride, FCC		
CAS Number	67-03-8		
Molecular Formula	$C_{12}H_{17}ClN_4OS.HCl$	Molecular Weight	337.27

Test	Specification		Result
	min	max	
ASSAY ($C_{12}H_{17}ClN_4OS.HCl$; ANHYDROUS BASIS)	98.0	102.0 %	(b) (4)
COLOR OF SOLUTION		TO PASS TEST	PASSES TEST
pH OF A 1 IN 100 SOLUTION	2.7 - 3.4		(b) (4)
LEAD (Pb)		2 mg/kg	
NITRATE (NO_3)		TO PASS TEST	PASSES TEST
RESIDUE ON IGNITION		0.2 %	(b) (4)
WATER		5.0 %	
IDENTIFICATION		TO PASS TEST	PASSES TEST
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			23-AUG-2020
DATE OF MANUFACTURE			24-AUG-2017
APPEARANCE			WHITE POWDER

(b) (4)

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

(b) (4)

Certificate of Analysis

(b) (4)

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

Customer PO No.: (b) (4)
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	(b) (4)
Ash %	AOAC 930.30	0	16.0	
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	
Moisture Loss on Drying %	AOAC 930.15	0	6.0	
pH (5% solution)	pH Meter	5.3	6.3	
Protein (N x 6.25) %	AOAC 990.03	55.0	100.0	
Salmonella /750g	AOAC RI 100201	NEGATIVE	NEGATIVE	
Salt as Chlorides %	AOAC 971.27	0	1.50	
Standard Plate Count cfu/g	AOAC 990.12	0	10000	
Yeast and Mold cfu/g	AOAC 121301	0	100	

(b) (4)

*ND = NOT DETECTED

Quality Assurance Manager

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

(b) (4)

(b) (4) Certificate of Analysis (b) (4)

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	(b) (4)
Ash %	AOAC 930.30	0	15.0	
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	
. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	
Moisture Loss on Drying %	AOAC 930.15	0	6.0	
pH (5% solution)	pH Meter	5.2	6.2	
Protein (N x 6.25) %	AOAC 990.03	70.0	100.0	
Salt as Chlorides %	AOAC 971.27	0	2.00	
Standard Plate Count cfu/g	AOAC 990.12	0	10000	
Yeast and Mold cfu/g	AOAC 121301	0	100	
Salmonella /375g	AOAC RI 100201	NEGATIVE	NEGATIVE	

(b) (4)

ND = NOT DETECTED

Quality Assurance Manager

Date: 01/25/19
Time: 15:28:53
Page 0 of 0

(b) (4)

Certificate of Analysis

(b) (4)

(b) (4)

d To: (b) (4)

Customer PO No.:
Customer Order No.: 157431
Item No.: SN2000027196 Amberferm 4210
50 LB Carton w/ Liner
Customer Item:
Lot No.: 3022424 300.000000 CT
Manufacture Date: 01/15/19
Lot Expiration Date: 01/15/21

Test Identification	Method	Min Value	Max Value	Test Value
MOISTURE METTLER POWDER		0	6.0	(b) (4)
PH (10% SOLUTION)		.5	5.5	
SALT AS CHLORIDES %		0	2.5	
AMINO NITROGEN/TOTAL NITROGEN		50.0	100.0	
ASH		0	12.0	
% EQUIV. PROTEIN (NX6.25)		74.0	100.0	
FLAVOR		PASS	PASS	
APPEAR		PASS	PASS	
ODOR		PASS	PASS	
AEROBIC PLATE COUNT (CFU/G)		<10000 /G	<10000 /G	
COLIFORM (CFU /G)		<10 /G	<10 /G	
YEAST & MOLD (CFU/G)		<100 /G	<100 /G	
SALMONELLA ELFA METHOD 375G		ND	ND	
E. COLI MPN/g		ND	ND	

*ND = NOT DETECTED

(b) (4)
Quality Assurance Manager

Date: 05/01/20
Time: 18:29:59
Page 0 of 0

(b) (4) **Certificate of Anal** (b) (4)

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

Customer PO No.: (b) (4)
Customer Order Nc
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	(b) (4)
Ash %	AOAC 930.30	0	20.0	
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	
steria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	
Moisture Loss on Drying %	AOAC 930.15	0	6.0	
pH (5% solution)	pH Meter	5.5	6.5	
Salt as Chlorides %	AOAC 971.27	0	1.00	
Standard Plate Count cfu/g	AOAC 990.12	0	10000	
Yeast and Mold cfu/g	AOAC 121301	0	50	
Salmonella /375g	AOAC OMA 2003.09	NEGATIVE	NEGATIVE	
Protein (N x 6.25) %	AOAC 990.03	50.0	100.0	

(b) (4)

*ND = NOT DETECTED

Quality Assurance Manager

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

(b) (4) Certificate of Anal (b) (4)

Sc o: ASCUS BIOSCIENCES INC
6450 LUSK BOULEVARD
SUITE E209
SAN DIEGO
CA
92121
US

Customer PO No.: (b) (4)
Customer Order N
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	(b) (4)
Ash %	AOAC 930.30	0	16.0	
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	
Moisture Loss on Drying %	AOAC 930.15	0	6.0	
pH (5% solution)	pH Meter	5.3	6.3	
Protein (N x 6.25) %	AOAC 990.03	55.0	100.0	
Salmonella /750g	AOAC RI 100201	NEGATIVE	NEGATIVE	
Salt as Chlorides %	AOAC 971.27	0	1.50	
Standard Plate Count cfu/g	AOAC 990.12	0	10000	
Yeast and Mold cfu/g	AOAC 121301	0	100	

(b) (4)

*ND = NOT DETECTED

Quality Assurance Manager

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

(b) (4) Certificate of Analy (b) (4)

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	(b) (4)
Ash %	AOAC 930.30	0	15.0	
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	
. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	
Moisture Loss on Drying %	AOAC 930.15	0	6.0	
pH (5% solution)	pH Meter	5.2	6.2	
Protein (N x 6.25) %	AOAC 990.03	70.0	100.0	
Salt as Chlorides %	AOAC 971.27	0	2.00	
Standard Plate Count cfu/g	AOAC 990.12	0	10000	
Yeast and Mold cfu/g	AOAC 121301	0	100	
Salmonella /375g	AOAC RI 100201	NEGATIVE	NEGATIVE	

(b) (4)

(b) (4)

Quality Assurance Manager

Date: 01/25/19
Time: 15:28:53
Page 0 of 0

(b) (4) Certificate of Analy (b) (4)

d To: (b) (4)

Customer PO No.:
Customer Order No.: (b) (4)
Item No.: SN2000027196 Amberferm 4210
50 LB Carton w/ Liner

Customer Item:
Lot No.: 3022424 300.000000 CT
Manufacture Date: 01/15/19
Lot Expiration Date: 01/15/21

Test Identification	Method	Min Value	Max Value	Test Value
MOISTURE METTLER POWDER		0	6.0	(b) (4)
PH (10% SOLUTION)		.5	5.5	
SALT AS CHLORIDES %		0	2.5	
AMINO NITROGEN/TOTAL NITROGEN		50.0	100.0	
ASH		0	12.0	
% EQUIV. PROTEIN (NX6.25)		74.0	100.0	
FLAVOR		PASS	PASS	
APPEAR		PASS	PASS	
ODOR		PASS	PASS	
AEROBIC PLATE COUNT (CFU/G)		<10000 /G	<10000 /G	
COLIFORM (CFU /G)		<10 /G	<10 /G	
YEAST & MOLD (CFU/G)		<100 /G	<100 /G	
SALMONELLA ELFA METHOD 375G		ND	ND	
E. COLI MPN/g		ND	ND	

*ND = NOT DETECTED

(b) (4)
Quality Assurance Manager

Date: 05/01/20
Time: 18:29:59
Page 0 of 0

(b) (4)

Certificate of Analys

(b) (4)

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

Customer PO No.: (b) (4)
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	(b) (4)
Ash %	AOAC 930.30	0	20.0	
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	
Moisture Loss on Drying %	AOAC 930.15	0	6.0	
pH (5% solution)	pH Meter	5.5	6.5	
Salt as Chlorides %	AOAC 971.27	0	1.00	
Standard Plate Count cfu/g	AOAC 990.12	0	10000	
Yeast and Mold cfu/g	AOAC 121301	0	50	
Salmonella /375g	AOAC OMA 2003.09	NEGATIVE	NEGATIVE	
Protein (N x 6.25) %	AOAC 990.03	50.0	100.0	

(b) (4)

*ND = NOT DETECTED

Quality Assurance Manager

Product Certificate of Analysis

Product Name	*Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulated Product
Batch Number	1801.2041
Date of Manufacture	09Dec2020
Expiration Date	N/A
Retest Date	09Dec2021
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.

(b)(6) 1/20/2021

Quality

Product Certificate of Analysis

Product Name	*Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulated Product
Batch Number	1801.2042
Date of Manufacture	09Dec2020
Expiration Date	N/A
Retest Date	09Dec2021
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.

(b)(6) 1/20/2021

Quality

Product Certificate of Analysis

Product Name	*Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulated Product
Batch Number	1801.2044
Date of Manufacture	10Dec2020
Expiration Date	N/A
Retest Date	10Dec2021
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.

(b)(6) 1/20/2021

Quality

Product Certificate of Analysis

Product Name	*Succinivibrio dextrinosolvens* ASCUSBF53 Freeze-dried Powder
Batch Number	1801.2041
Date of Manufacture	27Nov2020
Expiration Date	N/A
Retest Date	27Nov2021
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.

(b)(6)

1/20/2021

Quality

Product Certificate of Analysis

Product Name	*Succinivibrio dextrinosolvens* ASCUSBF53 Freeze-dried Powder
Batch Number	1801.2042
Date of Manufacture	27Nov2020
Expiration Date	N/A
Retest Date	27Nov2021
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.

(b)(6)

1/20/2021

Quality

Product Certificate of Analysis

Product Name	*Succinivibrio dextrinosolvens* ASCUSBF53 Freeze-dried Powder
Batch Number	1801.2044
Date of Manufacture	27Nov2020
Expiration Date	N/A
Retest Date	27Nov2021
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.

(b)(6) 1/20/2021

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53

Confidential Manufacturing Information

The raw materials used in the manufacture of *S. dextrinosolvens* ASCUSBF53 are listed in Table 1 below. Specifications for the raw materials are provided in Appendices 009A to 009ZG.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *S. dextrinosolvens* ASCUSBF53

(b) (4)			

Table Continued on next page.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *S. dextrinosolvens* ASCUSBF53

(b) (4)

Table Continued on next page.

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *S. dextrinosolvens* ASCUSBF53

(b) (4)

1 Overview

(b) (4)

(b) (4)

(b) (4)

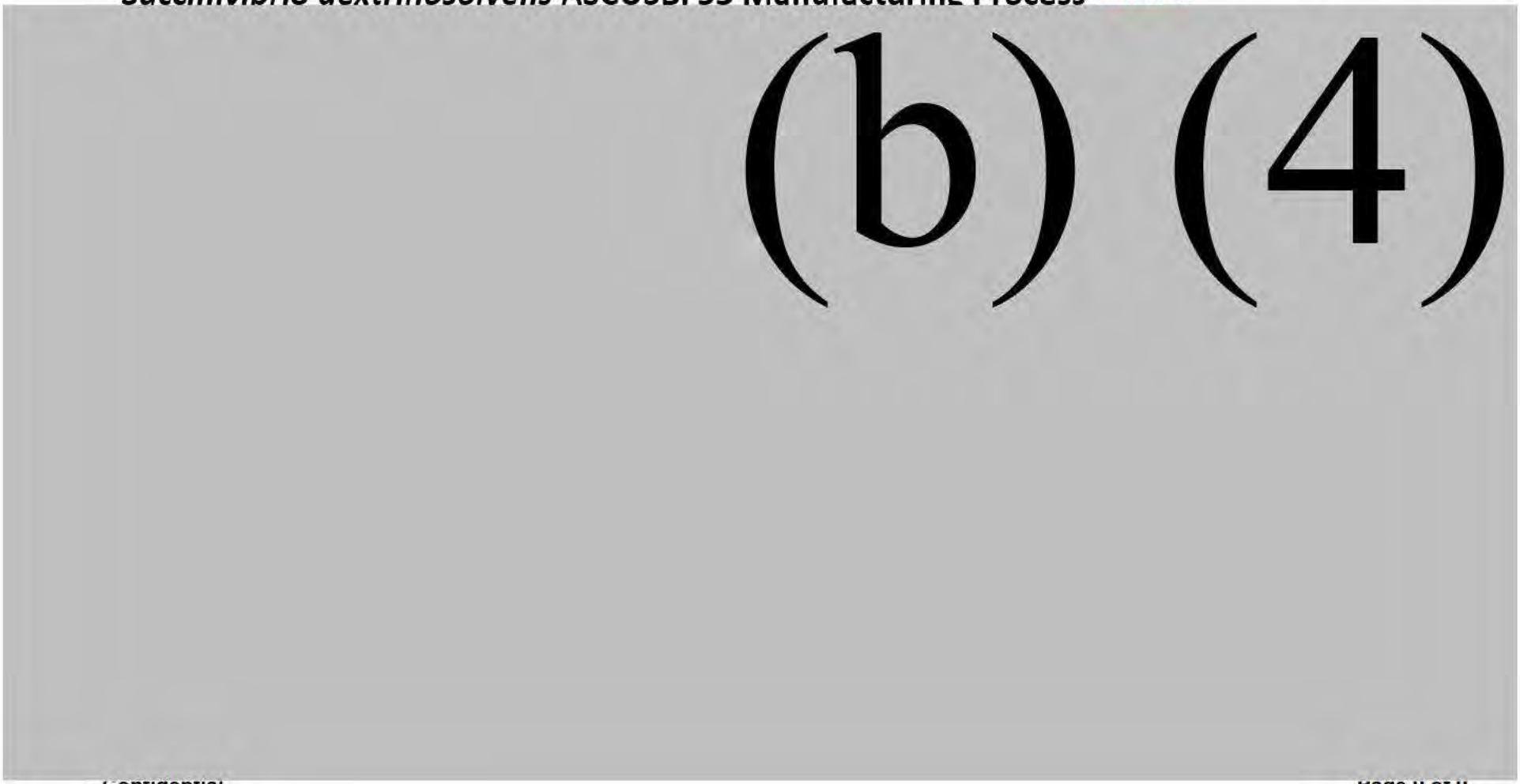
(b) (4)

(b) (4)

(b) (4)

Appendix A. Process Diagram of the Production of Fat Encapsulated *S. dextrinosolvens* ASCUSBF53

***Succinivibrio dextrinosolvens* ASCUSBF53 Manufacturing Process** CONFIDENTIAL 11 Jan 2021

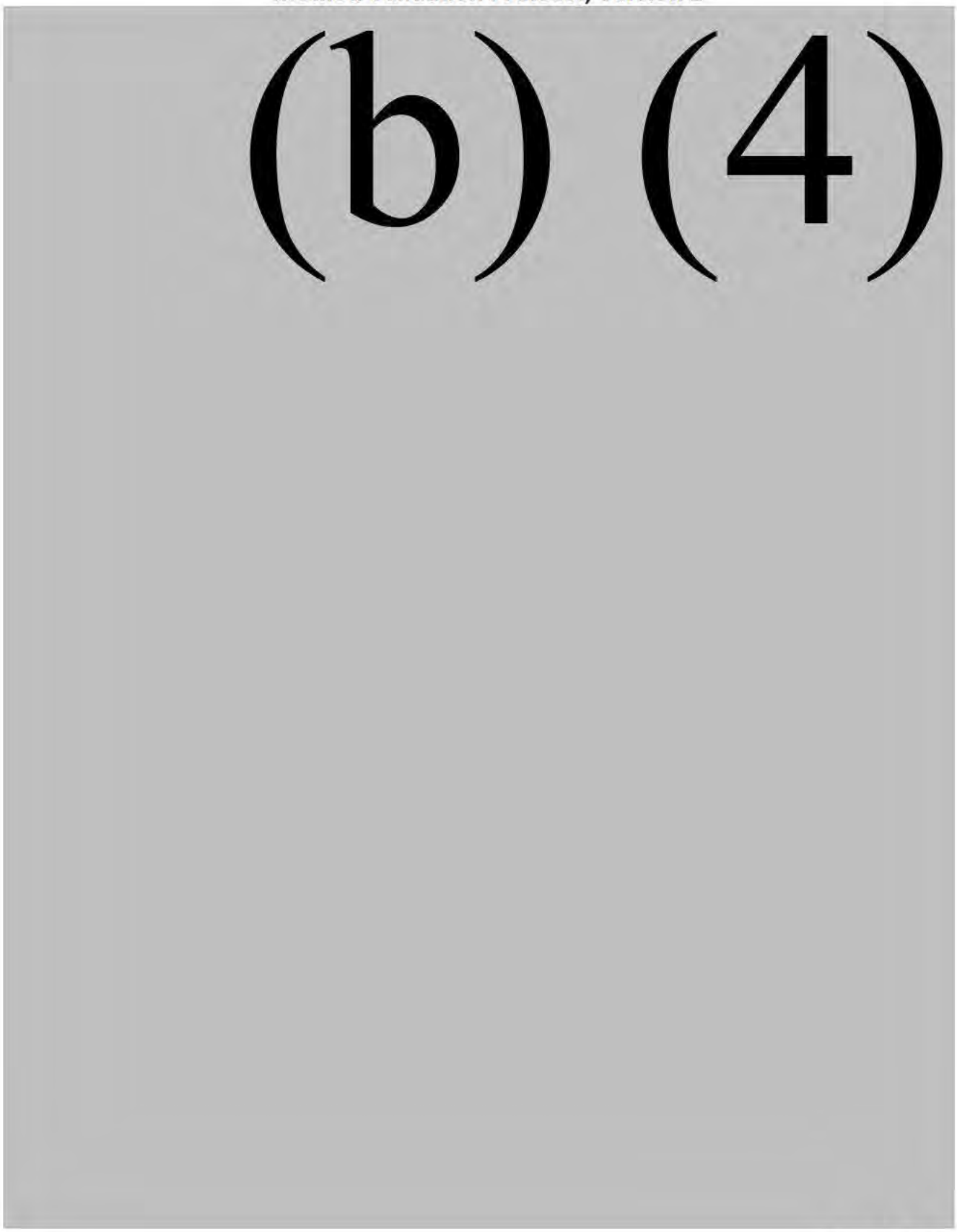


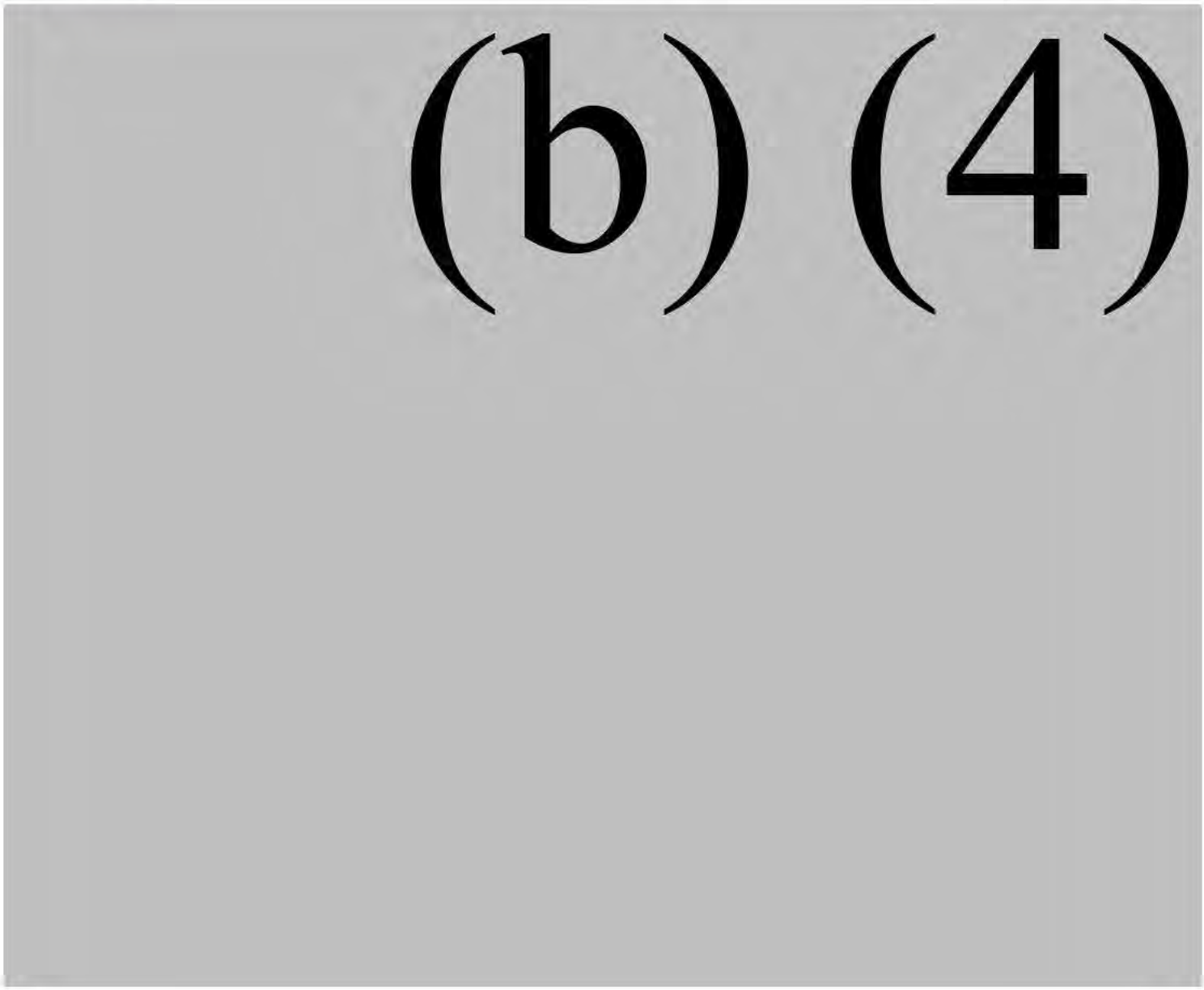
Appendix 011

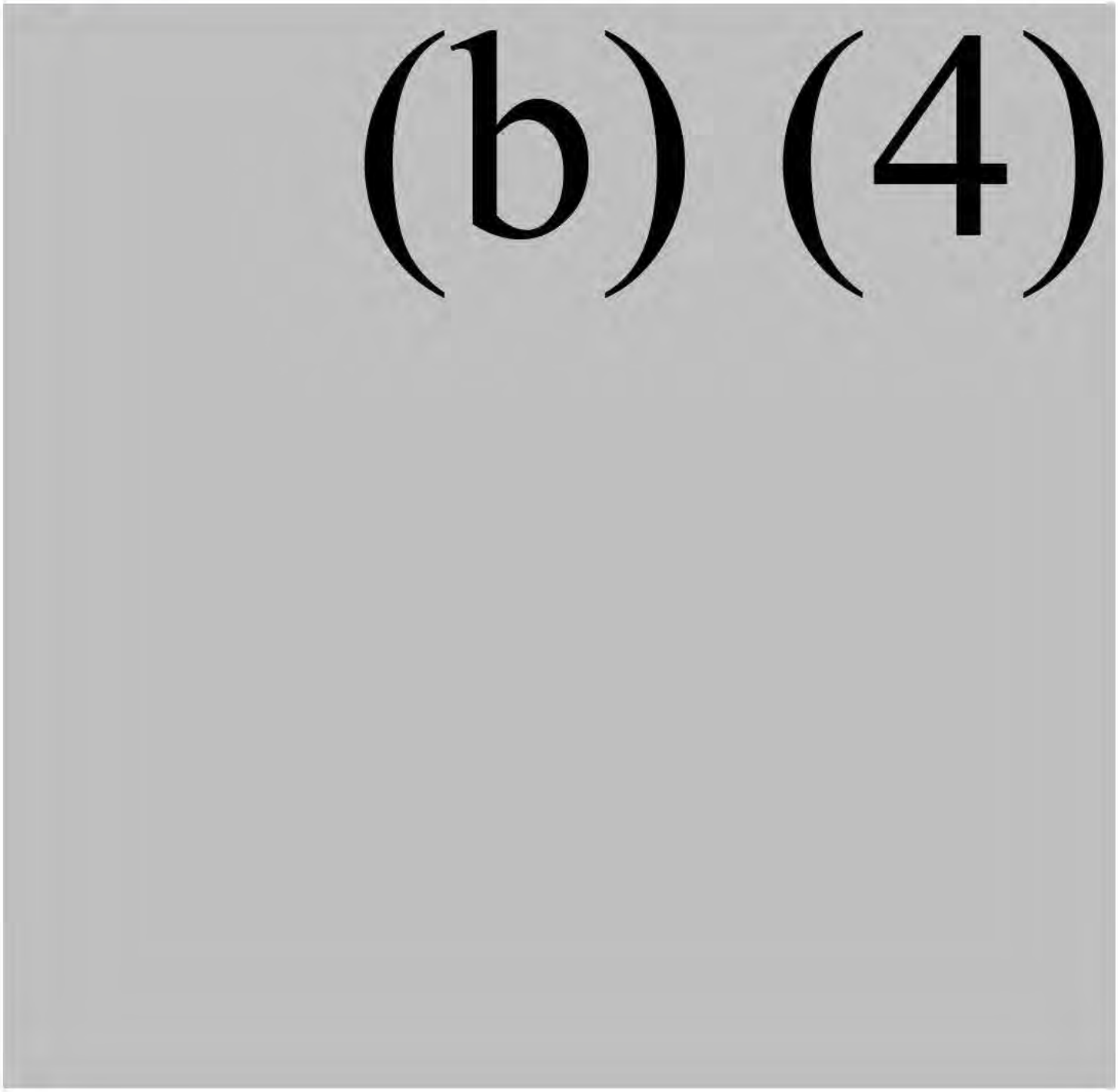
Comparison of Physical Properties of Fat Encapsulated Powder *Succinivibrio dextrinosolvens* ASCUSBF53 to recent prior submission (AGRN 38) *Pichia kudriavzevii* ASCUSDY21

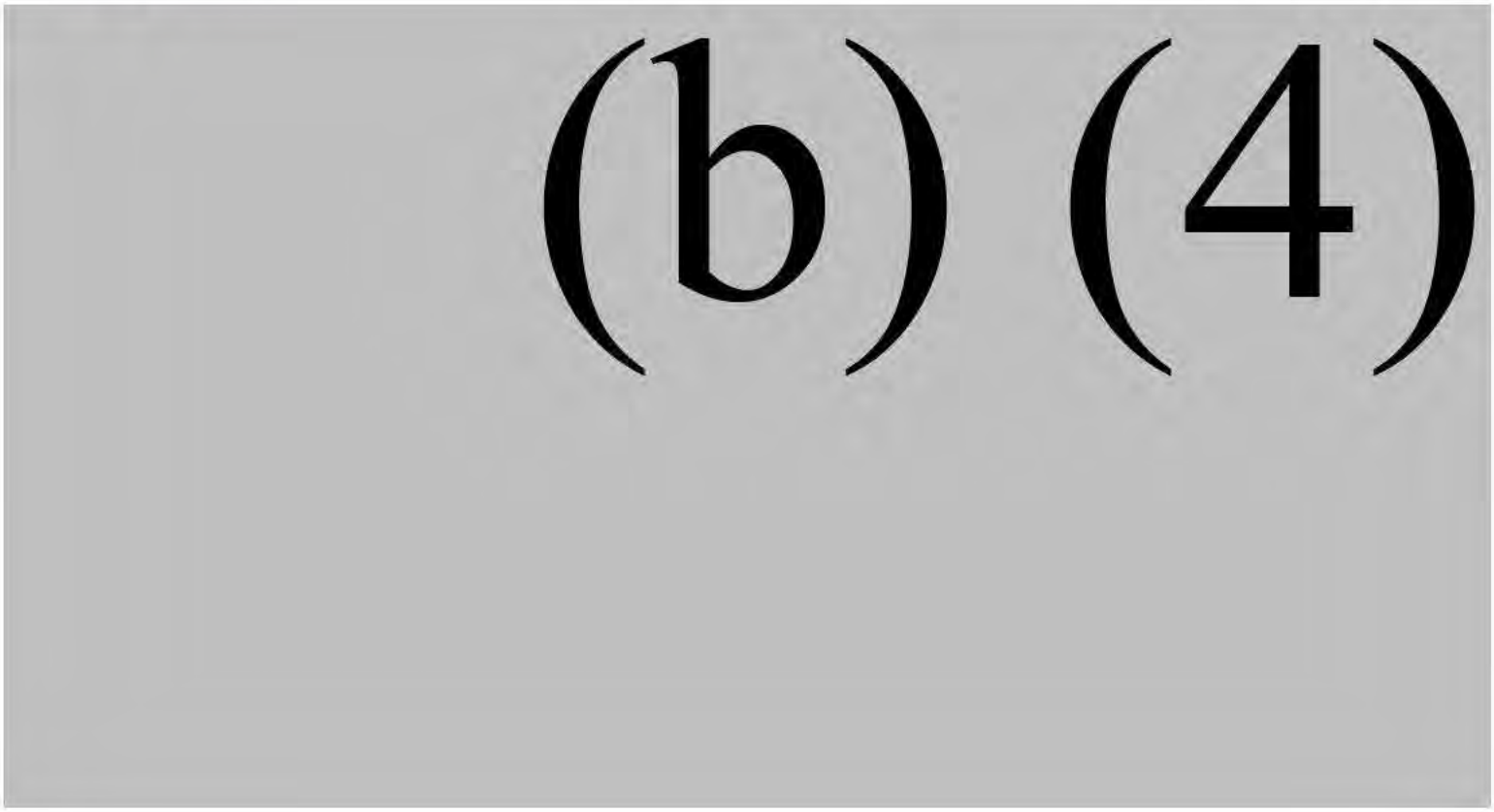
Physical Attribute	*S. dextrinosolvens* ASCUSBF53	(AGRN 38) *P. kudriavzevii* ASCUSDY21	Method
Organism concentration	(b) (4)		
Particle size (d_{50})			
Particle size (d_{90})			
Milled foam dried organism composition (g/kg in in final formula)			
Sodium Sulfate composition (g/kg in final formula)			
Hydrogenated glycerides composition (g/kg in final formula)			
Moisture content			

Method Validation Protocol, Version 1









(a)(4)

(b)(4)

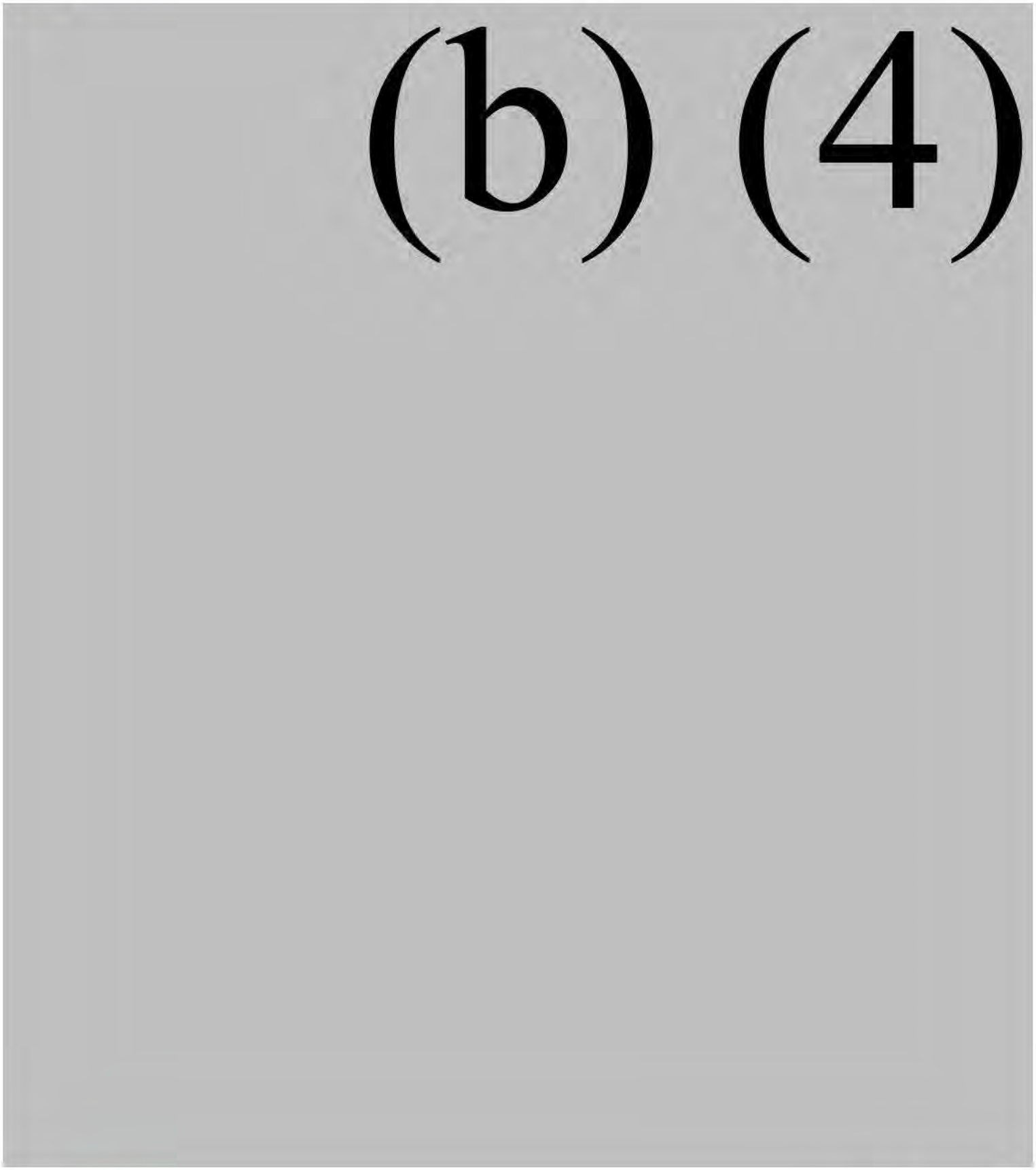
(b) (4)

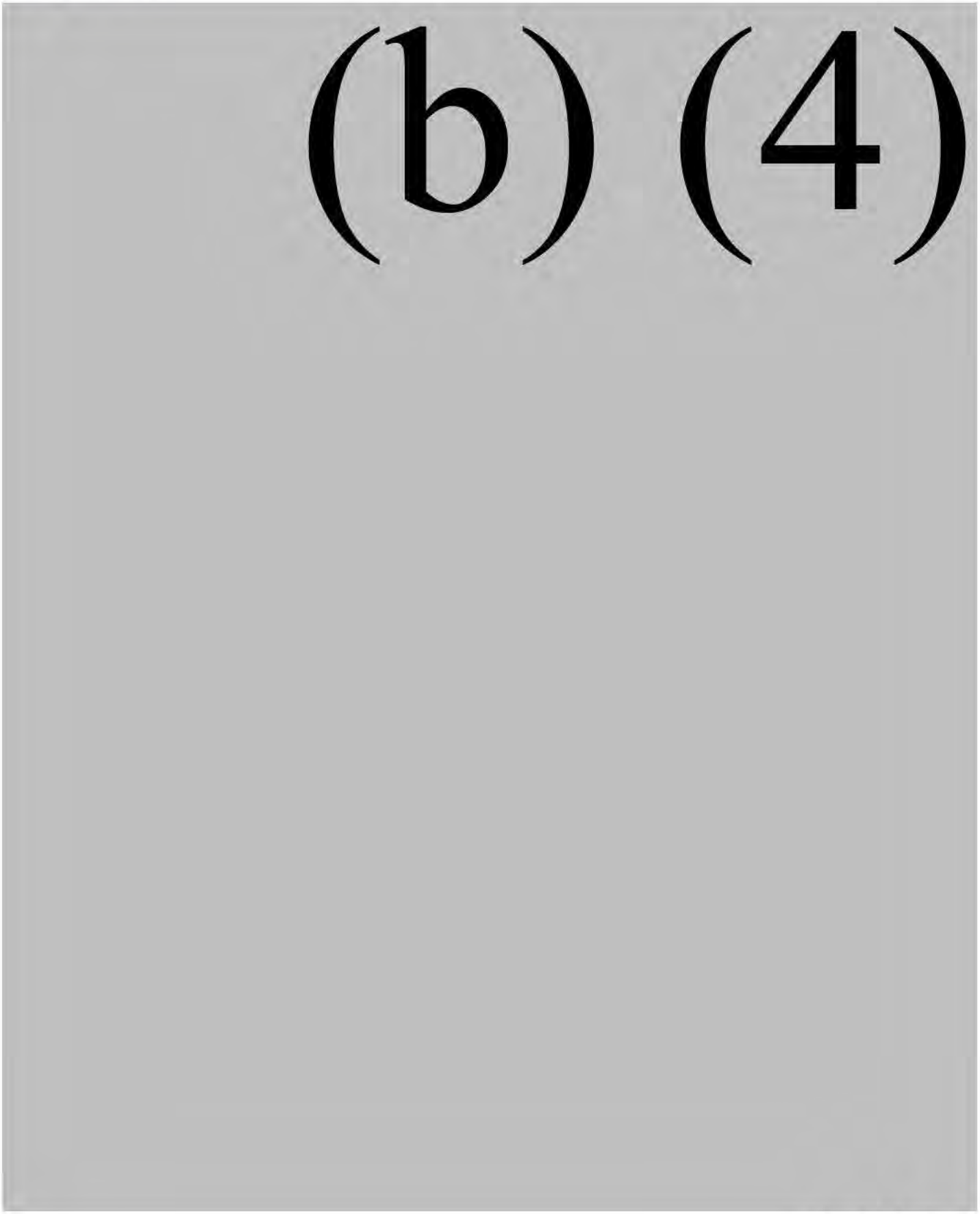
(a)(4)

(b) (4)

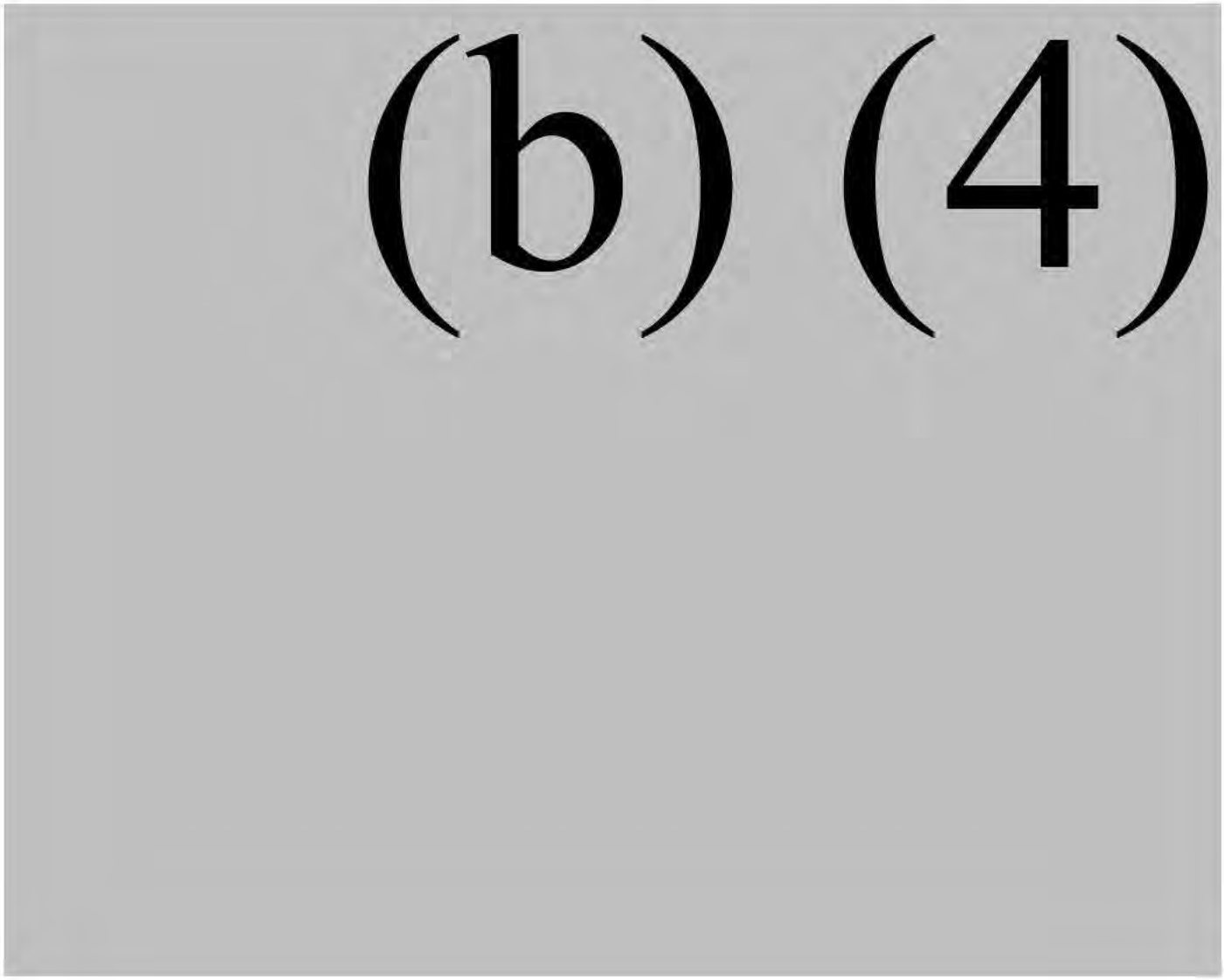
(b) (4)


(a)
(4)





(b) (4)



BF65 Solid Intermediate Microbe Enumeration Validation Summary Report

Method

BF65 Solid Intermediate Microbe Enumeration, V1

(b) (4)

(b) (4)

(b) (4) with

Name and Title	Signature and Date
Martin Mayhew VP – Process Development and Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 1/13/2021
(b)(6) Quality	DocuSigned by: (b)(6) 5B301285A10843D... 1/13/2021

Method

Title	Beef-53 Solid Intermediate Microbe Enumeration
Version	01
Effective Date	15Jan2021
Author	(b)(6)
Approver (Signature & Date)	DocuSigned by: (b)(6) 1/13/2021 Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Succinivibrio dextrinosolvens* in Beef-53 solid intermediates in samples from:

(b) (4)

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. Analysts 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
>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

(b) (4)

Method

1. (b) (4)

(b) (4)

(b) (4)

(b) (4)

Method Validation Protocol, Version 1

Method Title and Versions

Title	BF53 Liquid Intermediate Microbe Enumeration
Version	01 Draft

Lab Performing the Validation: Native Microbials Inc.

Pre-Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP-Process Development & Manufacturing	(b)(6)	9/30/2020
(b)(6)		9/30/2020

Post Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP-Process Development & Manufacturing	(b)(6)	10/15/2020
(b)(6)		10/15/2020

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

Printed Name	Signature	Tasks Performed
(b) (6)		Analyst 1
		Analyst 2

Purpose:

(b) (4)

(b) (4)

Background:

(b) (4)

Method Overview:

(b) (4)

Primary Dilution Preparation

Sample #	Sample Type (EoF or CC)	Sample Lot Number/ID	Approximate Viability
1		(b) (4)	
2			
3			
4			
5			

Validation Approach:

(b) (4)

Acceptance Criteria:

(b) (4)

Summary and Conclusions:

(b) (4)

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nativemicrobials.com

Data Collection – Analyst 1 Name [redacted]

Sample ID	Replicate	Dilution	Colonies	Initial/Date	Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-1	1	[redacted]							
	2								
	3								

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nativemicrobials.com

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1-3	1			(b) (4), (b)(6)
	2			
	3			

Sample ID	Replicate	Dilution	Colonies	Initial/Date
2	1			(b) (4), (b)(6)
	2			
	3			

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Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	1			(b) (4), (b)(6)
	2			
	3			

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	1			(b) (4), (b)(6)
	2			
	3			

Sample ID	Replicate	Dilution	Colonies	Initial/Date
5	1			
	2			
	3			

(b) (4), (b)(6)

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CFU Results

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

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CFU Results

Sample			Initial/Date		Sample			Initial/Date
3	Final Result (CFU/mL)	(b)(4), (b)(6)			5	Final Result (CFU/mL)	(b) (4), (b)(6)	
	Standard Deviation					Standard Deviation		
4	Final Result (CFU/mL)							
	Standard Deviation							

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Data Collection – Analyst 2 Name Sean Gilmore

Sample ID	Replicate	Dilution	Colonies	Initial/Date
1	1			(b)(4), (b)(6)
	2			
	3			

Sample ID	Replicate	Dilution	Colonies	Initial/Date
2	1			(b)(4), (b)(6)
	2			
	3			

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Sample ID	Replicate	Dilution	Colonies	Initial/Date
3	1	[redacted]		
	2			
	3			

Sample ID	Replicate	Dilution	Colonies	Initial/Date
4	1	[redacted]		
	2			
	3			

Sample ID	Replicate	Dilution	Colonies	Initial/Date
5	1			(b) (4), (b)(6)
	2			
	3			

native

nativemicrobials.com

CFU Results

Sample			Initial/Date	Sample			Initial/Date
1	Final Resul (CFU/mL)	(b)(4), (b)(6)		3	Final Result (CFU/mL)	(b)(4), (b)(6)	
	Standard Deviation				Standard Deviation		
2	Final Resul (CFU/mL)			4	Final Result (CFU/mL)		
	Standard Deviation				Standard Deviation		

CFU Results

Sample			Initial/Date		Sample			Initial/Date
5	Final Result (CFU/mL)	(b) (4), (b)(6)						
	Standard Deviation							

Draft Method

Title	Beef-53 Liquid Intermediate Microbe Enumeration
Version	01
Effective Date	Draft
Author	(b)(6)
Approver (Signature & Date)	Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable cells of *Succinivibrio dextrinosolvens* in Beef-53 liquid intermediates in samples from:

- (b) (4)

Safety

(b) (4)

Media & Reagents

(b) (4)

Method

NOTE: *Step 1 should be performed at least 24 hours prior to commencement of testing.*

(b) (4)

2. Sample Dilution

(b) (4)

3.

4.

(b) (4)

Reasons for Revision

1. Initial version.

BF52 Liquid Intermediate Microbe Enumeration Validation Summary Report

Methods

Beef-53 Liquid Intermediate Microbe Enumeration, V1

Objective

(b) (4)

Results

Repeatability

(b) (4)

Table 1: Summary table of DY19 (b) (4) method validation results

		Average CFU/mL	STDEV	CV
Analyst 1	Sample 1-1	(b) (4)		
	Sample 1-2			
	Sample 1-3			
	Sample 2			
	Sample 3			
	Sample 4			
	Sample 5			
Analyst 2	Sample 1			
	Sample 2			
	Sample 3			
	Sample 4			
	Sample 5			

Robustness

(b) (4)

Table 2: Summary of Repeatability, Linearity, and Robustness

(b) (4)

Linearity

(b) (4)

(b) (4)

(b) (4)

Conclusion

(b) (4)

(b) (4)

Approvals

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: (b)(6) 5B301285A10643D... 10/15/2020
(b)(6) Quality	10/15/2020

Method

Title	Beef-53 Liquid Intermediate Microbe Enumeration
Version	01
Effective Date	20Oct2020
Author	(b)(6)
Approver (Signature & Date)	DocuSigned by: (b)(6) 10/15/2020 Martin Mayhew – VP Product Development & Manufacturing

Scope

(b) (4)

(b) (4)

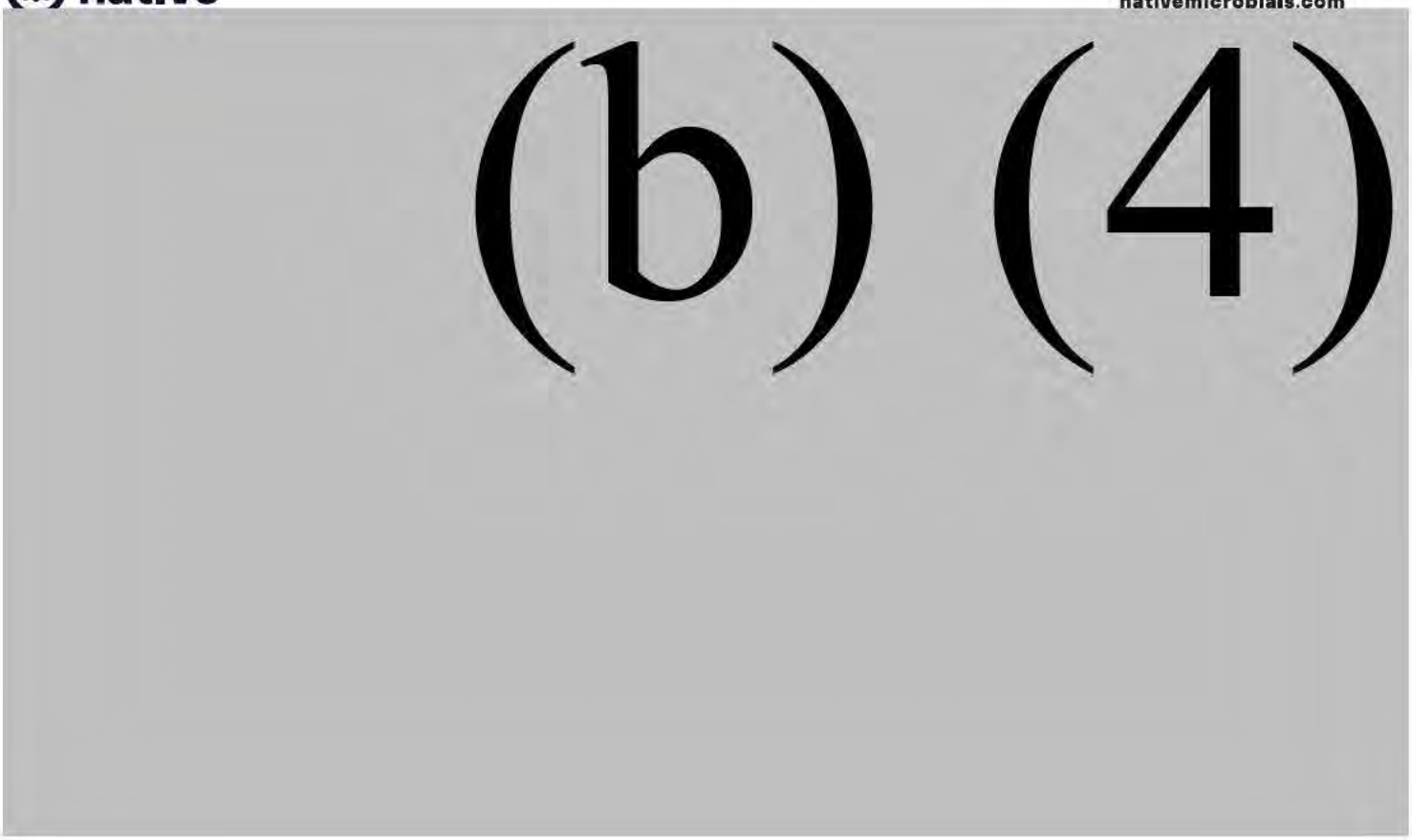
(b) (4)

Method

NOTE: *Step 1 should be performed at least 24 hours prior to commencement of testing.*

(b) (4)

(b) (4)



ASCUS BIOSCIENCES

Method

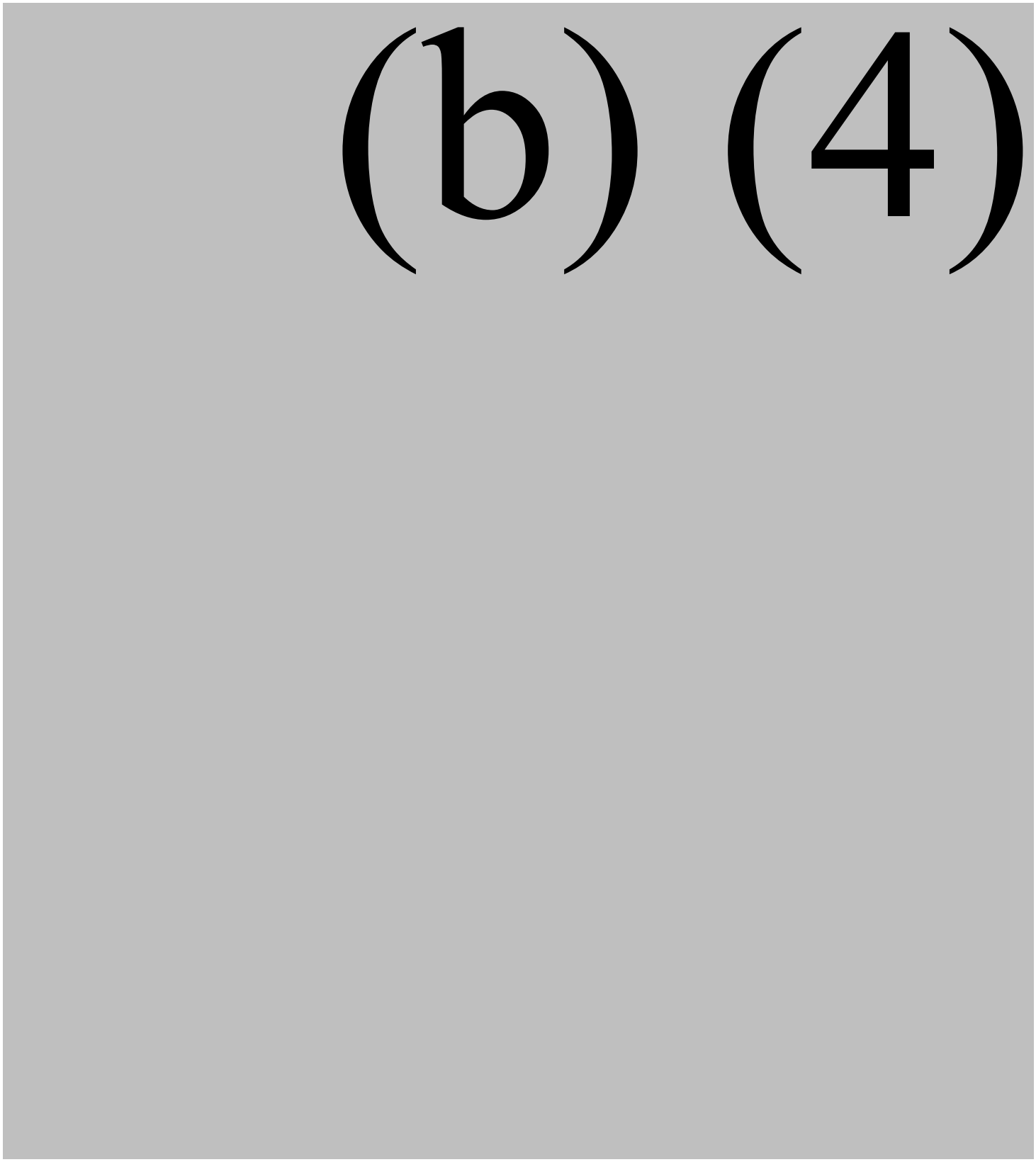
Title	DY21-POE Microbe Enumeration
Version	05
Effective Date	15May2020
Author	Miranda Striluk
Approver (Signature & Date)	(b)(6) 5/8/2020 Martin Mayhew VP – Process Development & Manufacturing

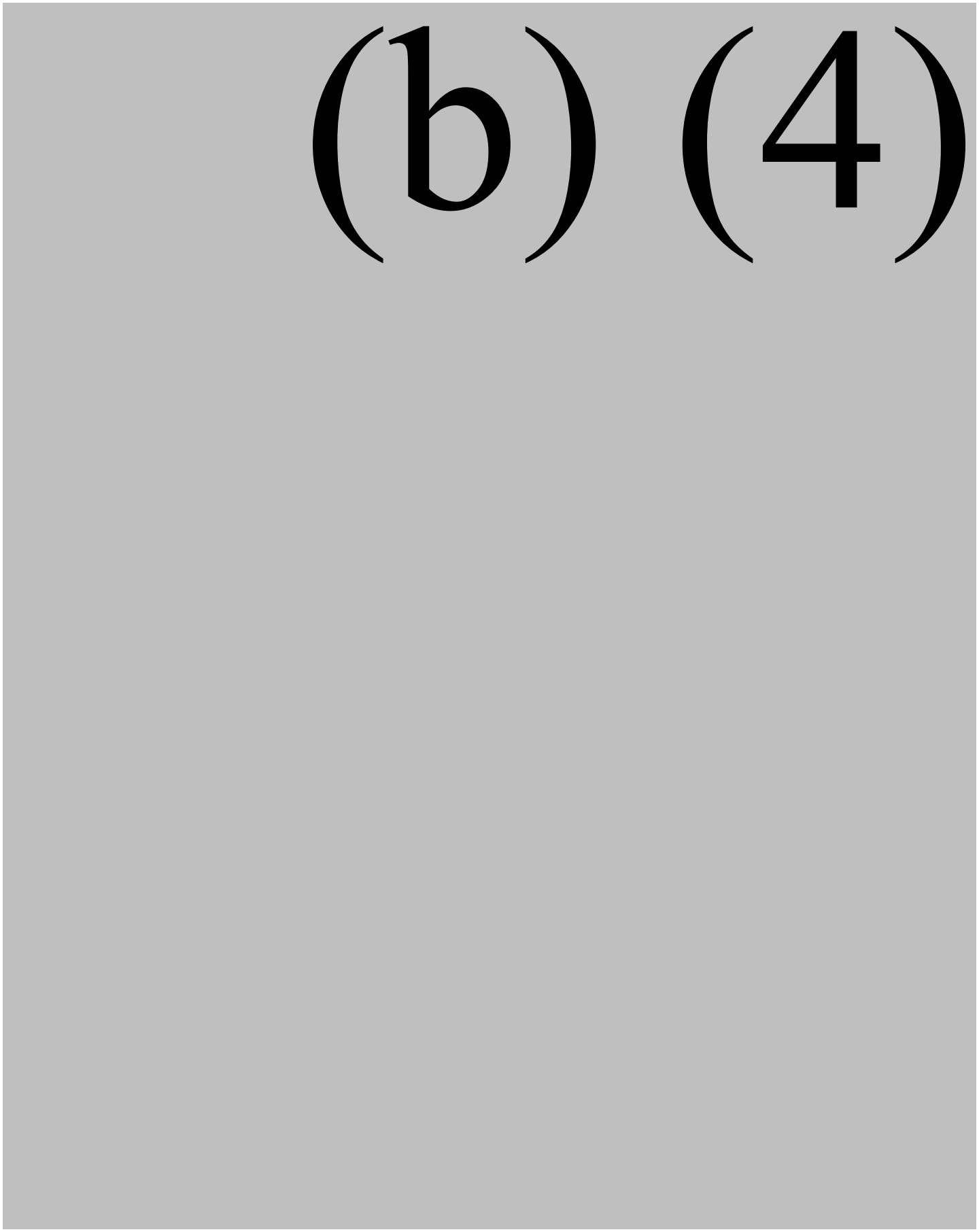
Scope

(b) (4)

(b) (4)

Method

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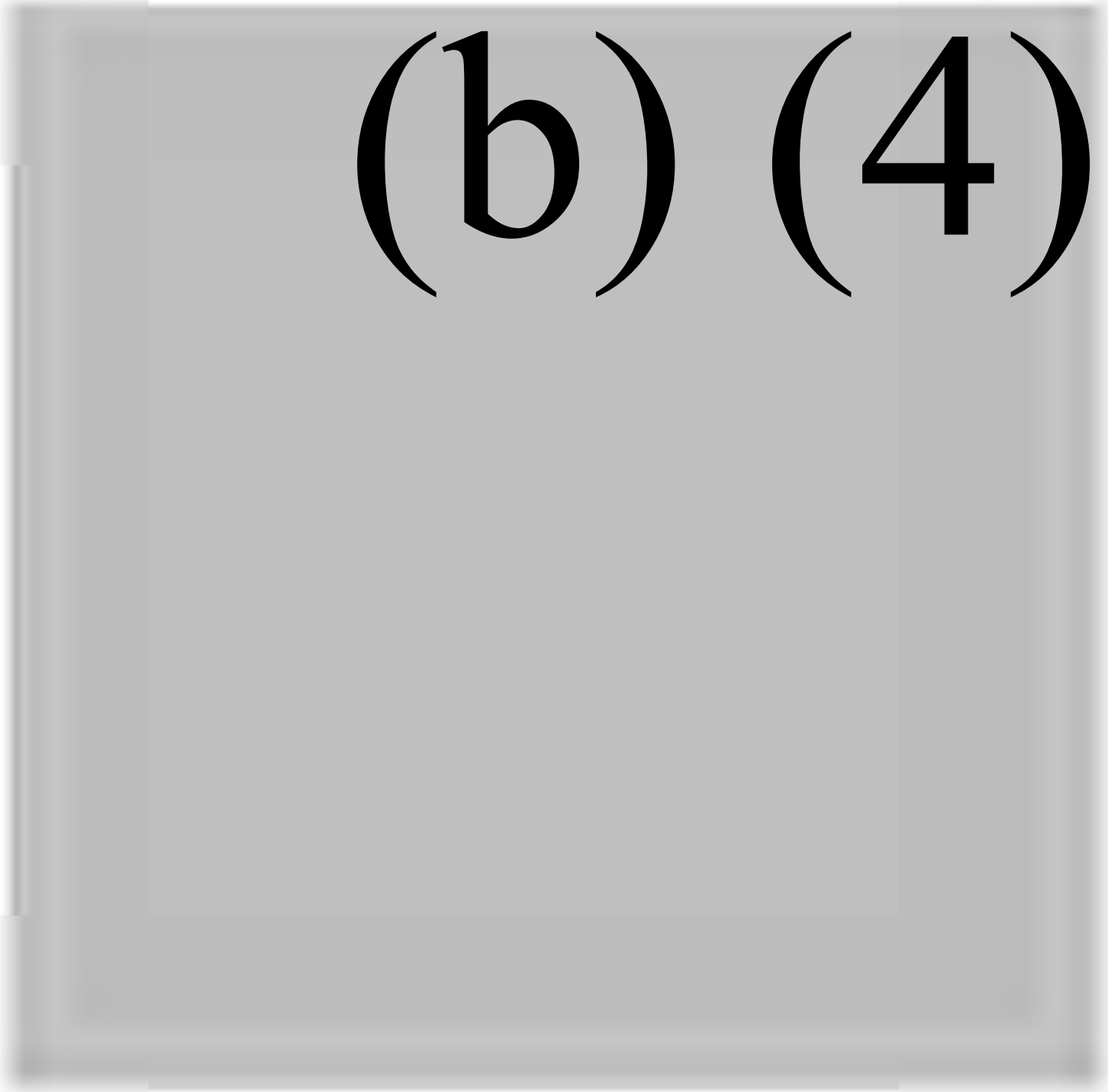
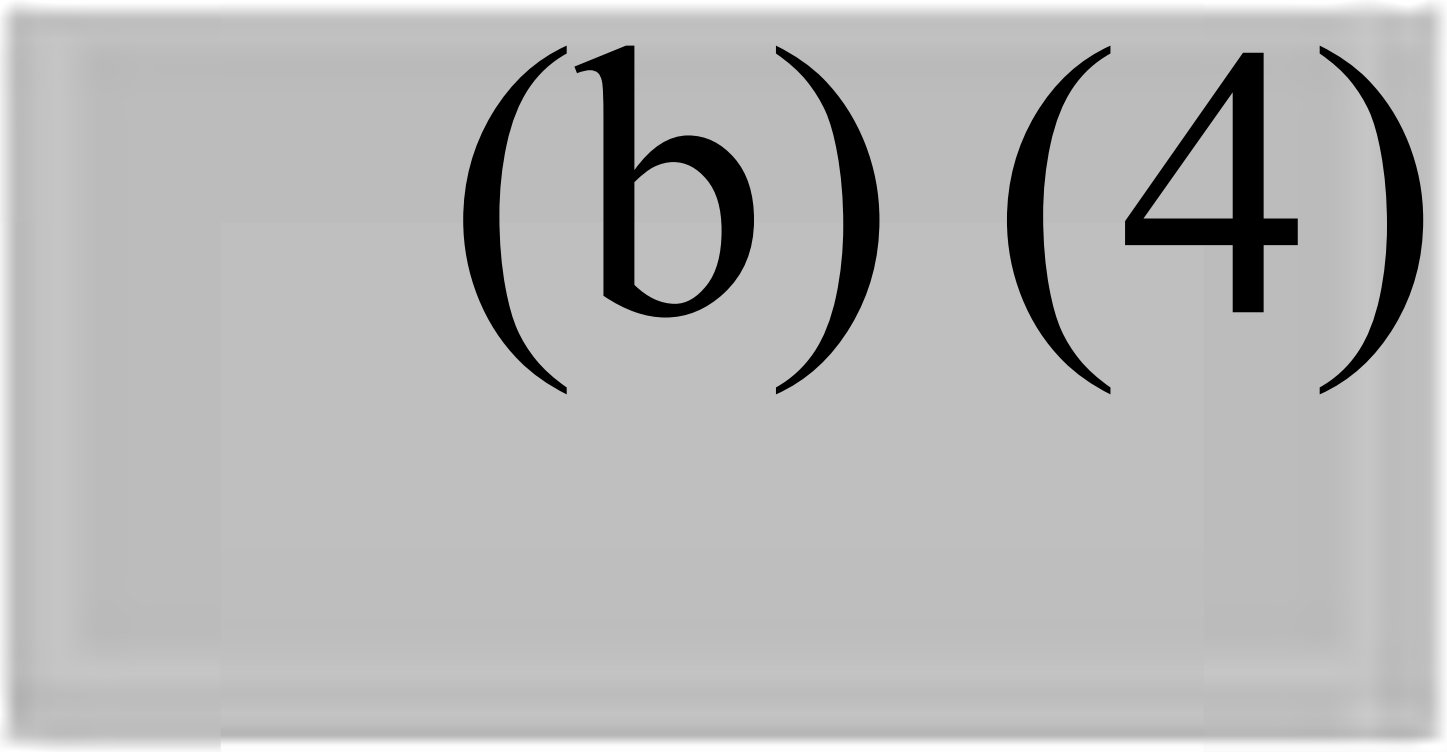

(b) (4)

ASCUS BIOSCIENCES

Method

Title	**Moisture Analysis**
Version	**01**
Effective Date	**15Dec2019**
Author	(b)(6)
Approver (Signature & Date)	DocuSigned by: (b)(6) D1605F1B4C3E49A... 12/3/2019 **Martin Maynew – VP – Process Development & Manufacturing**

(b) (4)



Analysis of *Succinivibro dextrinosolvens* ASCUSBF53 POE (BF53) for Heavy Metals & Microbial Contamination

Approvers:

DocuSigned by:
(b)(6)
CA3DAF452B8A47C...

1/11/2021

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
(b)(6)
5B301285A10643D...

1/11/2021

(b)(6)
Quality

Date

DocuSigned by:
(b)(6)
1C2149273B2345F...

1/12/2021

(b)(6)
Regulatory

Date

Prepared by
Native Microbials, Inc
San Diego, CA

January 2021

Analysis of *Succinivibro dextrinosolvens* ASCUSBF53 POE for Heavy Metals & Microbial Contamination

Three lots of ***Succinivibro dextrinosolvens* ASCUSBF53 POE** were sent for heavy metal and microbial contamination analysis at (b) (4) (b) (4). (Note: *S. dextrinosolvens* ASCUSBF53 is listed on certificate of analysis as Beef-53 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 *Succinivibro dextrinosolvens* ASCUSBF53 POE

Lot Number	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
Detection Limit	0.004	0.0008	0.001	0.001
BF53 1801.2041	ND	ND	ND	ND
BF53 1801.2042	ND	ND	ND	ND
BF53 1801.2044	ND	ND	ND	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 *Succinivibro dextrinosolvens* ASCUSBF53 POE

Lot Number	Coliform, CFU/g	E. coli, CFU/g	Salmonella, per 25g	Listeria, per 25g
Requirement	<10	<10	Negative	Negative
BF53 1801.2041	<10	<10	Negative	Negative
BF53 1801.2042	<10	<10	Negative	Negative
BF53 1801.2044	<10	<10	Negative	Negative

S. dextrinosolvens ASCUSBF53 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/hd/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 *S. dextrinosolvens* ASCUSBF53 POE.

Attachment 1. Certificate of Analysis – Heavy Metal Analysi (b) (4) Sample No. 1067697)

(b) (4)

Certificate of Analysis

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

Order No (b) (4)
Sample No. 1067697

SAMPLE INFORMATION

Description	Beef-53 Fat Encapsulate
Lot Number	1801.2041
Received	December 22, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 22, 2020 to December 29, 2020

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

Reported by

(b) (4)

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

(b) (4)

Attachment 2. Certificate of Analysis – Heavy Metal Analysis (b) (4)Sample No. 1067698)

(b) (4)

Certificate of Analysis

December 29, 2020

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

Order No. (b) (4)
Sample No. 1067698

SAMPLE INFORMATION

Description	Beef-53 Fat Encapsulate
Lot Number	1801.2042
Received	December 22, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 22, 2020 to December 29, 2020

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

Reported by

(b) (4)

December 29, 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. 1067699)

(b) (4)

Certificate of Analysis

December 29, 2020

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

Order No (b) (4)
Sample No. 1067699

SAMPLE INFORMATION

Description	Beef-53 Fat Encapsulate
Lot Number	1801.2044
Received	December 22, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 22, 2020 to December 29, 2020

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

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

(b) (4), (b)(6)

December 29, 2020

(b) (4)

Attachment 4. Certificate of Analysis – Microbial Contamination Testing For All Three lots

(b) (4)

Certificate of Analysis

December 30, 2020

(b) (4)

Native Microbials, Inc.
10255 Science Center Drive, Suite C2
San Diego, CA 92121

FINAL REPORT

SAMPLE INFORMATION

Product: **FINISHED PRODUCT**
Date Received: December 22, 2020

Method: AOAC 2018.13 – Coliforms/E. coli
AOAC 2013.01 – Salmonella
AOAC 2013.10 - Listeria
Analyses Date: December 22, 2020 to December 30, 2020

ANALYTICAL RESULTS

Sample ID	Description	Lot No.	Coliforms cfu/g	E. coli cfu/g	Salmonella per 25g	Listeria per 25g
1067697	Beef-53 Fat Encapsulate	1801.2041	<10	<10	Negative	Negative
1067698	Beef-53 Fat Encapsulate	1801.2042	<10	<10	Negative	Negative
1067699	Beef-53 Fat Encapsulate	1801.2044	<10	<10	Negative	Negative
1067700	Beef-65 Fat Encapsulate	1801.2039	<10	<10	Negative	Negative
1067701	Beef-65 Fat Encapsulate	1801.2043	<10	<10	Negative	Negative
1067702	Beef-65 Fat Encapsulate	1801.2045	<10	<10	Negative	Negative

(b) (4), (b)(6)

(b) (4)

Fat Encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53 Accelerated Stability Report

Approvers:

DocuSigned by:
(b)(6)
CA3DAF452B8A47C...

2/2/2021

Martin Mayhew
Vice President – Product Development & Manufacturing

Date

DocuSigned by:
(b)(6)

2/3/2021

Quality

Date

(b)(6)

2/2/2021

Regulatory

Date

DocuSigned by:
(b)(6)

2/2/2021

Scientist

Date

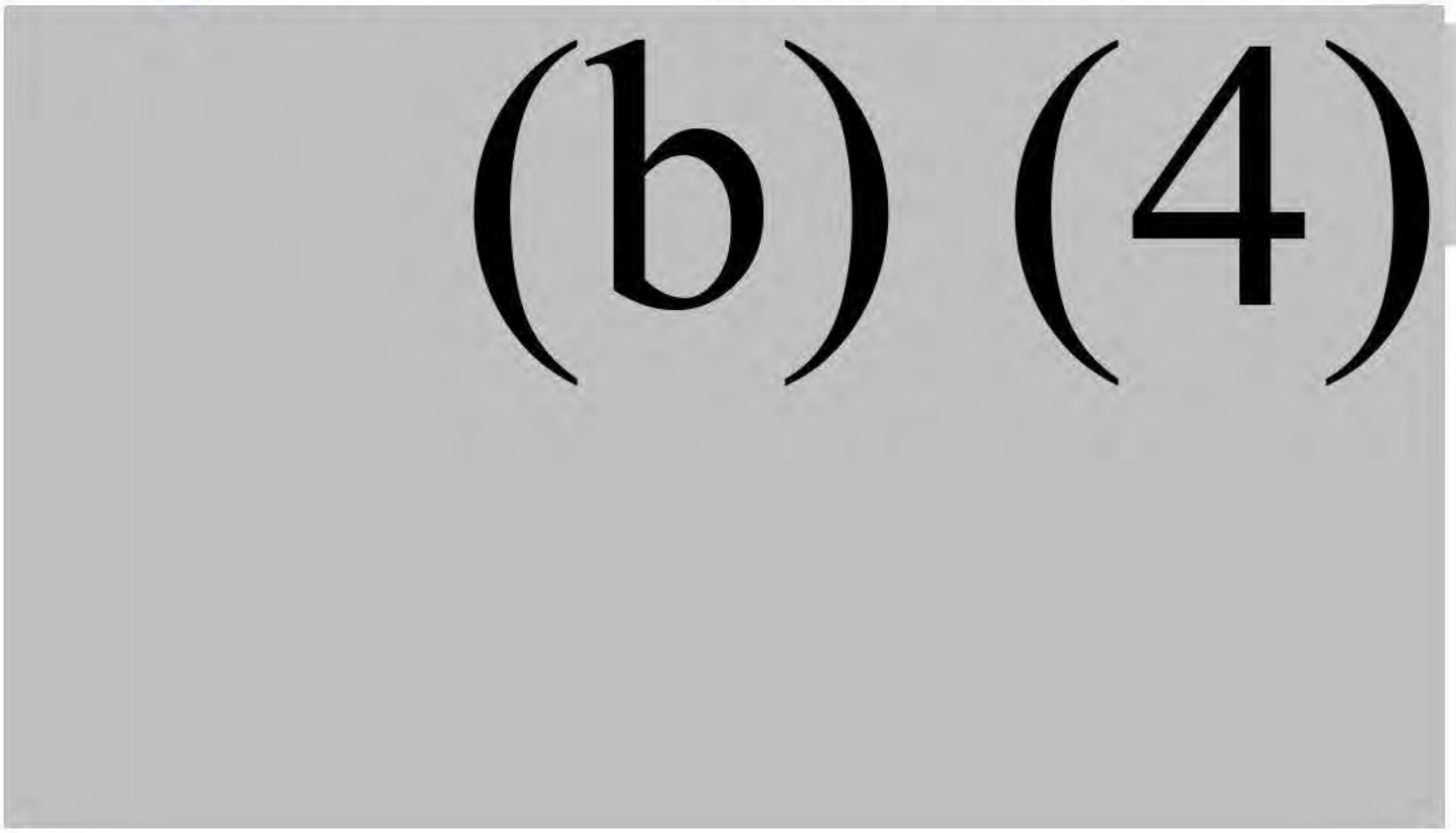
Prepared by
Native Microbials, Inc
San Diego, CA

January 2021

(b) (4)

(b) (4)

(b) (4)



Stability Protocol Titles:	*Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate (b) (4) *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate
Organism	*Succinivibrio dextrinosolvens* ASCUSBF53
Purpose:	All storage conditions are to support prediction of product stability at 2-10°C for one year.
Number of Samples to Place on Stability:	7 samples per temperature condition.
Sample Storage Container:	(b) (4)
Temperature Conditions:	
Acceptance Criteria:	See acceptance criteria section
Method:	BF53 Solid Intermediate Microbe Enumeration method

1. **Introduction**

(b) (4)

2. **Sampling Plan**

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

12. Protocol Approvals

Name & Title	Signature & Date
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: (b)(6) 12/29/2020
(b)(6) Regulatory	12/29/2020
(b)(6) Quality	12/29/2020

Appendix 016

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 *Succinivibrio dextrinosolvens* ASCUSBF53

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 *S. dextrinosolvens* ASCUSBF53 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.

native MICROBIALS

Appendix 016 Pariza Decision Tree

native MICROBIALS

Appendix 017: Literature Search Strategy

A literature search was conducted by Native Microbials on January 7, 2021 in order to identify potential information related to the safety and utility of *Succinivibrio dextrinosolvens* as a direct fed microbial (DFM) strain for cattle. The overall search strategy is described in Table 1. Google Scholar 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 genus. 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: *Succinivibrio dextrinosolvens*.

Table 1: Literature Search and Selection Strategy		
Step 1	Records identified using selected literature databases	Google Scholar
	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: Web of Science]	Term 1	*Succinivibrio dextrinosolvens*
		Term 2	Toxi*(n=3) Pathogen* (n=83) Safe*(n=67) Infection (n=125) Disease (n=218) Mortal* (n=2)
Search strategy for safety of *Succinivibrio dextrinosolvens* for cattle [Target Animal Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Succinivibrio dextrinosolvens*
		Term 2	Cattle (n=424) Cow* (n=253) Bovine (n=379) Ruminant* (n=374) Calf (n=85) Calves (n=168) Bull* (n=78) Heifer* (n=37)
Search strategy for history of use of *Succinivibrio dextrinosolvens* for use in food and feed [History of Use Search]	Keywords/search terms [Database: Web of Science]	Term 1	*Succinivibrio dextrinosolvens*
		Term 2	Food* (n=396) Feed* (n=429)

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

Microbiome Safety for *Succinivibrio dextrinosolvens* ASCUSBF53

Objectives

The objective of this review is to:

a) Demonstrate that the typical microbial composition and diversity of the rumen microbial community of beef cattle is robust and stable across various diets and regions. We will demonstrate this by:

   i) Showing internal datasets (e.g. data and analyses created by Native Microbials)

   ii) Presenting data via external datasets (e.g. data published in peer reviewed manuscripts).

b) Present data that shows the feeding of native microorganisms does not negatively alter the microbiome composition. Specifically, that daily administration of *Succinivibrio dextrinosolvens* ASCUSBF53 does not increase its own abundance nor the overall composition of the microbiome beyond typically observed ranges.

Robust Nature of the Dairy Rumen Microbiome

Native Microbials Animal Experiments: A series of experiments were conducted 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.

Microbiome Survey : A survey experiment was conducted to identify the rumen composition of 50 Angus steers over a period of 70 days in (b)(6). The animals were fed a typical local diet for measuring feed efficiency (see Attachment 1). Rumen samples were taken every 7 days throughout the study to analyze and characterize the rumen microbiome. The study has been peer reviewed and published (Clemmons, Martino, Powers, et al. 2019; Clemmons, Martino, Schneider, et al. 2019).

Findings: The results of the survey experiment are summarized in Table 1, showing the average rumen bacterial phyla abundances. In all of these experiments, the abundances of the most predominant phyla were comparable to the ranges observed in the independent literature studies (presented below). The typical abundance of *S. dextrinosolvens*, specifically, in the rumen of an Angus steer based on Native Microbials survey was found to be 8.84% on average (ranged from 1.3% to 19.99%) of the rumen bacterial population.

Table 1. Abundance of Rumen Bacterial Phyla from Native Microbials Survey Experiment, Reported as a Percent

Phylum	Average Abundance (%)	Abundance Range (%)
Bacteroidetes	48.02	29.91 - 60.71
Proteobacteria	26.86	8.70 - 46.13
Firmicutes	21.99	8.86 - 40.70
Cyanobacteria/Chloroplast	1.60	0.44 - 7.94
Actinobacteria	1.16	0.55 - 2.65
Spirochaetes	0.8871	0.1866 - 2.2603
Lentisphaerae	0.3829	0.0098 - 1.0838
Tenericutes	0.3032	0.0307 - 1.0240
Fibrobacteres	0.1736	0.0050 - 0.7579
TM7 (Candidatus Saccharibacteria)	0.1663	0.0034 - 2.2466
Verrucomicrobia	0.1382	0.0007 - 1.0695
Chloroflexi	0.1212	0.0061 - 0.3732
Acidobacteria	0.0920	0.0150 - 0.5587
Planctomycetes	0.0781	0.0039 - 0.5447
Synergistetes	0.0739	0.0109 - 0.3845
Elusimicrobia	0.0662	0.0001 - 1.7201
Armatimonadetes	0.0450	0.0007 - 0.2095
Fusobacteria	0.0426	0.0004 - 1.4855
Deinococcus-Thermus	0.0214	0.0008 - 0.1972
Thermotogae	0.0162	0.0014 - 0.0914
SR1	0.0058	0.0004 - 0.0258
Chrysiogenetes	0.0053	0.0005 - 0.0297

Cloacimonetes	0.0047	0.0012	-	0.0195
Aquificae	0.0044	0.0007	-	0.0216
Chlorobi	0.0030	0.0030	-	0.0030
Chlamydiae	0.0021	0.0005	-	0.0043
Parcubacteria	0.0007	0.0005	-	0.0008

Product Study:

Study 1: In this study, six native rumen microorganisms were administered directly to the rumen of 16 cannulated Angus heifers daily to determine the effect of microorganism supplementation on the native rumen microbial community. The animals were located in (b)(6) and were fed a typical feedlot diet (see Attachment 2) over 110 days. Eight control animals received a saline buffer solution, while eight experimental animals received the same buffer solution containing multiple rumen microorganisms. The microbial blend consisted of *S. dextrinosolvens* ASCUSBF53, *Chordacoccus ruminofurens* ASCUSBF65, *Prevotella albensis* ASCUSBF41, *Bacteroides xylanisolvens* ASCUSBF52, and *Clostridium sp.* ASCUSBF26. The animals were transitioned from a low-grain diet (<50% concentrate) to a high-grain diet (≥50% concentrate) (see Attachment 2) over 21 days following a standard step-up procedure. The animals were also challenged with a more fermentable diet to induce acidosis. Rumen samples were taken periodically throughout the study to analyze and characterize the rumen microbiome.

Findings: In this administration experiment, it can be seen that addition of *S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, *P. albensis* ASCUSBF41, *B. xylanisolvens* ASCUSBF52, *Clostridium sp.* ASCUSBF26 to Angus heifers did not significantly alter the rumen bacteria microbiome composition when compared to the control group within each diet condition (Table 2). Abundances of all bacterial phyla are within standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum tended to be similar across experimental groups.

Table 2. Abundance of Rumen Bacterial Phyla in the Rumen from Native Microbials Product Study 1, Reported as a Percent

	Low-grain Diet		High-grain Diet		Acidosis Challenge	
Phylum	No Microbes	Six Microbes	No Microbes	Six Microbes	No Microbes	Six Microbes
Bacteroidetes	32.90	(b) (4)	40.69	(b) (4)	37.86	(b) (4)
Firmicutes	40.32		24.23		28.07	
Proteobacteria	4.20		20.11		17.22	
Actinobacteria	0.54		0.96		2.34	
Synergistetes	0.10		1.52		1.04	
Spirochaetes	0.75		0.27		0.11	
Fibrobacteres	1.29		0.23		0.07	
Tenericutes	0.30		0.08		0.03	
TM7 (Candidatus Saccharibacteria)	0.21		0.08		0.02	
Cyanobacteria/Chloroplast	0.00		0.03		0.02	
Armatimonadetes	0.05		0.04		0.01	
Lentisphaerae	0.00		0.00		0.01	
Fusobacteria	0.03		0.61		0.00	
SR1	0.33		0.04		0.00	

Study 2: In the second experiment, three native rumen microorganisms were added to diet and fed to 75 Angus steers daily over 168 days to determine the effect of microorganism supplementation on the native rumen microbial community. The study took place in (b)(6). The animals were transitioned from a low-grain diet (<50% concentrate) to a high-grain diet (≥50% concentrate) (see Attachment 3). The study consisted of two study groups with 50 animals receiving a microbial blend (*S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41) and 25 animals receiving no microbes (control). Rumen samples were taken periodically throughout the study to analyze and characterize the rumen microbiome.

Findings: In this administration experiment, it can be seen that addition of *S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41 to Angus steers did not significantly alter the rumen bacteria composition when compared to the control group (Table 3). Abundances of all bacterial phyla are within standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum did not differ significantly across experimental groups.

Table 3. Abundance of Rumen Bacterial Phyla in the Rumen from Native Microbials Product Study 2, Reported as a Percent.

	Low-grain Diet		High-grain Diet	
Phylum	**Control**	**Three Microbes**	**Control**	**Three Microbes**
Bacteroidetes	71.43	(b) (4)	39.52	(b) (4)
Firmicutes	14.29		14.96	
Proteobacteria	11.51		44.40	
Cyanobacteria/Chloroplast	0.6596		0.3146	
Synergistetes	0.4559		0.2401	
Actinobacteria	0.3855		0.1982	
Spirochaetes	0.3725		0.1654	
TM7 (Candidatus Saccharibacteria)	0.3661		0.0347	
Tenericutes	0.2262		0.0670	
Lentisphaerae	0.0716		0.0141	
Planctomycetes	0.0461		0.0131	
Fibrobacteres	0.0395		0.0573	
Chloroflexi	0.0368		0.0102	
Verrucomicrobia	0.0298		0.0130	
Elusimicrobia	0.0285		0.0120	
Acidobacteria	0.0245		0.0133	

Armatimonadetes	0.0190	(b) (4)	0.0167	(b) (4)
Fusobacteria	0.0190		0.0136	
Deinococcus-Thermus	0.0188		0.0093	
Candidate Division WPS-2	0.0134		0.0046	
SR1	0.0092		0.0066	

Study 3: In the third experiment, three native rumen microorganisms were added to the ration and fed to Angus steers daily over 109 days. This study took place in (b)(6). The animals were fed typical local farm diets and transitioned from a low-grain diet (<50% concentrate) to a high-grain diet (≥50% concentrate)(see Attachment 4). A blend of microbes (*S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41) were administered to 100 animals, while the other 100 animals received none and served as controls. Rumen samples were taken periodically from a subset of animals throughout the study to analyze and characterize the rumen microbiome.

Findings: In this administration experiment, it can be seen that addition of *S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41 to Angus steers did not significantly alter the rumen bacteria composition when compared to the control group (Table 4). Abundances of all bacterial phyla are within standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum tended to be similar across experimental groups.

Table 4. Abundance of Rumen Bacterial Phyla in the Rumen from Native Microbials Product Study 3, Reported as a Percent.

	Low-grain Diet		**High-grain Diet**	
Phylum	**Control**	**Three Microbes**	**Control**	**Three Microbes**
Firmicutes	48.05	(b) (4)	37.67	(b) (4)
Bacteroidetes	43.82		28.56	
Proteobacteria	3.26		30.81	
Actinobacteria	1.0844		0.9479	
Spirochaetes	0.6173		0.2481	
Lentisphaerae	0.4313		0.0188	
Tenericutes	0.3916		0.1768	

TM7 (Candidatus Saccharibacteria)	0.3542	(b) (4)	0.1278	(b) (4)
Chloroflexi	0.3519		0.0211	
Fibrobacteres	0.3474		0.1324	
Cyanobacteria/Chloroplast	0.2635		0.7363	
Synergistetes	0.2120		0.4497	
SR1	0.1686		0.0184	
Elusimicrobia	0.1158		0.0149	
Fusobacteria	0.1122		0.0450	
Acidobacteria	0.1104		0.0514	
Planctomycetes	0.0944		0.0000	
Verrucomicrobia	0.0825		0.1003	
Armatimonadetes	0.0484		0.0220	
Deferribacteres	0.0374		0.0019	
Candidate Division WPS-2	0.0316		0.0000	
Dictyoglomi	0.0102		0.0000	
Thermodesulfobacteria	0.0087		0.0000	
Deinococcus-Thermus	0.0050		0.0159	
Poribacteria	0.0049		0.0000	
Aquificae	0.0045		0.0245	
Thermotogae	0.0039		0.0000	
Chrysiogenetes	0.0024		0.1505	

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 *S. dextrinosolvens.* Several bacterial analyses conducted by academic institutions were found for beef cattle including: R. M. Petri et al. 2013; Myer et al. 2016; Ribeiro et al. 2017; Khafipour et al. 2009; Stewart et al. 2019, 2018; Kocherginskaya, Aminov, and White 2001). 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:

a) Ribeiro et al. (2017)) 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 transfer microbiome composition are reported in the manuscript, only the pre-transfer results are presented here.

b) Petri et al. (2013) 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.

c) Seshadri et al. (2018) reported an effort on culturing rumen representative microorganisms from global ruminants. The collection represents ~75% of the rumen microbiome at genus level.

d) Myer et al. (2016) studied the rumen microbiome of 3 steers. The animals were cross-breeds of a variety of feedlot cattle on a high-grain diet. The animals were selected based on their similar feed efficiency phenotype and minimal deviation among each other.

e) Stewart et al. (2018) sequenced the rumen samples from 42 Scottish beef cattle and identified 913 representative microorganisms. The cattle were fed a high-concentrate diet. The microbiome was profiled on all samples based on the representative microorganisms.

f) Stewart et al. (2019) sequenced the rumen samples from 283 Scottish beef cattle. The animals were on a high-concentrate diet. The study identified 4,941 representative microorganisms. The microbiome was profiled on all samples based on the representative microorganisms.

g) Auffret et al. (2017) studied the rumen microbiome from 50 beef cattle. The animals were either on a high-concentrate diet or a forage-based diet. The microbiome was profiled for each diet.

h) Myer et al. (2016), R. M. Petri et al. (2013), Ribeiro et al. (2017), and Stewart et al. (2019) have also identified the abundance of *Succinivibrio* in the rumen microbiome. *Succinivibrio* was particularly abundant in animals fed with a high-grain diet, ranging from 0% to 1.6%,

i) Kocherginskaya, Aminov, and White (2001) and Khafipour et al. (2009) also evaluated the rumen microbiome of cattle fed with a high-grain diet. Although their microbiome analysis was not robust enough to include in the analysis here, their results are consistent with others' and Native Microbials' findings.

Findings:

i) 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, and season. Additionally, there are groups of microorganisms that are unique to particular breeds of cattle, 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 the 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 cattle (see Table 5).

Table 5. Abundance of bacterial phyla in the rumen from independent studies, reported as a percent. Empty cells indicate that data was not reported.

	Seshadri et al. 2018	Petri et al. 2013a	Myer et al. 2016	Petri et al. 2013				Ribeiro et al. 2017	Stewart et al. 2019	Stewart et al. 2018	Auffret et al. 2017	
Phylum	(Global Rumen Representatives)	(Rumen Core[a])	(High grain)	(Forage)	(High grain)	(Acidotic)	(Recovery)	(Barley straw)	(High grain)	(High grain)	(Forage)	(High-grain)
Bacteroidetes	12.78	32.8	68.64	25.7	40.3	40	31.5	20.29	49.85	36	31-61	46-61
Firmicutes	68.06	43.2	21.58	55.2	37	33.6	43.7	40.53	33.73	50	20-55	24-76
Proteobacteria	6.19	14.3	0.51	4.7	17.9	16.5	15.2	1.64	7.21	3.1	3-11	3-11
Fibrobacteres	0.4		< 1	7.1				25.04	0.59		0-7	0-2
Spirochaetes	1.2		< 1	2.8				6.13	0.43	1	0-2	0-2
Tenericutes			< 1						0.14			
Actinobacteria	6.59		< 1		1.6			1.78	1.8	3.5	2-21	2-12
Genus												
Succinivibrio	0.60[b]		0.26	0	0.39	0.56	0		1.6			

a. "Rumen core" values reported in Petri et al. (2013a) were sourced from Jouany (1991).

b. The abundance of *S. dextrinosolvens*

ii) 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 and global ruminant microbiome effort were found for cattle fed a variety of diets (Seshadri et al. 2018; Ribeiro et al. 2017; Petri et al. 2013; Petri et al. 2013a; Myer et al. 2016; Stewart et al. 2019, 2018; Auffret et al. 2017). These manuscripts were further investigated to determine prevalence of the overall bacterial taxonomic composition of the typical rumen microbiome. These studies showed that diet formulation has the greatest impact on microbiome composition.

iii) Cumulatively, these independent studies investigated the microbial community across a variety of breeds, diets, and feed management regimes. Table 5 (above) summarizes the findings from Seshadri et al. (2018), Ribeiro et al. (2017), Petri et al. (2013), Petri et al. (2013a), Myer et al. (2016), Stewart et al. (2019), Stewart et al. (2018), and Auffret et al. (2017) 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. Proteobacteria is the third most prevalent phylum in ruminants and its abundance is directly positively correlated with the amount of corn in diet (Kocherginskaya, Aminov, and White 2001). As can be seen from this data, there is a broad range of abundances. *S. dextrinosolvens* ASCUSBF53 falls into the Proteobacteria phylum, which was found to comprise 0.51% - 17.9% of the rumen microbial community. Kocherginskaya, Aminov, and White (2001) found the abundance of ruminal Proteobacteria is enriched in animals on a corn based high-grain diet. The study reported that Proteobacteria can comprise up to 27% of the rumen microbiome.

iv) Based on the global rumen microbiome effort, *S. dextrinosolvens* represents 0.6% of the rumen microbial populations. The abundance of genus *Succinivibrio* ranged from 0.26% to 1.6% in rumen content of animals fed a high-grain diet but few were detected in animals on a forage based diet (Table 5). This is consistent with the findings of Khafipour et al. (2009) that the abundance of *S. dextrinosolvens* is associated with a high-grain diet.

v) 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 12.78%-68.64%, while the observed abundance of Firmicutes ranged from 20%-76%. Proteobacteria ranged from 3%-27% and could be higher, depending on the corn content in the diet. Other phyla did appear, but often represented less than 10% of the total bacterial population. These ranges were utilized to describe the “average” rumen in comparative analyses.

Conclusion

This summary covers the Native Microbial studies as well as published data to assess the potential microorganisms shift in microbiome that may raise safety concerns. Information presented demonstrated that the normal microbial community in the rumen is robust and not adversely affected by the addition of native external microbes, including *S. dextrinosolvens*. Hence, it is clear that the dietary addition of *S. dextrinosolvens* will not cause a safety concern based on changes in the microbiome**.**

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

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Attachment 1: Knoxville, TN Survey details

Diet: The survey took place in (b)(6) and utilized the following diet:

As-fed Ingredients	%
Corn Silage	80
Cracked Corn	10
Protein Supplement	10
DM	%
Crude Protein	11.57
Total Digestible Nutrients	76.93

Rumen samples were collected every 7 days via oro-gastric tubes. Steers were observed daily for overall clinical health throughout the study.

Attachment 2: (b) (4) **Product Study details**

Diet: The study took place in (b)(6) and utilized the following diet:

As-fed Ingredients (%)	Low-grain	High-grain	Acidosis Challenge
Hay	60.8	2.3-40.6	2.38-2.54
Corn Silage	17.6	14.8-17.1	7.02-7.87
Dry Rolled Corn	13	25.5-50.9	53.12-54.19
MDGS	7.2	14.1-25.3	--
Reconstituted DDGS	--	--	18.43-19.48
DDGS	--	10.99-11.04	11.01-11.06
Vitamin and Mineral Premix	1.4	2.7-5	5
water	--	14.38-15.85	11.28-21.16
Roughage Dry Matter (%)	**92.31**	**1.97-42.61**	**0.9-2.05**
Concentrate Dry Matter (%)	**7.69**	**57.39-98.03**	**97.95-99.1**

Animals were transitioned from a low-grain diet to a high-grain diet. Rumen acidosis was induced twice by increasing the amount of grain 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 every 3 to 4 days. Heifers were observed daily for overall clinical health throughout the study.

Attachment 3: Parma, ID product study

Diet: The survey took place in (b)(6) and utilized the following diet:

As-fed Ingredients (%)	Low-grain Diet	High-grain Diet
Alfalfa Hay	--	2.5-33.8
Corn (Rolled)	--	10.2-12
Corn Syrup	15	14.6-21
Distillers	--	0-7
Earlage	--	22-44.2
Grass Hay	77.5	0
Vitamin and Mineral Mix	2.5	3-4.6
Tallow	--	0-1.8
Wheat	--	10-27
Wheat Straw	--	0-4
Water	5	0
Roughage Dry Matter (%)	**91.42**	**10.23-44.63**
Concentrate Dry Matter (%)	**8.58**	**55.37-89.77**

Rumen samples were collected via oro-gastrictubing on days 0, 13, 28, 56, 88, 127, 153, and 168. Animals were observed daily for overall clinical health throughout the study.

Attachment 4: Native Microbial's third product study experiment

Diet: The survey took place in (b)(6) and utilized the following diet:

Ingredients (% DM)	Low-grain Diet	High-grain Diet
Alfalfa hay	100	6-35
Dry rolled corn	--	54-81
Molasses (cane 64)	--	6
CA23.00 Early Pel	--	5-9.04
Roughage Dry Matter (%)	**100**	**6-35**
Concentrate Dry Matter (%)	**0**	**65-94**

Rumen samples were collected via oro-gastrics tubing on days 7, 15, 29, 57, 83, and 109. Animals were observed daily for overall clinical health throughout the study.

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 ((b) (4), (b)(6) and 5% (b) (4), (b)(6), 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.

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 (b) (4) Environmental RNA/DNA Isolation Kit (b) (4), (b)(6), USA). The 16S rRNA gene was amplified using 27F and 534R (LANE and J 1991; Muyzer, de Waal, and Uitterlinden 1993) primers modified for (b) (4) sequencing, following standard protocols (b) (4), (b)(6) USA). Following amplification, PCR products were verified with a standard 2% agarose gel electrophoresis and purified using (b) (4), (b)(6), USA). The purified amplicon library was quantified and sequenced on the (b) (4), (b)(6) USA) according to standard protocols using a 2x300 v3, 600-cycle kit. Raw fastq reads were de-multiplexed on the (b) (4), (b)(6), USA). All samples were sequenced at a depth such that each sample file contained at least 10,000 sequences after processing.

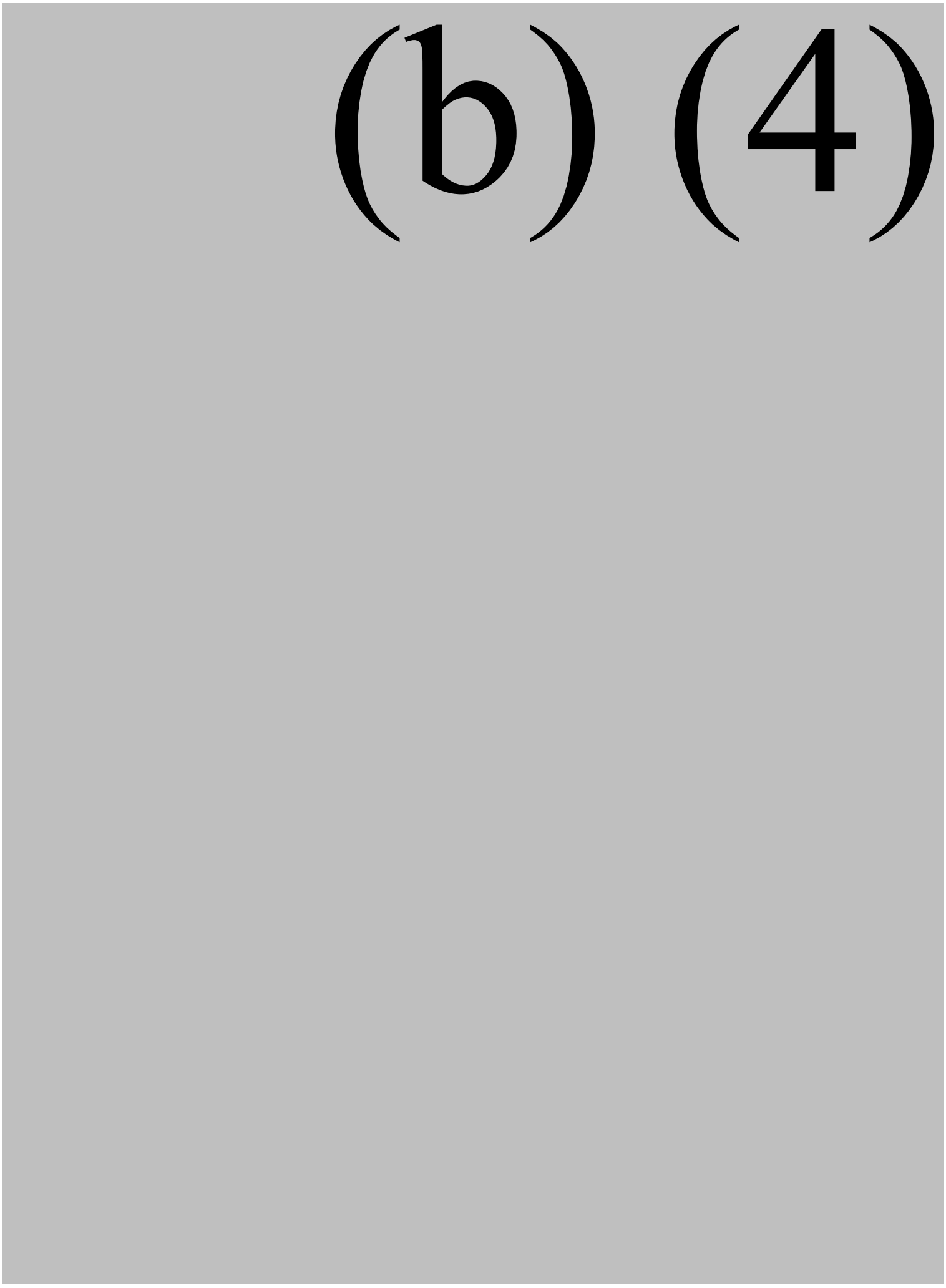
Analysis Method

All raw sequencing data was trimmed of adapter sequences and phred33 quality filtered at a cutoff of 20 using Trim Galore (Krueger and Others 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 2016; Edgar and Flyvbjerg 2015; Edgar, 2016a) 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.

(b) (4)

(b) (4)

(b) (4)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FINAL STUDY REPORT

A comparison of novel microbial products administered in feed to improve beef steers' performance

PROTOCOL NUMBER:	ASC1901
COMPOUND NAME:	Magnius
AUTHOR:	(b)(6)
STUDY INITIATION DATE:	15Apr19
STUDY COMPLETION DATE:	Date of Study Director's signature on Final Report

FINAL STUDY REPORT APPROVAL -

Dated: 13 FEB 2020 Signed: (b)(6)

Dated: 14FEB2020 Signed: (b)(6)

Mallory Embree, Ph.D.
Sponsor Representative
Ascus Biosciences, Inc.

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1 OBJECTIVE

To determine the effects of Ascus microbes, when administered in feed as a top dress, on average daily gain, feed efficiency, and rumen content parameters. Specifically, this inclusion in feed for feedlot cattle was administered throughout the entire feeding period, including during transition to a high concentrate ration.

1.1 Experimental Design

This study was a randomized complete block design with weight as the blocking factor consisting of seventy-five individually penned angus or cross-bred beef steers. During individual housing, feed consumption was monitored, and scheduled rumen samples and body weights were collected.

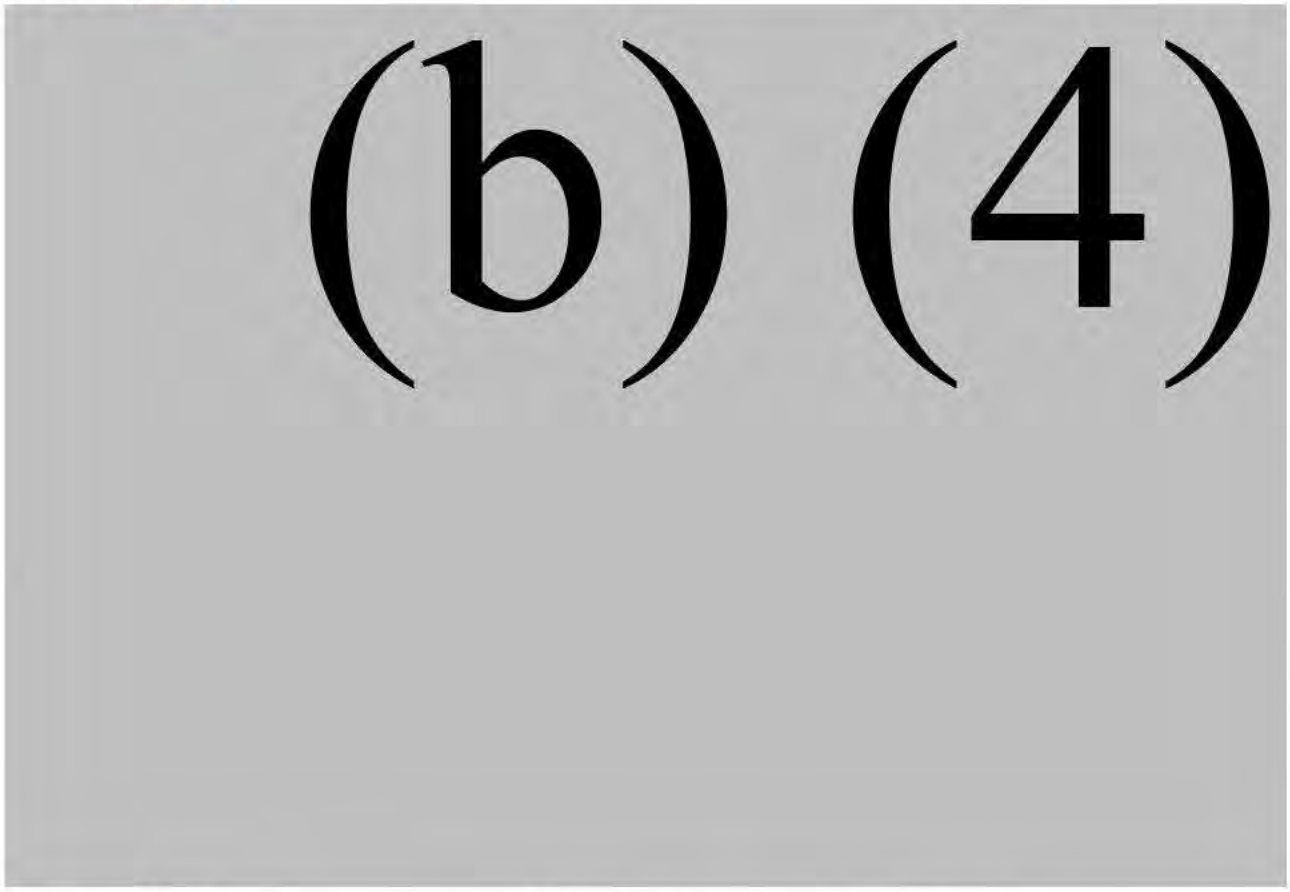
Table 1 Treatment Groups

Treatment Group Description	Gender	Dose	Route / Frequency	Animals per Treatment
Control- not inoculated	(b) (4)			
Microbial-inoculate				
Microbial-inoculate				

1.2 Assignment to Study and Randomization

(b) (4)

1.3 Masking



	RED			GREEN			YELLOW		
Pen number	120	121	122	123	124	125	126	127	128
Weight	Heavy	Middle	Light	Heavy	Middle	Light	Heavy	Middle	Light
[n= number per pen]	7	8	8	8	8	9	8	8	9

2 STUDY SCHEDULE

2.1 Acclimation and Treatment Period

Table 3. Acclimation and Treatment Period Study Schedule

Activity	Date	Study Day
Animal selection and individual pen acclimation	Friday, June 14, 2019	-7
Initial body weight, Rumen Sample, Weigh back	Thursday, June 20, 2019	-1
Second Day 0 Body weight; Start Step up Ration 1.	Friday, June 21, 2019	0
Bodyweight, Rumen Sample;	Wednesday, July 03, 2019	12
Weigh back with Dry Matter; Start Step up Ration 2.	Thursday, July 04, 2019	13
Start Step up Ration 3.	Friday, July 12, 2019	21
Bodyweight, Rumen Sample; Start Finish Ration.	Thursday, July 18, 2019	27
Bodyweight, Rumen Sample	Thursday, August 15, 2019	55
Bodyweight, Rumen Sample, Dry Matter on Weigh back	Monday, September 16, 2019	87
Bodyweight, Dry Matter on Weigh back	Tuesday, September 17, 2019	88
NEW FINISH RATION- Sample, DM, Weigh back	Monday, September 23, 2019	94
3 Pens (Middle Weights) collect rumen samples post feeding 2 hours.	Tuesday, October 01, 2019	102
Ration change to increased concentrate if needed.	Thursday, October 24, 2019	125
Bodyweight, Rumen Sample, Weigh back	Friday, October 25, 2019	126
Bodyweight, Rumen Sample, Weigh back	Wednesday, November 20, 2019	152
Final Rumen sample and Bodyweight	Friday, December 06, 2019	168
Secondary Final Bodyweight	Monday, December 09, 2019	171

3 ANIMAL SELECTION AND IDENTIFICATION

Table 4. Test Animal Description

TEST ANIMAL DESCRIPTION	
Source	(b) (4)
IACUC Site Number	(b) (4)
Number of Animals Received / Date	82 steers/ 13May19 15 steers/ 03Jun19
Age	7-10 months

Sex and Physiological Status	Steers
Species / Breed	Bovine; Crossbred beef
Implants	Existing implants removed
Initial Body Weight	~769 lbs
Identification Method	Duplicate ear tags (one in each ear) with an individualized numerical identifier for each animal with a separate tag with color that corresponded to pen.
Pre-Study Processing	Revalor XS/ Lot:A210A01 / Exp date: Mar2020 Cydectin/ Lot: AH02LLH / 07-20 Bovi-Shield Gold 5/ Lot:331442 / 14Jan20 Cavalry 9/ Lot: 302 / 20Aug2021 Safeguard/ Lot: E772A01 / 12-2020

4 STUDY FACILITIES AND ANIMAL MANAGEMENT

Table 5. Study Facilities and Animal Management

<table>
<tr><th colspan="2">STUDY FACILITIES AND ANIMAL MANAGEMENT</th></tr>
<tr><td>Housing</td><td>57ft by 9.0 ft. (bunk variation from 8-9 ft) =513 ft^2 per animal in individual pen study.
75ft by 25.0 ft. = 2025 ft^2 total in group pen study.</td></tr>
<tr><td>Diet and feeding method</td><td>Cattle were housed in individual pens and fed a transition ration as follows until 16Sep19:
(b) (4)</td></tr>
<tr><td>Water analysis</td><td>Fresh, potable, non-medicated water was available ad libitum. A copy of the most recent water analysis was included in the study notebook. Water analysis included total coliforms, arsenic, lead, mercury, nitrates, and sulphate levels and results were included in the study records. Water testing results indicated water was acceptable for livestock.</td></tr>
<tr><td>Concomitant Medications</td><td>Two instances of concomitant medication intervention were required on trial.
<table>
<tr><td>Animal</td><td>Medication</td><td>Route</td><td>Diagnosis</td></tr>
<tr><td>1861</td><td>Oxytetracycline</td><td>Subcutaneous</td><td>Pink-eye</td></tr>
<tr><td>1807</td><td>Tildipirosin</td><td>Subcutaneous</td><td>BRD</td></tr>
</table></td></tr>
</table>

Environmental conditions	Data collected at the local U.S. Bureau of Reclamation included, daily mean (°F), minimum (°F), and maximum air temperatures (°F), and mean daily humidity (%). Average Temperature over the entirety of the study was 58.12 °F Average Relative Humidity was 58.37%. Full documentation is recorded in the study files.

5 TEST MATERIALS

The testing facility-maintained records of test article receipt and use and these records are included in the raw data.

5.1 Investigational Test Article (TA)

Table 6. Investigational Test Article (TA) Characteristics Low Dose (Green)

TEST ARTICLE (TA)	
Generic name:	(b) (4) Low Dose
Organism type: CFU	*Prevotella albensis*: 2 x 10^7 CFU/g (1-10%) *Succinivibrio dextrinosolvens*: 2 x 10^7 CFU/g (1-10%) Chordacoccus ruminofurens: 2 x 10^7 CFU/g (1-10%) Calcium Carbonate (70-97%)
Storage conditions:	Storage excursions to 8°C were permitted. Actual storage conditions daily averages were between 2-8°C.
Special precautions:	Safety procedures for handling the test article were done in accordance with the applicable standard operating procedures (SOPs) and Material Safety Data Sheets (MSDSs). No inhalation exposure, skin contact, eye contact, or ingestion occurred working with this material.

Table 7. Investigational Test Article (TA) Characteristics High Dose (Red)

TEST ARTICLE (TA)	
Generic name:	(b) (4) High Dose
Organism type: CFU	*Prevotella albensis*: 2 x 10^8 CFU/g (1-10%) *Succinivibrio dextrinosolvens*: 2 x 10^8 CFU/g (1-10%) Chordacoccus ruminofurens: 2 x 10^8 CFU/g (1-10%) Calcium Carbonate (70-97%)
Storage conditions:	Storage excursions to 8°C were permitted. Actual storage conditions daily averages were between 2-8°C.

Special precautions:	Safety procedures for handling the test article were done in accordance with the applicable standard operating procedures (SOPs) and Material Safety Data Sheets (MSDS). No inhalation exposure, skin contact, eye contact, or ingestion occurred working with this material.

6 Data Collection

6.1 Daily Observations

Steers were evaluated daily for signs of lethargy, in-appetence, diarrhea, or general abnormalities common in feedlot beef steers. Any problems were relayed to the study investigator or site veterinarian for clinical evaluation. Based on clinical interpretation of the disease, intervention was prescribed accordingly. Documentation of abnormalities were recorded on the daily health observation record and adverse event records.

6.2 Physical Exams

All steers were evaluated prior to study start. Veterinary exam included all body systems of importance and relevance to the study. The primary goal of interest was utilization of healthy animals that would complete the study. Organ systems evaluated were: Respiratory, Gastrointestinal, Cardiovascular, Musculoskeletal, Integument, and Neurologic. Animals were confirmed castrated and primarily of British descent.

6.3 Body Weight Measurements

Steers were weighed using a certified scale that was accurate from 1 to 1800 pounds. Prior to each weighing event when bodyweights were captured, a scale check was performed to ensure the scale was functioning properly. Certification via a third party for the weights and the scale was performed.

6.4 Rumen Sample Collection and Processing

Cattle on study were physically restrained in a commercial chute. An (b) (4) tube was passed orally into the rumen and a manual hand pump was attached to the end of the tubing. Pumping removed rumen fluid and content that was collected in a disposable cup. After collection, ruminal pH was immediately determined (b) (4), and subsequently % and mmHg of dissolved carbon dioxide of the sample was then quantified via (b) (4) A separate (b) (4) tube was used for each treatment group and care was used to wash the (b) (4) tubes with water in between each animal.

6.5 Feed Intake Monitoring

Feed intakes were monitored daily via certified scales and dry matter from ration samples were quantified periodically throughout the study. Scale certification occurred daily and feed refusals were also collected via certified scale. Dry matters were determined on feed refusals.

7 ADVERSE EVENTS (AE)

Table 8 Adverse Event

Animal ID	Event Description
1828	Animal removed from trial due to anorexia
1861	Pinkeye
1844	Animal removed from trial due to Anorexia and Cachexia
1807	Bovine respiratory disease
1888	Musculoskeletal lameness

8 STUDY COMPLETION AND ANIMAL DISPOSITION

8.1 Food Use Authorization and Holding Requirements

No food use authorization was allowed for the test article products of investigation. Upon in-life completion, animals that were within the control group re-entered the commercial herd. All animals administered test article (low or high dose (b) (4) were euthanized and disposed of at local landfill.

9 STUDY PERSONNEL and CONTACT INFORMATION

A listing of key study personnel and contact information is summarized in the following table.
Table 9: Key Personnel

Study Location	**Key Personnel**
Testing Facility	
	Study Investigator:
	(b) (4), (b)(6)
Sponsor Representative:	
Ascus Biosciences, Inc. 6450 Lusk Blvd. Suite E109 San Diego, CA 92121	Mallory Embree, Ph.D. Phone: 877-696-8945 ext. 701

10 ARCHIVING AND RETENTION

10.1 Testing Facility

Back-up rumen content samples were returned to the sponsor from the testing site. Shipment to the sponsor occurred on 09Jan2020.

10.2 Investigational Veterinary Product (TA)

No investigational veterinary product was retained. All material used for test article administration was disposed of or returned to sponsor.

11 PROTOCOL AMENDMENTS AND DEVIATIONS

11.1 Protocol Amendments

Table 10. Protocol Amendment Summary

Amendment No.	Protocol section	Date (s)	Amendment Summary	Study Impact
1	Randomization	21May19	Added in an assigned color to each treatment to maintain blinding	positive
	Bodyweight	21May19	Secondary bodyweight collections for initiation and at completion of the study.	positive
	Feed Administration	21May19	During treatment, feeding was amended to strive for 95% ad libitum through visual bunk call. Tylosin was also added to the diet.	neutral
	Ration and Feeding schedule	21May19	Each ration was scheduled to be saved at -20 degrees Celsius	positive
	Weigh backs	21May19	Changed to a visual bunk call and weigh backs done as needed or at the least once a week.	neutral
2	Disposition and Accountability	06Sep19	Steers treated with test article were not allowed to enter the food chain due to lack of food use authorization.	neutral
3	Study Continuation	16Sep19	The study was continued. Animals assigned to specific treatments continued to receive their	neutral

			treatments during transition to a group housing. Bodyweights and rumen sampling continued	

11.2 Protocol Deviations

Table 11. Protocol Deviation Summary

Deviation No.	Date (s)	Protocol Sections Affected	Deviation Summary	Study Impact
1	09Jul19	Feed administration and TA Delivery	During training of a new employee two animals from the yellow treatment group were inadvertently missed and did not receive TA and the 2 lbs of feed for TA delivery	Minimal
2	13Aug19	Weigh back collection	On 27Jun19 and 30 Jun19 weigh back samples were not attained for Dry Matter. Both were individual pens 57C and 59B respective to previous dates.	Minimal

12 RESULTS

Where possible, results were calculated using a two-way analysis of variance or repeated measures analysis of variance using (b) (4) to generate LS means, standard errors, and confidence intervals. Animals 1844 and animals 1828 were removed from analysis due to early removal from study as a non-test article related removals.

12.1 General Animal Health

Overall, general health was within normal limits across all treatment groups for both individual housing and group housing. There were no adverse events attributable to or consistent with a specific test article. Health events were not outside of normal limits for cattle transitioning to high concentrate diets in the feed yard.

12.2 Ruminal Fluid CO_2

Figure 1. Percent Ruminal Dissolved CO2 Over Time

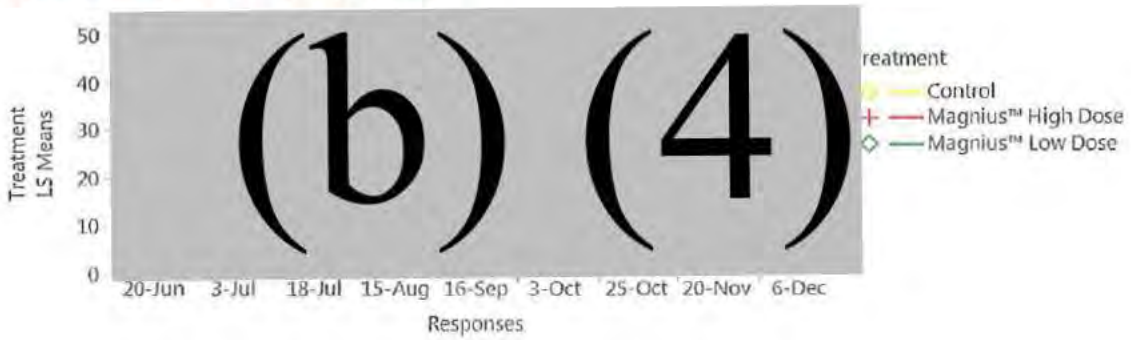


Table 12. Dissolved Carbon Dioxide Data

Treatment	Dissolved CO_2 over time								
	20-Jun	3-Jul	18-Jul	15-Aug	16-Sep	3-Oct*†	25-Oct†	20-Nov†	6-Dec†
Control	(b) (4)								
(b) (4) ow Dose									
igh Dose									

*Subset population of animals sampled post feed delivery at peak ruminal fermentation
†Group housing in small pens with ration change to a higher caloric / higher rapidly fermentable feed ingredient

12.3 Ruminal Fluid pH

Figure 2. Ruminal pH Over Time

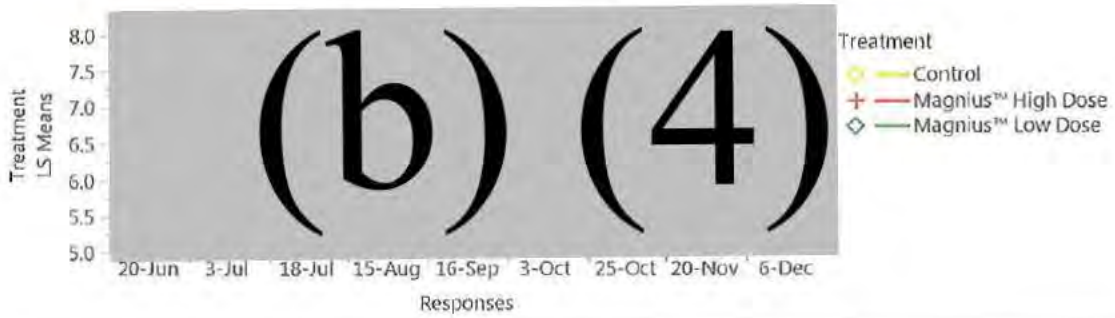


Table 13. Ruminal pH Data

Treatment	Ruminal pH over time								
	20-Jun	3-Jul	18-Jul	15-Aug	16-Sep	3-Oct*†	25-Oct†	20-Nov†	6-Dec†
Control									
(b) (4) Low Dose									
High Dose									

*Subset population of animals sampled post feed delivery at peak ruminal fermentation
†Group housing in small pens with ration change to a higher caloric / higher rapidly fermentable feed ingredient

12.4 Palatability

Cattle intakes were not different across treatment groups in either the individual housing study or the group housing study. Observations of animals at the time of administration for the test article indicated that there were no palatability issues with the test article.

Table 14. Feed Consumption Data

Individual Housing					
Treatment	Number	Individual Total Feed consumed (lbs)	Standard Error	Lower 95%	Upper 95%
Control					
(b) (4) Low Dose					
High Dose					
Group Housing (pens)					
Treatment	Number	Individual Total Feed consumed (lbs)	Standard Error	Lower 95%	Upper 95%
Control					
(b) (4) Low Dose					
High Dose					

12.5 Body Weight (ADG)

Table 15. Gain Data

Individual Housing					
Treatment	Number	Mean ADG (lbs)	Standard Error	Lower 95%	Upper 95%
Control					
(b) (4) Low Dose					
High Dose					
Group Housing (pens)					
Treatment					
Control					
(b) (4) Low Dose					
High Dose					
Overall					
Treatment					
Control					
(b) (4) Low Dose					
High Dose					

12.6 Feed Efficiency (F:G)

Table 16. Feed Efficiency Data

Individual Housing					
Treatment	Number	Mean F:G (lbs)	Standard Error	Lower 95%	Upper 95%
Control					
(b) (4) Low Dose					
High Dose					
Group Housing (pens)					
Treatment	Number	Mean F:G (lbs)	Standard Error	Lower 95%	Upper 95%
Control					
(b) (4) Low Dose					
High Dose					

13 CONCLUSION

Use of (b) (4) in feedlot steers appears safe and well tolerated when assessed via normal feed yard practice of observation for health abnormalities. Effects were limited and did not result in treatment differences for ADG. High dose steers had slightly less efficient feed conversion during individual housing. Overall, Feed efficiency was not different between groups during group housing. During group housing there was an evident treatment effect of (b) (4) on ruminal pH. Control treated steers had a ruminal pH lower than that of the treated groups. This difference was not reflective in % dissolved CO_2.

Cerrito, Chelsea

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Wednesday, August 10, 2022 5:58 PM
To: Animalfood-premarket
Cc: Kevin Korth
Subject: [EXTERNAL] RE: Status Update for GRAS Notice AGRN # 45 Succinivibrio dextrinosolvens ASCUSBF53
Attachments: GRAS Amendment - AGRN 45 Sdextrinosolvens ASCUSBF53 07-2022--FINAL.pdf; AGRN45 _Amendment_Combined_Attachments.pdf; Section_6_Amendment_References_Combined_AGRN45 _.pdf

In response to the July 21, 2022 request for additional information to support the GRAS Notice 45, for the use of Succinivibrio dextrinosolvens ASCUSBF53 in beef cattle feed, we are providing the attached information.

This email has attached the narrative of the amendment, copies of the amendment attachments, and the revised Section 6 (attachment 12) references. The narrative reference material will be sent by a subsequent email.

We appreciate the opportunity to amend the GRAS notice. Please confirm that you have received this information.

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: Animalfood-premarket [mailto:Animalfood-premarket@fda.hhs.gov]
Sent: Thursday, July 21, 2022 2:57 PM
To: Kristi Smedley
Cc: Animalfood-premarket
Subject: Status Update for GRAS Notice AGRN # 45 Succinivibrio dextrinosolvens ASCUSBF53

Dear Dr. Smedley,

This is in regards to Native Microbials Inc.'s GRAS notice for *Succinivibrio dexrinosolvens* ASCUSBF53, as a viable microorganism in diets of beef cattle, designated as GRAS Notice No. AGRN 45 that was filed on March 16, 2021. At this point in our evaluation of the notice, CVM has questions on the following sections:

Chemistry, Manufacturing and Controls (CMC)

1. Manufacturing Process and Controls

- The appendix 10, App_010_Manufacturing Process (CONFIDENTIAL).pdf is titled ASCUSBF65. The notifier needs to clarify if it is a typographical error, and whether the manufacturing is for ASCUSBF53.

- The notifier should clarify what % or concentration of (b) (4) is used as an antioxidant for maintaining the fermentation broth.

- It is not clear what concentration of (b) (4) is used in the pre-prepared preservation mixture. The notifier should provide these details.

- In Section 4 – Biomass Harvest by Centrifugation in Appendix 010, (b) (4) s.

2. Starting Materials

- In Table 1 of Appendix 010, the 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	

- 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.

- The specification unit for cadmium, lead and arsenic in (b) (4) (Appendix 009C) are listed as g/g, the notifier needs to clarify the unit (g/g, mg/kg, µg/g or ppm?).

- The notifier provides a product data sheet for defoamer (b) (4) from (b) (4) in Appendix 009B. The data sheet does not list components of (b) (4) but it states that (b) (4) contains ingredients included in the "no objection" letter that was issued by FDA to the (b) (4) regarding the use of (b) (4) (page 4 in Appendix 010). However, in Table 1 of Appendix 010, " (b) (4) The notifier should clarify this discrepancy and provide quantity and revised table with specific function.
- The notifier refers to AAFCO OP 33.19 for the definition of the hydrogenated glycerides that is used to encapsulate the cryo-milled freeze-dried *S. dextrinosolvens* ASCUSBF53. However, AAFCO OP 33.19 - Hydrogenated Glycerides has been withdrawn. Current Tentative definition OP T73.311 (A) – Hydrogenated

Glycerides requires that vegetable oils used to produce the hydrogenated glycerides must meet the requirements listed in AAFCO OP 33.2. It is the notifier's responsibility to ensure that their hydrogenated glycerides meet the current requirement.

3. Specifications of the Notified Substance

- The notifier states that three batches of *S. dextrinosolvens* ASCUSBF53 cell concentrate were analyzed for botulinum toxins. The notifier needs to clarify why the batches were being tested for botulinum toxins. 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.

- The notifier states that the batches tested to establish specifications are representative of the commercial materials. Same batches were also used in the stability study. However, necessary information is not provided to justify how the tested batches can represent the expected quality of the commercial products.
  The same batch IDs are used for *S. dextrinosolvens* ASCUSBF53 cell concentrate, freeze dried powder and final fat encapsulated product as listed in the table below:

Batch # (Lot #)	Manufacturing date		
	Cell concentrate	Freeze dried powder	Fat encapsulated
1801.2041 (2041)	unknown	11/27/2020	12/09/2020
1801.2042 (2042)	unknown	11/27/2020	12/09/2020
1801.2044 (2044)	unknown	11/27/2020	12/10/2020

The notifier needs to clarify the relationship among these products bearing the same batch ID, e.g. whether fermentation batch 1801.2041 was processed to produce only freeze dried and fat encapsulated batch 1801.2041. The notifier also needs to clarify whether batches 1801.2041, 1801.2042, and 1801.2044 were three independent fermentation batches.

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 *S. dextrinosolvens* ASCUSBF53 cell concentrate, freeze dried powder and final fat encapsulated product. Considering that 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 of 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 *S. dextrinosolvens* ASCUSBF53.

4. Stability

The submitted data collected at 40°C, 50°C and 60°C are not adequate to demonstrate/estimate the stability of Fat Encapsulated *S. dextrinosolvens* ASCUSBF53 at 2-10°C. Using the Arrhenius equation to predict the stability or viability of microorganisms at different temperatures have been explored but presented 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.

To support the claimed shelf life for the Fat Encapsulated *S. dextrinosolvens* ASCUSBF53 at 2-10°C, the notifier should provide real time stability data under the recommended storage conditions using representative pilot or commercial batches.

5. Analytical Methods

- The in-house enumeration method approach is acceptable to determine the viable cell counts of solid and liquid intermediates of *S. dextrinosolvens* ASCUSBF53 in freeze-dried and fat encapsulated product. However, I note that Appendix 012B summarizes a microbe enumeration validation report for BF65 solid intermediate and not for *S. dextrinosolvens* ASCUSBF53. The notifier should provide a justification or the summary of BF53 solid enumeration method validation results. Appendix 012E is titled "BF52 Liquid Intermediate Microbe Enumeration Validation Summary Report" instead of BF53 and Table 1 in the appendix 012E is titled as "Summary table of DY19 liquid enumeration method validation results". The notifier should address these discrepancies and provide a justification or the summary of BF53 liquid enumeration validation results.

- The notifier refers to the 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.

Microbial Safety

Genome safety

1. The notifier's cut-off setting for database searches is too stringent and would not allow identification of homologs for toxins and virulence factors 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.

2. Regarding the databases used by the notifier, the notifier should 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.

3. Clarification is needed about the notifier's conclusion that IslandViewer 4 web server did not identify any pathogenicity islands in *Succinivibrio dextrinosolvens* ASCUSBF53, e.g., how many genomic islands are predicted for the *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 did not raise safety concerns, e.g., being associated with pathogenicity? The firm should address all these points in a revised narrative.

Microbial safety

1. In the case any potential toxins or virulence factors are identified using appropriate databases and cut-off setting, the notifier should revise its narrative and address how those toxins or virulence factors will not raise safety concerns.

2. It has been reported in the scientific literature that, under certain conditions, increased abundance of lactate producing bacteria including *S. dextrinosolvens* may be associated with metabolic/digestive disorders, including ruminal acidosis and frothy bloat. (PMID: 31811042, PMID: 23584771, PMID: 30862851). However, none of the above-mentioned articles are included and discussed in the notice. For microbial safety, the notifier is responsible to provide a balanced view of the scientific literature, including both positive and negative information. Thus, the notifier should include those articles and address any associated safety concerns in its revised narrative.

Utility

1. Native Microbials states in Section 1.3 and Section 2.5 of its GRAS notice (page 9) that the intended purpose of *Succinivibrio dextrinosolvens* ASCUSBF53 supplementation is to "augment the digestion of feed in the rumen". However, it also states in Section 2.5 (page 37) that *S. dextrinosolvens* ASCUSBF53 will act only to support normal ruminal function of digestion of animal feed. This conclusion is also supported by the statement in Section 6 (page 43) of its notice, "the microorganism [referring to *S. dextrinosolvens*] has the potential to support digestion by aiding fermentation of forages and partially degraded digesta in the rumen." Thus, the description of the intended conditions of use of the additive is not consistent and the intended technical effect may be acceptable if as described elsewhere in the notice, the notifier indicates that the use is to support rumen fermentation. CVM notes that the terms "support" and "augment" have different meanings.

2. Native Microbials describes in Section 2.5 that "the technical effect of *S. dextrinosolvens* ASCUSBF53 when fed to beef cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety." However, the notifier incorporates numerous statements in Section 2.5 that describe how modifying the microbiome could influence rumen fermentation processes and provides examples of ways that *S. dextrinosolvens* might alter end-products of digestion and subsequently these end-products have altered composition of animal products or animal productivity. Further, there are claims made in this section that are not consistent with use as an animal food product. The notifier needs to address how supplementing *S. dextrinosolvens* ASCUSBF53 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. The notifier should recognize that it is contradictory to argue that safety does not relate to utility, but then to include a discussion outlining expected benefits associated with feeding the viable microorganism, such as increased digestion, improved animal productivity. Some of these do relate to safety. This issue could be addressed by removal of this type of information from this section.

Target Animal Safety

Based on the information contained in the notice submitted by Native Microbials, Inc., CVM has questions on the target animal safety of the notified substance, S. dextrinosolvens ASCUSBF53 strain, and its intended use as a direct-fed microbial for beef cattle at a use rate of $1x10^8$ CFU/head/day.

The firm should provide a robust narrative describing how reasonable certainty of no harm for target animal safety can be derived from data and information included in the sections on genomic and microbial safety and discuss how this conclusion is corroborated by publicly available and other target animal data. In addition, the firm should ensure it addresses the following:

1. The firm includes a proposed specification for botulinum toxins in the cell concentrate (Table 2.18; Pages 28-29 of M-000106-N-0001_sub_001.pdf). The firm should clarify why botulinum toxins are tested.

2. In section 6.5 of the firm's notice, two studies were cited to support the safety narrative. In both articles, an unspecified strain of *S. dextrinosolvens* was administered to ruminants. However,
    a. The Bello et al. (2019) article is written in Spanish. An English translation should be provided.
    b. The Rigobelo et al. (2016) article does not specify which strain of S. dextrinosolvens was administered to sheep. Additionally, this was not a safety study for the microbe but a clinical challenge with shiga toxin-producing *E. coli*. Given this, it appears that this study does not support the firm's safety conclusion. This firm should clearly describe how this study provides information for TAS of their *S. dextrinosolvens* ASCUSBF53 strain.

3. Exposure calculations for the target animals provided on page 40 of the notice are inconsistent and appear to contain errors.

4. A recently published article reported a correlation between growth of a small group of bacteria, one of which was *S. dextrinosolvens*, and development of ruminal acidosis.[1] Another publication indicates that levels of Succinivibrio (and Myxococcales) are consistently enriched in rumens of cattle with alfalfa-induced frothy bloat [2]. The notifier needs to address safety of *S. dextrinosolvens* ASCUSBF53 in beef cattle transitioning to high grain diets and the conditions of intended use since Native Microbials states on page 46 of the notice, "internal studies have shown that this species can comprise up to 20% of the rumen population". The firm should use available data and literature to address if there is potential for the S. dextrinosolvens ASCUSBF53 strain to induce frothy bloat. The firm should be sure to discuss implications of potential frothy bloat occurrences for TAS.

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[1] Dai and coworkers, 2019; J. Dairy Sci. 102:334–350: doi:10.3168/jds.2018-14807; Dai and coworkers, 2020; Applied and Environmental Microbiology 86:e02193-19. doi:10.1128/AEM.02193-19; Mizuguchi and coworkers, 2021; J. Vet. Med. Sci. 83(6):905-910: doi-org.fda.idm.oclc.org/10.1292/jvms.21-0037.

[2] E Azad, H Derakhshani, RJ Forster, RJ Gruninger, S Acharya, TA McAllister, E Khafipour. 2019. Characterization of the rumen and fecal microbiome in bloated and non-bloated cattle grazing alfalfa pastures and subjected to bloat prevention strategies. 2019. Scientific Reports 9:4272

CVM requests that the information be sent within two weeks from the receipt of this email which will be on August 04, 2022 in order to complete the evaluation of the GRAS notice.

Thank you,

Wasima

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FDA
U.S. FOOD & DRUG
ADMINISTRATION

AGRN # 45 *Succinivibrio dextrinosolvens* ASCUSBF53
GRAS Notice Amendment

The following represents the Native Microbials, Inc. response to the FDA-CVM questions in the email dated July 21, 2022 from Wasima Wahid, M.S. The contents of the email are represented below, with the response below each question in blue text. Supporting documentation is contained in referenced attachments.

Native Microbials, Inc. continues to conclude that S. *dextrinosolvens* ASCUSBF53 is generally recognized as safe as a direct fed microbial in beef cattle at the intended rate of inclusion.

List of Attachments:

1. [Revised] Dossier Appendix 10 - Confidential Detailed Manufacturing Summary of Fat Encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53
2. USDA Condensed Fermented Corn extractive monograph.
3. Monopotassium Phosphate Animal Feed Safety Regulatory Review.
4. (b) (4) (b) (4) Letter and CofA Showing Correct Units.
5. [Revised] Dossier Sections 1.3, 2.1 and 2.5
6. Botulinum test method letter.
7. [Revised] Dossier Section 3.1.2.
8. BF53 Solid Intermediate Microbe Enumeration Method Validation Report.
9. BF53 Liquid Intermediate Microbe Enumeration Validation Summary Report.
10. Ambient (5°C) Stability Data summary for *S. dextrinosolvens* ASCUSBF53
11. Accelerated (25°C) Stability Data summary for *S. dextrinosolvens* ASCUSBF53
12. [Revised] Dossier Section 6
13. [Revised] Dossier Appendix 18 Microbiome Safety.
14. GRAS Safety Summary and Target Animal Safety for the Direct Fed Microbial *Succinivibrio dextrinosolvens* ASCUSBF53

Content of email from Animalfood-premarket@fda.hhs.gov and associated response

Chemistry, Manufacturing and Controls (CMC)

Manufacturing Process and Controls

- The Appendix 10, App_010_Manufacturing Process (CONFIDENTIAL).pdf is titled ASCUSBF65. The notifier needs to clarify if it is a typographical error, and whether the manufacturing is for ASCUSBF53.
  Response: Native Microbials confirms that the header had not been properly updated from an earlier template and was erroneously showing the proper organism's name but incorrect strain number. The correct strain number is ASCUSBF53 and the Dosser Appendix 10 page header has been revised to correct this error (see Attachment 1)

- The notifier should clarify what % or concentration of (b) (4) is used as an antioxidant for maintaining the fermentation broth.
  Response: Less than (b) (4) is used in the fermentation broth. Dossier Appendix 10 (Attachment 1) has been updated to reflect this

- It is not clear what concentration of (b) (4) is used in the pre-prepared preservation mixture. The notifier should provide these details.
  Response: Dossier Appendix 10 (Attachment 1) has been revised to provide the target range of (b) (4) concentrations, up (b) (4) respectively.

- In Section 4 – Biomass Harvest by Centrifugation in Appendix 010, the notifier states that the centrifuge (b) (4) . *S. dextrinosolvens* ASCUSBF53 is a non-spore-forming bacterium. The notifier needs to explain why a (b) (4) For any (b) (4) and justify whether the residual level of such ingredient(s) in the concentrate and final product Fat Encapsulated *S. dextrinosolvens* ASCUSBF53 would raise safety concerns.

  Response: Although *S. dextrinosolvens* ASCUSBF53 does not produce spores, we considered it a best practice (b) (4)

  (b) (4)

  that meet the criteria stated above do not raise safety concerns for the *S. dextrinosolvens* ASCUSBF53, because residues would not be present in the final product as they are non-residual in nature and centrifuges are rinsed out with validated rinsing procedures.

Starting Materials

- In Table 1 of Appendix 010, the regulatory status citations for the following starting materials are incorrect:

Ingredient	Citation in Table 1	Correct citation
		(b) (4)

(b) (4)

Response: Appendix 10 has been amended (see Attachment 1) to update the incorrect references, noting (b) (4) (see Attachment 2) and the corrected citation (AAFCO 48.24) has been added.

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: Regarding monopotassium phosphate, we acknowledge that it is not currently listed as permitted in animal use in the US, however, based on the safety assessment (Attachment 3) we have supported the safe use. Below is a summary of that assessment.

Monopotassium phosphate is used as a buffering agent in the main fermentation media and preservation solution of *S. dextrinosolvens* ASCUSBF53 and is not used in any subsequent step of the process. Monopotassium phosphate (Potassium Phosphate, monobasic) meets the current Food Chemical Codex specifications, which describes the requirements for food use. Calculation of worst-case residual monopotassium phosphate is estimated as follows: The main fermentation media contains (b) (4) monopotassium phosphate. After biomass harvest by centrifugation, at worst the concentration remains at (b) (4) (b) (4) If the projected 5 g daily dose is composed entirely of fat-encapsulated *S. dextrinosolvens* ASCUSBF53, the quantity of monobasic phosphate remaining ends up being 16.5 mg, corresponding to a maximum of 1.65 parts-per-million (ppm) of monobasic potassium phosphate residue in a minimal daily feed intake of 10 kg per day (dry matter intake of beef cattle can be as high as 20 kg per day). Based on the exposure assessment and the fact that the Monopotassium phosphate is food grade, there is no safety concern.

The specification unit for cadmium, lead and arsenic in (b) (4) (Appendix 009C) are listed as g/g, the notifier needs to clarify the unit (g/g, mg/kg, µg/g or ppm?).

Response: The manufacturer ((b) (4)) confirms that they had a typo on the CofA we used in our Dossier Appendix 009C. They have clarified that the units are ppm (see Attachment 4) and a more current CofA is included in Attachment 4 showing proper units.

The notifier provides a product data sheet for defoamer (b) (4) from (b) (4) in Appendix 009B. The data sheet does not list components of (b) (4) , but it states that (b) (4) contains ingredients included in the "no objection" letter that was issued by FDA to the Enzyme Technical Association (ETA)

regarding the use of permitted defoaming agents in the manufacture of enzyme preparations (fermentation, enzyme recovery, and enzyme formulation) used in food. (b) (4) in Section 3 Fermentation (page 4 in Appendix 010). However, in Table 1 of Appendix 010, " (b) (4) . The notifier should clarify this discrepancy and provide quantity and revised table with specific function.

Response: Table 1 in the Revised Dossier Appendix 10 (Attachment 1) has been updated to show the function as (b) (4) Appendix 10, Table 1 and on page 4 of this appendix.

The notifier refers to AAFCO OP 33.19 for the definition of the hydrogenated glycerides that is used to encapsulate the cryo-milled freeze-dried *S. dextrinosolvens* ASCUSBF53. However, AAFCO OP 33.19 - Hydrogenated Glycerides has been withdrawn. Current Tentative definition OP T73.311 (A) – Hydrogenated Glycerides requires that vegetable oils used to produce the hydrogenated glycerides must meet the requirements listed in AAFCO OP 33.2. It is the notifier's responsibility to ensure that their hydrogenated glycerides meet the current requirement.

Response: The specific hydrogenated glyceride used to encapsulate the cryo-milled freeze-dried *S. dextrinosolvens* ASCUSBF53 is a hydrogenated vegetable oil that meets the definition AAFCO OP in 33.2. All hydrogenated glycerides used in production will be ensured to meet the requirements of AAFCO OP T73.311(a).

3. Specifications of the Notified Substance

The notifier states that three batches of *S. dextrinosolvens* ASCUSBF53 cell concentrate were analyzed for botulinum toxins. The notifier needs to clarify why the batches were being tested for botulinum toxins. 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 (b) (4), not because *S. dextrinosolvens* ASCUSBF53 is expected to be or even capable of producing botulinum toxins, rather the bacteria that do produce botulinum toxins are also (b) (4). By testing for the toxins we can rule out the contamination of the batch by botulinum toxin producing bacteria.

(b) (4)

(b) (4)

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The notifier states that the batches tested to establish specifications are representative of the commercial materials. Same batches were also used in the stability study. However, necessary information is not provided to justify how the tested batches can represent the expected quality of the commercial products.

Response: The three independent batches of fat encapsulated *S. dextrinosolvens* ASCUSBF53 were conducted in a manner consistent with manufacturing scale relevance. (b) (4)

Key performance and quality attributes at scale are expected to meet or exceed those achieved with the three pilot scale batches. (b) (4)

The freeze-drying, milling, and fat encapsulation steps were conducted (b) (4) 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 AGRN 45. Batches that do not meet quality specifications will not be considered for release.

The same batch IDs are used for *S. dextrinosolvens* ASCUSBF53 cell concentrate, freeze dried powder and final fat encapsulated product as listed in the table below:

Batch # (Lot #)	Manufacturing date		
	Cell concentrate	Freeze dried powder	Fat encapsulated
1801.2041 (2041)	unknown	11/27/2020	12/09/2020
1801.2042 (2042)	unknown	11/27/2020	12/09/2020
1801.2044 (2044)	unknown	11/27/2020	12/10/2020

The notifier needs to clarify the relationship among these products bearing the same batch ID, e.g. whether fermentation batch 1801.2041 was processed to produce only freeze dried and fat

encapsulated batch 1801.2041. The notifier also needs to clarify whether batches 1801.2041, 1801.2042, and 1801.2044 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.

1801.2041: November 5, 2020

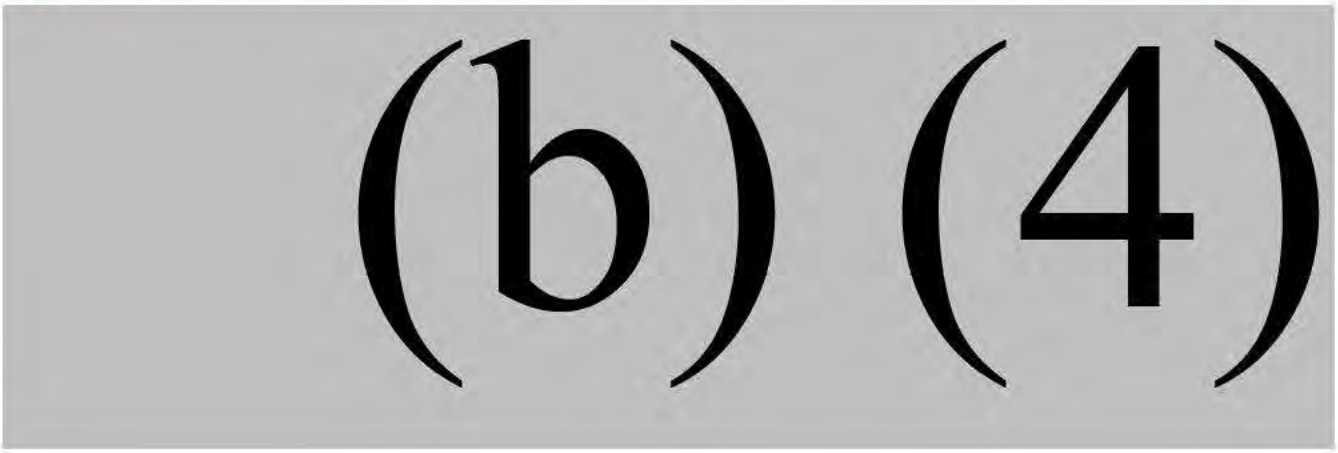
1801.2042: November 12, 2020

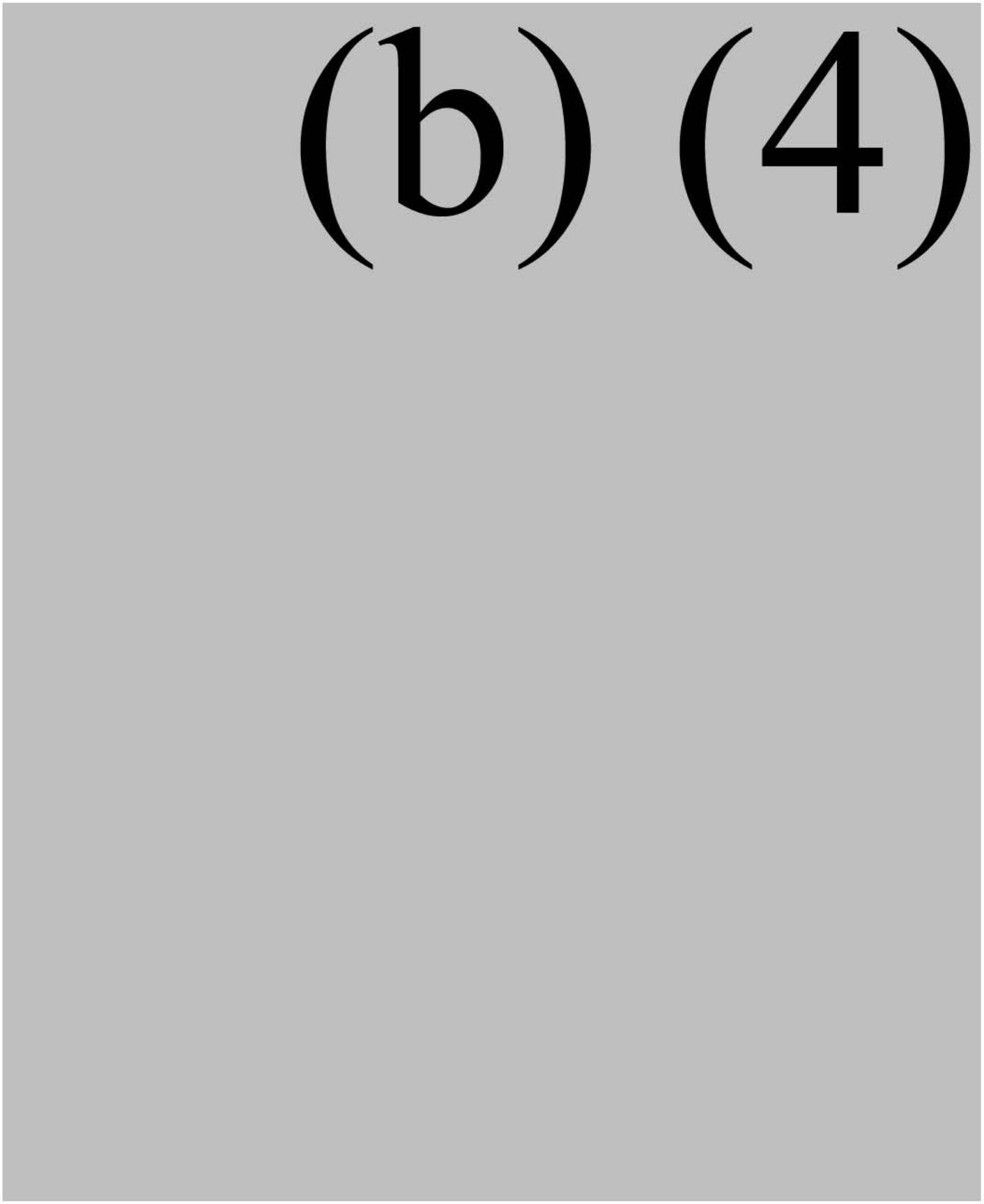
1801.2044: November 19, 2020

For example, each 1801.2041 cell concentrate batch became the 1801.2041 Freeze dried powder batch, which became the 1801.2041 batch of Fat Encapsulate. Only the batches of the same number were converted into batches of the same number. There was no co-mingling, mixing or splitting.

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 *S. dextrinosolvens* ASCUSBF53 cell concentrate, freeze dried powder and final fat encapsulated product. Considering that 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 of 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 *S. dextrinosolvens* ASCUSBF53.

Response: Following are the batch sizes and parameters for the three pilot-scale runs used to create the batches used in the AGRN 45 dossier. Details on how the runs were done are found in Appendix 10




(b) (4)

Stability

The submitted data collected at (b) (4) and (b) (4) are not adequate to demonstrate/estimate the stability of Fat Encapsulated *S. dextrinosolvens* ASCUSBF53 at (b) (4). Using the Arrhenius equation to predict the stability or viability of microorganisms at different temperatures have been explored but presented 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 (b) (4)

To support the claimed shelf life for the Fat Encapsulated *S. dextrinosolvens* ASCUSBF53 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 AGRN 45 dossier has been completed through 12 months for recommended storage conditions of ambient refrigerated storage (b) (4) and for room temperature (b) (4) which is an accelerated temperature for the declared ambient.

Attachment 10 shows that little change (b) (4)

Analytical Methods

The in-house enumeration method approach is acceptable to determine the viable cell counts of solid and liquid intermediates of *S. dextrinosolvens* ASCUSBF53 in freeze-dried and fat encapsulated product. However, I note that Appendix 012B summarizes a microbe enumeration validation report for BF65 solid intermediate and not for *S. dextrinosolvens* ASCUSBF53. The notifier should provide a justification or the summary of BF53 solid enumeration method validation results. Appendix 012E is titled "BF52 Liquid Intermediate Microbe Enumeration Validation Summary Report" instead of BF53 and Table 1 in the appendix 012E is titled as "Summary table of DY19 liquid enumeration method validation results". The notifier should address these discrepancies and provide a justification or the summary of BF53 liquid enumeration validation results.

Response: The correct BF53 solid intermediate method validation report has been attached (Attachment 8). The typographical errors have been corrected in the BF53 liquid intermediate

microbe enumeration validation summary report and the newest version has been attached (Attachment 9).

- The notifier refers to the 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 6

Microbial Safety

Genome safety

The notifier's cut-off setting for database searches is too stringent and would not allow identification of homologs for toxins and virulence factors 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.

Response: We have re-evaluated our thresholds for amino acid alignment and edited the narrative in Section 2.1.6 and 2.1.8 to provide justification (see Attachment 5).

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):

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 2.1.6 and 2.1.8 narrative.

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 *Gammaproteobacteria* specific toxin database with an e-value cutoff of 1E-04. The results are presented in the revised Section 2.1.8 narrative. No features in the *S. dextrinosolvens* ASCUSBF53 genome aligned to protein toxins from *Gammaproteobacteria* at the 1E-04 threshold.

Regarding the databases used by the notifier, the notifier should 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.

Response: We acknowledge that entries from VFDB and Victors are not fully integrated into PATRIC. We have evaluated these databases by downloading the complete original databases from source and aligning them to amino acid sequences from *S. dextrinosolvens* ASCUSBF53. We have amended section 2.1.8 in the dossier (Attachment 5) to reflect these changes. Two features not presented in the original dossier aligned to *S. dextrinosolvens* ASCUSBF53. This included a translational elongation protein, EF-TU, and a flagellar regulation protein, cheY. Both proteins are commonly found in pathogenic and non-pathogenic species and do not directly impart pathogenicity or virulence.

Clarification is needed about the notifier's conclusion that IslandViewer 4 web server did not identify any pathogenicity islands in *Succinivibrio dextrinosolvens* ASCUSBF53, e.g., how many genomic islands are predicted for the *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 did not raise safety concerns, e.g., being associated with pathogenicity? The firm should address all these points in a revised narrative.

Response: We have amended section 2.1.8 (Attachment 5) to contain more detailed information about the method and results generated by IslandViewer4. In short, IslandViewer4 Identified 9 genomic islands, none of which contain any virulence, pathogenicity, or antimicrobial resistance genes. Therefore, the conclusion remains that there are no pathogenicity islands in the *S. dextrinosolvens* ASCUSBF53 genome.

Microbial safety

In the case any potential toxins or virulence factors are identified using appropriate databases and cut-off setting, the notifier should revise its narrative and address how those toxins or virulence factors will not raise safety concerns.

Response: We have amended the narrative in section 6 (Attachment 12) to reflect the revised analysis done in Dossier section 2.1.8 (Attachment 5).

It has been reported in the scientific literature that, under certain conditions, increased abundance of lactate producing bacteria including *S. dextrinosolvens* may be associated with metabolic/digestive disorders, including ruminal acidosis and frothy bloat. (PMID: 31811042, PMID: 23584771, PMID: 30862851). However, none of the above-mentioned articles are included and discussed in the notice. For microbial safety, the notifier is responsible to provide a balanced view of the scientific literature, including both positive and negative information. Thus, the notifier should include those articles and address any associated safety concerns in its revised narrative.

Response: We have amended the narrative in section 6.7.3 (Attachment 12) to include additional literature review and discussed *S. dextrinosolvens* in context of high grain diets and rumen acidosis.

Utility

1. Native Microbials states in Section 1.3 and Section 2.5 of its GRAS notice (page 9) that the intended purpose of *Succinivibrio dextrinosolvens* ASCUSBF53 supplementation is to "augment the digestion of feed in the rumen". However, it also states in Section 2.5 (page 37) that *S. dextrinosolvens* ASCUSBF53 will act only to support normal ruminal function of digestion of animal feed. This conclusion is also supported by the statement in Section 6 (page 43) of its notice, "the microorganism [referring to *S. dextrinosolvens*] has the potential to support digestion by aiding fermentation of forages and partially degraded digesta in the rumen." Thus, the description of the intended conditions of use of the additive is not consistent and the intended technical effect may be acceptable if as described elsewhere in the notice, the notifier indicates that the use is to support rumen fermentation. CVM notes that the terms "support" and "augment" have different meanings.

   Response: Everywhere the terms "augment" or "augments" appears, it has been replaced by "support" or "supports" when referring to supporting rumen function and fermentation. These changes are reflected in Attachment 5, which includes revised dossier sections, 1.3, 2.1 and 2.5.

   Native Microbials describes in Section 2.5 that "the technical effect of *S. dextrinosolvens* ASCUSBF53 when fed to beef cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety." However, the notifier incorporates numerous statements in Section 2.5 that describe how modifying the microbiome could influence rumen fermentation processes and provides examples of ways that *S. dextrinosolvens* might alter end-products of digestion and subsequently these end-products have altered composition of animal products or animal productivity. Further, there are claims made in this section that are not consistent with use as an animal food product. The notifier needs to address how supplementing *S. dextrinosolvens* ASCUSBF53 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. The notifier should recognize that it is contradictory to argue that safety does not relate to utility, but then to include a discussion outlining expected benefits associated with feeding the viable microorganism, such as increased digestion, improved animal productivity. Some of these do relate to safety. This issue could be addressed by removal of this type of information from this section.

   Response: Dossier Section 2.5 (Attachment 5) has been revised to remove claims inconsistent with an animal food products.

Target Animal Safety

Based on the information contained in the notice submitted by Native Microbials, Inc., CVM has questions on the target animal safety of the notified substance, S. dextrinosolvens ASCUSBF53 strain, and its intended use as a direct-fed microbial for beef cattle at a use rate of $1x10^8$ CFU/head/day.

The firm should provide a robust narrative describing how reasonable certainty of no harm for target animal safety can be derived from data and information included in the sections on genomic and microbial safety and discuss how this conclusion is corroborated by publicly available and other target animal data.

Response: We have provided a robust and concise safety summary in Attachment 14. This complements the more detailed safety narrative in revised section 6 (attachment 12).

In addition, the firm should ensure it addresses the following:

The firm includes a proposed specification for botulinum toxins in the cell concentrate (Table 2.18; Pages 28-29 of M-000106-N-0001_sub_001.pdf). The firm should clarify why botulinum toxins are tested.

Response: As stated previously, Botulinum toxins are tested out of an abundance of caution, considering the fermentation batch is (b) (4) not because *S. dextrinosolvens* ASCUSBF53 is expected to be or even capable of producing botulinum toxins, rather the bacteria that do produce botulinum toxins are also (b) (4) By testing for the toxins we can rule out the contamination of the batch by botulinum toxin producing bacteria. We have added a statement in Dossier Section 6.9 (Attachment 12) reflecting this.

2. In section 6.5 of the firm's notice, two studies were cited to support the safety narrative. In both articles, an unspecified strain of *S. dextrinosolvens* was administered to ruminants. However,

   The Bello et al. (2019) article is written in Spanish. An English translation should be provided.

   The Rigobelo et al. (2016) article does not specify which strain of S. dextrinosolvens was administered to sheep. Additionally, this was not a safety study for the microbe but a clinical challenge with shiga toxin-producing *E. coli*. Given this, it appears that this study does not support the firm's safety conclusion. This firm should clearly describe how this study provides information for TAS of their *S. dextrinosolvens* ASCUSBF53 strain.

   Response: The dossier section 6 has been updated to address these issues and is included as Attachment 12. Regarding points 2a and 2b, the Bello et al. (2019) manuscript has been translated and included with the references. Strain information is frequently unpublished. Further, section 6.5 highlights the historical use of the species *S. dextrinosolvens* in food or feed ingredients and not necessarily just target animal safety. These published studies are demonstrations of tolerance and that the species has been fed to live animals as DFM and generally considered safe.

   Regarding the agency's concern on TAS of *S. dextrinosolvens* ASCUSBF53, the agency has previously communicated to Native Microbials that "TAS studies for this product class are unusual" and that "a traditional TAS study would not be appropriate." (from the May 21, 2020 FDA/Native Microbials meeting minutes, pg 2, 5th and 2nd bullet respectively). Hence, the Rigobelo et al. (2016) study was intended to demonstrate the species *S. dextrinosolvens* has

been fed to live animals as a DFM and is generally considered safe, not to provide information for TAS.

Exposure calculations for the target animals provided on page 40 of the notice are inconsistent and appear to contain errors.

Response: Section 3.1.2 has been revised to present the calculations of target animal exposure with greater clarity. This revised section is included as Attachment 7. In summary, expected exposure of non-organism elements (encapsulation materials: hydrogenated glycerides + Sodium Sulfate) represent less than 0.013% of the overall dry matter intake of typical beef cattle and poses no risk from a sodium or sulfur intake contribution nor would it have any impact from a nutritional standpoint, positively or negatively.

A recently published article reported a correlation between growth of a small group of bacteria, one of which was *S. dextrinosolvens*, and development of ruminal acidosis.[1] Another publication indicates that levels of Succinivibrio (and Myxococcales) are consistently enriched in rumens of cattle with alfalfa-induced frothy bloat [2]. The notifier needs to address safety of *S. dextrinosolvens* ASCUSBF53 in beef cattle transitioning to high grain diets and the conditions of intended use since Native Microbials states on page 46 of the notice, "internal studies have shown that this species can comprise up to 20% of the rumen population". The firm should use available data and literature to address if there is potential for the S. dextrinosolvens ASCUSBF53 strain to induce frothy bloat. The firm should be sure to discuss implications of potential frothy bloat occurrences for TAS.

Response: As stated in greater detail in the revised dossier Microbiome Safety section (see Attachment 12), correlation between *S. dextrinosolvens* and acidosis or frothy bloat is not causative. The presence of *S. dextrinosolvens* under these conditions is likely due to its capnophilic metabolism. Detailed arguments have been amended in revised section 6.7.3 (Attachment 12).

[1] Dai and coworkers, 2019; J. Dairy Sci. 102:334–350: doi:10.3168/jds.2018-14807; Dai and coworkers, 2020; Applied and Environmental Microbiology 86:e02193-19. doi:10.1128/AEM.02193-19; Mizuguchi and coworkers, 2021; J. Vet. Med. Sci. 83(6):905-910: doi-org.fda.idm.oclc.org/10.1292/jvms.21-0037.

[2] E Azad, H Derakhshani, RJ Forster, RJ Gruninger, S Acharya, TA McAllister, E Khafipour. 2019. Characterization of the rumen and fecal microbiome in bloated and non-bloated cattle grazing alfalfa pastures and subjected to bloat prevention strategies. 2019. Scientific Reports 9:4272

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native MICROBIALS

Succinivibrio dextrinosolvens ASCUSBF53 AGRN #45 Dossier

AMENDMENT ATTACHMENTS

List of Attachments

ATTACHMENT 1

[Revised] Dossier Appendix 10 - Confidential Detailed Manufacturing Summary of Fat Encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53

Confidential Detailed Manufacturing Summary of Fat Encapsulated *Succinivibrio dextrinosolvens* ASCUSBF53

(b) (4)

(b) (4)

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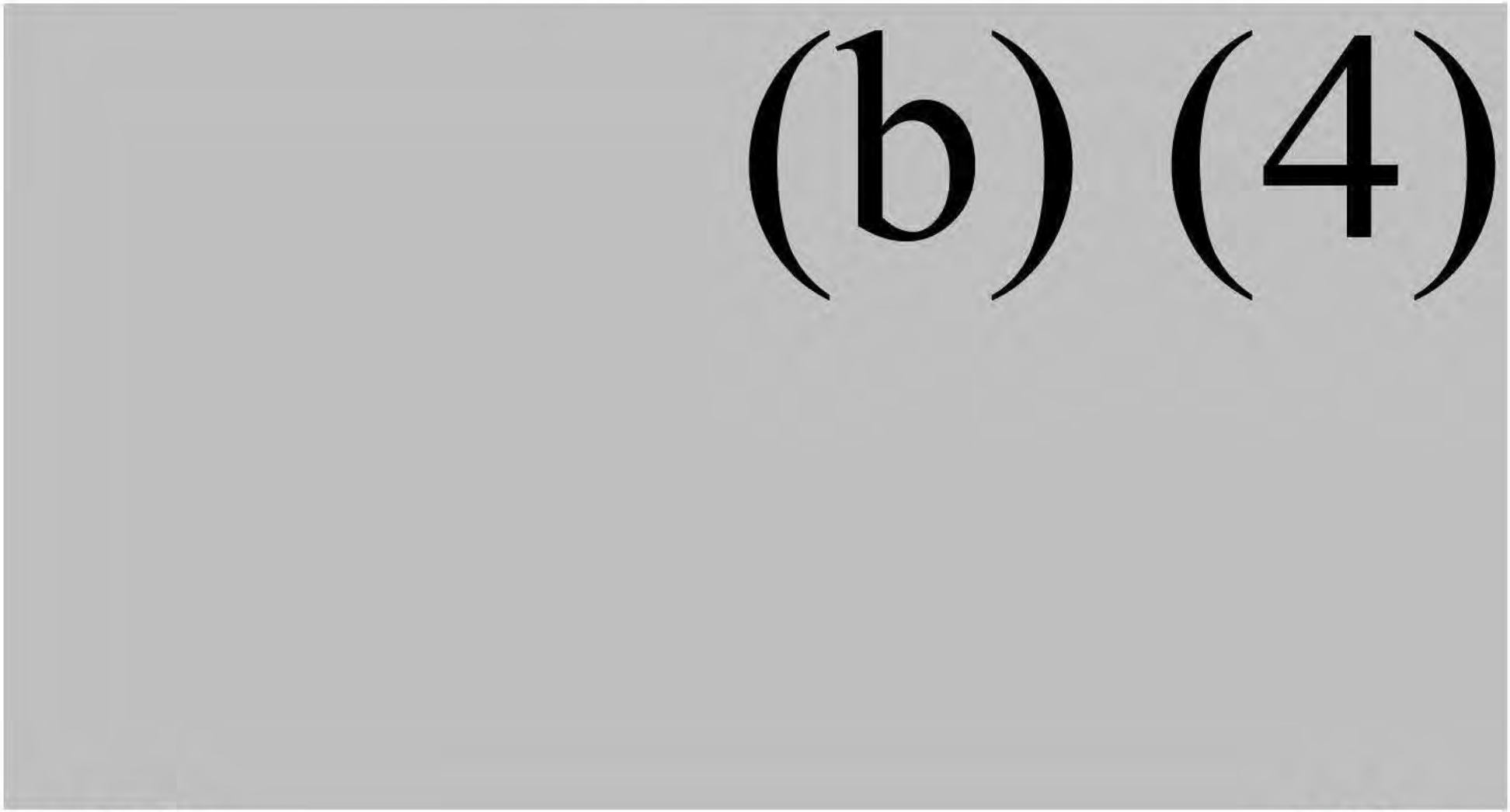
(b) (4)

(b) (4)

(b) (4)

(b) (4)

Appendix A. Process Diagram of the Production of Fat Encapsulated *S. dextrinosolvens* ASCUSBF53



ATTACHMENT 2

USDA Condensed Fermented Corn Extractive Monograph

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).

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ATTACHMENT 3

Monopotassium Phosphate Animal Feed Safety 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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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

4.1 Exposure of Dairy Cows to Monopotassium Phosphate in ASCUSDY19 in Typical Conditions

Calculation of worst-case residual monopotassium phosphate, using fat encapsulated ASCUSDY19 *Butrivibrio fibrisolvens* is estimated as follows. The main fermentation media contains 1.2 g/L monopotassium phosphate. After biomass harvest by centrifugation, at worst the concentration remains at 1.2 g/L. After addition of preservation solution to the cell concentrate and drying, the concentration would then be 3.6 g/kg. After coating with hydrogenated glycerides, the concentration is further diluted to 0.72 g/kg. If the projected 5g daily dose is composed entirely of fat-encapsulated *B. fibrisolvens* ASCUSDY19, the quantity of monobasic phosphate remaining ends up being 3.6 mg, corresponding to 16 parts-per-billion (ppb) of monobasic potassium phosphate residue in a daily Total Mixed Rations (TMR) intake of 100 lb per day.

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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NRC, 1990. National Research Council. Mineral Tolerances of Animals. The National Academies Press.

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ATTACHMENT 4

native
MICROBIALS

(b) (4) Letter and CofA Showing Correct Units

(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.

If you have any further questions, please contact us by telephone at (b)(6) Option 2, or by email at (b) (4).

Sincerely,

(b) (4)

(b) (4), (b)(6)

(b) (4)

Catalog : AS102 Ascorbic Acid, USP **Lot :** 2П0075

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			< 3000 ppm

(b) (4)

Certificate of Analysis Results Certified By:

(b) (6)

(b) (4), (b) (6)

ATTACHMENT 5

[Revised] Dossier Sections 1.3, 2.1 and 2.5

[Revised]

1.3 Intended Conditions of Use

S. dextrinosolvens ASCUSBF53 is intended for use as a supplemental source of viable microorganisms in the feed of beef 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 beef cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing fat encapsulated *S. dextrinosolvens* ASCUSBF53 may be incorporated into the diet of 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 *S. dextrinosolvens* ASCUSBF53 will be incorporated into feed at a recommended level of $1x10^8$ CFU/head/day.

[Revised]

PART 2 – IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

2.1.1 Taxonomic Classification

The current taxonomic classification of the microbial strain, *S. dextrinosolvens* ASCUSBF53, is provided in Table 2.1. *S. dextrinosolvens* is a prominent member of the rumen of both cattle and sheep and is enriched in animals on high grain diets where it acts to degrade starch and produce volatile fatty acids (VFAs) (Bryant and Small 1956; Wozny et al. 1977a; Hespell 1992; Hippe et al. 1999).

Table 2.1: Taxonomic Classification of *S. dextrinosolvens*

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Aeromonadales
Family	*Succinivibrionaceae*
Genus	*Succinivibrio*
Species	*dextrinosolvens*

2.1.2 Source of the Microorganism

S. dextrinosolvens ASCUSBF53 was identified and isolated to axenicity from the rumen content of a healthy steer by Native Microbials. The isolate was deposited in the NRRL, Agricultural Research Service Culture Collection, and referenced as B-67550.

2.1.3 Description of the Microorganism

S. dextrinosolvens ASCUSBF53 is an anaerobic, non-spore forming bacterium composed of helically twisted, curved rods with 1 to 3 cells per grouping (Figure 2.1). Cells are motile and stain gram-negative (Figure 2.2). Our observations of *S. dextrinosolvens* ASCUSBF53 are consistent with the original description of the species by (Bryant and Small 1956).

Figure 2.1: *S. dextrinosolvens* ASCUSBF53 Methylene Blue Stain after 48 hours of incubation (1000x magnification)

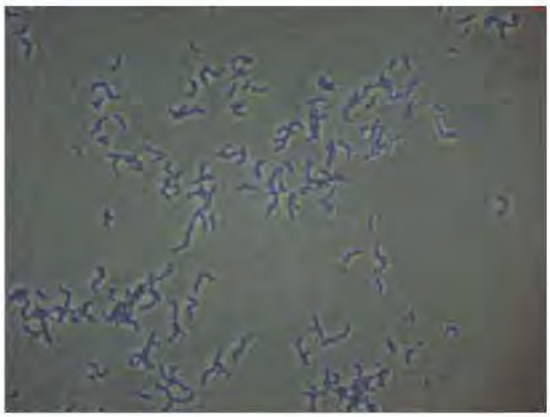

Figure 2.2: *S. dextrinosolvens* ASCUSBF53 Gram Stain after 48 hours of incubation (1000x magnification)

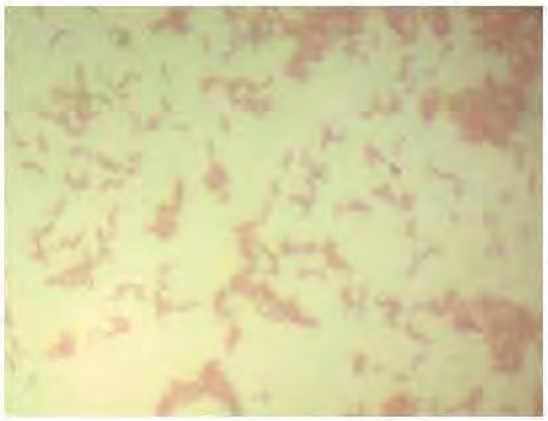

Table 2.2: Growth of *S. dextrinosolvens* ASCUSBF53 on Different Carbon Sources

Carbon Source	Growth	Carbon Source	Growth

No Carbon Control	No Growth	(b) (4)	No Growth
Glycerol	No Growth		No Growth
Erythritol	No Growth		Growth
D-Arabinose	No Growth		No Growth
L-Arabinose	Growth		No Growth
D-Ribose	Growth		No Growth
D-Xylose	Growth		No Growth
L-Xylose	No Growth		No Growth
D-Adonitol	No Growth		No Growth
Methyl-BD-xylopyranoside	No Growth		No Growth
D-Galactose	Growth		No Growth
D-Glucose	Growth		Growth
D-Fructose	Growth		Growth
D-Mannose	No Growth		Growth
L-Sorbose	No Growth		Growth
L-Rhamnose	No Growth		No Growth
Dulcitol	No Growth		No Growth
D-Melezitose	No Growth		No Growth
D-Raffinose	No Growth		No Growth
Starch	Growth		No Growth
Glycogen	No Growth		Growth

Xylitol	No Growth	(b) (4)	No Growth
Gentiobiose	No Growth		No Growth
D-Turanose	No Growth		No Growth
D-Lyxose	No Growth		

In vitro assays demonstrate that *S. dextrinosolvens* ASCUSBF53 grows on a variety of monosaccharides, disaccharides, and sugar alcohols including (b) (4). Additionally, the strain ferments starch. Carbon source utilization results are summarized in Table 2.2. Fermentation of (b) (4) are consistent with observations of the species, though there is some interspecies variability (Bryant 2015; Bryant and Small 1956). The results align with the proposed function of *S. dextrinosolvens* in the rumen as a key degrader of starch and dextrins in ruminants on high grain diets (Bryant 2015; Cotta 1988).

Metabolite production of *S. dextrinosolvens* ASCUSBF53 was measured at 40 hours elapsed fermentation time using an (b) (4) series (b) (4) with refractive index (RI) detector. The results are summarized in Table 2.3 and Appendix 002. Major fermentation products include succinate, lactate, and acetate.

Table 2.3: Metabolite Production of *S. dextrinosolvens* ASCUSBF53 on Complex Media with (b) (4)

Metabolite	Production (g/L)
Pyruvic acid	(b) (4)
Succinic acid	
Lactic acid	
Glycerol	
Acetic acid	
Propionic acid	
Butyric acid	
Ethanol	
1-Butanol	

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 (b) (4) (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. The results indicated that *S. dextrinosolvens* ASCUSBF53 was most closely related to *Succinivibrio dextrinosolvens* CA76 (99.8%), followed by *Succinivibrio dextrinosolvens* CG79 (99.8%), and *Succinivibrio dextrinosolvens* Z6 (99.2%). The closest match not from the *Succinivibrio* genus is *Anaerobiospirillum thomasii* DSM 11806 (92%).

2.1.4.2 Whole Genome Sequence Assembly and Annotation

Genomic DNA was isolated from a pure culture of *S. dextrinosolvens* ASCUSBF53 and sequencing libraries were prepared using the (b) (4) and in parallel, long-read libraries were prepared from the same extracted DNA using (b) (4) following the protocol outlined by Jain *et al*. (2018) and (b) (4) (Jain et al. 2018). (b) (4) Read quality and genome coverage was evaluated using (b) (4) . Assembly statistics can be found in Table 2.4. The full details of the assembly are provided in Appendix 003C.

Protein coding genes were predicted through (b) (4) 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 (Lowe and Eddy 1997) and rRNA genes (Aziz et al. 2008).

The *S. dextrinosolvens* ASCUSBF53 genome contains (b) (4) coding sequences which were subsequently built into a metabolic reconstruction describing 210 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 CP068345 for the main chromosome and CP068346 for the chromid.

Table 2.4: Assembly Statistics for *S. dextrinosolvens* ASCUSBF53	
# of Contigs	2
# of Contigs ≥ 5,000 bp	2
Longest Contig (bp)	(b) (4)

Assembly Length	(b) (4)
N50	
N75	
GC%	39.1

2.1.4.3 Whole Genome Sequence Comparison

To determine relatedness of *S. dextrinosolvens* ASCUSBF53 to other closely related species at a higher resolution, whole genomes were compared using ANI. Candidate genomes for genome-genome comparison to *S. dextrinosolvens* ASCUSBF53 were selected by full length 16S rRNA similarity and downloaded from the NCBI database. (b) (4) 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 (b) (4) alignment can be found in Table 2.5.

The only ANI matches to *S. dextrinosolvens* ASCUSBF53 above the 95% ANI cutoff to be considered the same species were two strains of *S. dextrinosolvens* (DSM 3072 and H5) (Richter and Rosselló-Móra 2009).

Table 2.5: Average Nucleotide Identity (ANI) of Related Species to *S. dextrinosolvens* ASCUSBF53 by (b) (4)

Genus species (assembly)	ANI (%)	Coverage (%)
Succinivibrio dextrinosolvens DSM 3072 (GCA_900167015)	97.9	74.9
Succinivibrio dextrinosolvens H5 (GCA_000702045)	96.8	72.9
Succinivibrio dextrinosolvens ACV-10 (GCA_900116345)	88.3	43.0
Succinivibrio dextrinosolvens 22B (GCA_900114195)	87.9	35.0
Succinivibrio dextrinosolvens Z6 (GCA_011065405)	87.9	31.7
Anaerobiospirillum succiniciproducens DSM 6400 (GCA_000482845)	82.4	0.64
Anaerobiospirillum thomasii NCTC13093 (GCA_900445225)	82.3	0.62
Succinatimonas hippei YIT12066 (GCA_000188195)	82	0.22

2.1.4.4 *Summary and Conclusions*

16S rRNA and whole genome analysis confirm that *S. dextrinosolvens* ASCUSBF53 is a strain of the species *S. dextrinosolvens.*

2.1.5 Plasmid Analysis

To confirm the presence/absence of plasmids, the assembly graph for the *S. dextrinosolvens* ASCUSBF53 was analyzed by (b) (4)(Wick et al. 2015). The assembly graph analysis confirmed that the *S. dextrinosolvens* ASCUSBF53 assembly was contained in 2 circular chromosomes with no unincorporated fragments, verifying the completeness of the assembly. The image of the assembly graph can be found in Figure 2.3.

The smaller chromosome (163,867 bp) was evaluated using three different methods to determine if it should be classified as plasmid or a chromid:

1. PlasmidFinder (Carattoli et al. 2014): The PlasmidFinder database utilizes 469 origin of replication sequences from species in the family *Enterobacteriaceae*.
2. Alignment to proteins in the NCBI plasmid database by BLAST (Brooks, Kaze, and Sistrom 2019): The NCBI plasmid database consists of 1,295,867 plasmid derived proteins from 29,505 plasmids, covering 5,161 species.
3. MOB-suite (Robertson and Nash 2018). The MOB-suite database consists of 23,240 plasmids, of which 10,224 are derived from Gammaproteobacteria, with 223 originating in *Aeromonadales.* The analysis with MOB-suite emphasizes origin of replication, relaxase, and mate-pair formation genes with the goal of identifying plasmids and determining their potential for mobility.

Characteristics of the databases can be found in Table 2.6. To ensure no hits were missed due to codon bias or sequencing error, protein alignments to the NCBI plasmid database were considered a hit if they have greater than 80% identity over more than 70% query coverage. PlasmidFinder and MOB-suite yielded no hits. Additionally, MOB-suite determined the smaller chromosome of *S. dextrinosolvens* ASCUSBF53 to be non-mobile. Alignment to the NCBI plasmid database yielded one hit to the *S. dextrinosolvens* ASCUSBF53 genome. NCBI plasmid alignment results can be found in Table 2.7.

The hit to the NCBI plasmid database was to a ubiquitous membrane potential regulating protein that aids in regulation of osmotic stress and maintains membrane potential, YqaE/PMP3 (Inada et al. 2005; Kwok et al. 2020; Navarre and Goffeau 2000; Raivio, Leblanc, and Price 2013). Homologues of this protein are ubiquitously found in plants, prokaryotes, yeasts and other eukaryotes (Kwok et al. 2020).

Given the lack of plasmid features on the secondary chromosome of *S. dextrinosolvens* ASCUSBF53 and the predicted immobility of the chromosome by MOB-suite, it should be designated as a chromid rather than a plasmid. Though there is no literature describing

chromids in *S. dextrinosolvens* to date, it is estimated that one in ten bacteria carry a chromid (Harrison et al. 2010). With only eight representative sequences from the species, only one of which is a closed sequence, the species has likely not been sequenced extensively enough to document chromid carrying members.

Figure 2.3: ***S. dextrinosolvens*** **ASCUSBF53 Assembly Graph as Generated by** (b) (4)

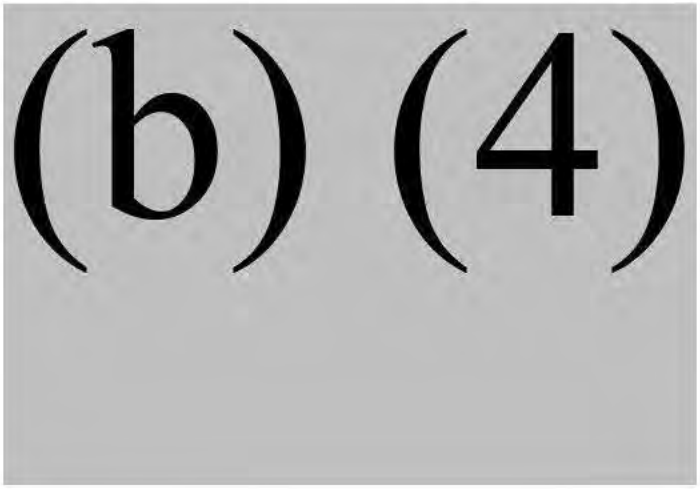

Table 2.6: Characteristics of Databases Used to Identify Plasmids			
Database Name	Number of Plasmid Entries	Features Evaluated	*S. dextrinosolvens* Entries
PlasmidFinder	No full Plasmids	Origin of replication	0
NCBI Plasmid DB	29,505	All plasmid proteins	0
MOB-suite	23,240	Origin of replication, relaxases, mate-pair formation genes	0

Table 2.7: *S. dextrinosolvens* ASCUSBF53 Hits to the NCBI Plasmid Database						
Source Organism	Gene	Genbank Accession #	Function	Query Coverage	identity	E-Value
Enterobacterales	MULTISPECIES: YqaE/Pmp3 family protein	WP_057393895	Proteolipid membrane potential modulator	92	81.4	5E-28

2.1.6 *In-vitro* and *In-silico* Analysis of Antibiotic Susceptibility

Phenotypic testing was conducted on *S. dextrinosolvens* ASCUSBF53 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials with relevance to

human and veterinary medicine. The full study report is provided in Appendix 004. The results were evaluated against the resistant breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram negative anaerobes," European Food Safety Authority (EFSA) for "gram negative bacteria," and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). Results for *S. dextrinosolvens* ASCUSBF53 can be found in Table 2.8. The MIC values reported for *S. dextrinosolvens* ASCUSBF53 were equal, or lower than, the cut-off values and break-points established by EFSA, EUCAST and/or CLSI for chloramphenicol, tetracycline, and ampicillin. MIC values reported for *S. dextrinosolvens* ASCUSBF53 were higher than the cutoff values and break-points established by EFSA, EUCAST and/or CLSI for gentamicin, kanamycin, clindamycin and streptomycin.

It should be noted that susceptibility to aminoglycosides and macrolides 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-negative organisms and should not be applied to *S. dextrinosolvens* due to its anaerobic nature. CLSI and EUCAST refrain from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. Furthermore, since *S. dextrinosolvens* ASCUSBF53 is considered to be a gram-negative bacteria, vancomycin will be ineffective against this organism, as gram-negative bacteria are known to be unresponsive to vancomycin (Antonoplis et al. 2019).

Table 2.8: *S. dextrinosolvens* ASCUSBF53 Antimicrobial Susceptibility in Relation to EUCAST, and CLSI Breakpoints

Antibiotic	Range Tested (ug/mL)	MIC (ug/mL) of *S. dextrinosolvens* ASCUSBF53	EFSA Interpretation	EUCAST Interpretation	CLSI Interpretation
Ampicillin	0.5 – 128	< 0.5	S	S	S
Vancomycin	0.125 – 32	> 32	N/A	N/A	N/A
Gentamicin	0.5 – 32	16	R	-	N/A
Kanamycin	0.5 – 64	16	R	N/A	N/A
Streptomycin	0.5 – 64	32	R	N/A	N/A
Erythromycin	0.5 – 16	16	N/A	-	N/A
Clindamycin	0.03 – 32	> 32	N/A	R	R
Tetracycline	0.0625 – 64	0.25	S	N/A	S
Chloramphenicol	0.5 – 64	< 0.5	N/A	S	S

To evaluate the presence of antimicrobial resistance genes in the *S. dextrinosolvens* ASCUSBF53 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 2.9.

Table 2.9: Characteristics of Databases Used to Assess Antimicrobial Resistance

Database Name	Number of Entries	Number of *Succinivibrio* Entries	*S. dextrinosolvens* 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.

No hits were identified by PATRIC or ResFinder. BLAST alignment to the AMRFinder database revealed one hit in the *S. dextrinosolvens* ASCUSBF53 genome. The hit corresponds to the tufA gene. This gene is ubiquitous and encodes for a translation elongation factor in bacteria (Pramanik and Schwartz 1984; Filer and Furano 1980, 1981). Point mutations in the tufA gene in some cases have resulted in resistance to the polyketides; kirromycin and pulvomycin (Kraal et al. 1995; Tubulekas, Buckingham, and Hughes 1991; Zeef et al. 1994). Results for the BLAST search to the AMRFinder database can be found in Table 2.10.

Table 2.10: *S. dextrinosolvens* ASCUSBF53 Antimicrobial Resistance by NCBI AMR BLASTp

Gene	E-value	Percent Identity (%)	Query Coverage (%)
tufA	0	82.9	99

2.1.6.1 Section Summary

In vitro testing demonstrated that *S. dextrinosolvens* ASCUSBF53 is resistant to gentamicin, kanamycin, streptomycin, and clindamycin. Resistance to aminoglycosides and macrolides such as is reflective of *S. dextrinosolvens* ASCUSBF53 being anaerobic rather than any specific resistance mechanism or genotype. Furthermore, being unresponsive to vancomycin is a function of *S. dextrinosolvens* ASCUSBF53 being gram-negative, rather than an organism-specific resistance. *In silico* analyses revealed the presence of tufA, a gene that can have point mutations that could lead to resistance to kirromycin and pulvomycin. *S. dextrinosolvens* ASCUSBF53 is susceptible to ampicillin, tetracycline, and chloramphenicol, suggesting that should *S. dextrinosolvens* ASCUSBF53 cause an opportunistic infection in a human or animal, it can be readily treated using standard antibiotics.

2.1.7 Antimicrobial Production

S. dextrinosolvens ASCUSBF53 supernatant 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 2.11. 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 factors were missed, amino acid sequences from *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53.

A more conservative alignment approach was taken with the alignment of *S. dextrinosolvens* ASCUSBF53 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 *Succinivibrio* to which *S. dextrinosolvens* ASCUSBF53 belongs, a custom database was generated that contained all protein toxin entries in the VFDB and DBETH databases from the class Gammaproteobacteria.

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 E-value, >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 2.11: Characteristics of Databases Used to Assess Virulence and Pathogenicity

Database Name	Number of Entries	Number of *Succinivibrio* Entries	*S. dextrinosolvens* Entries	Contains Redundant Protein ID entries
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	1	1	Yes
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	0	0	Yes
VFDB	**28,982 (3,580 curated entries)**	**0**	**No**	**No**
Victors	5,304	**0**	**No**	**No**
DBETH (Gammaproteobacteria)	108	0	No	No
PATRIC_VF	38,791(1,570 non-redundant proteins)	1	1	Yes
Phi-Base	6,780	0	0	No
IslandViewer4	4,065 pathogenicity islands	1	1	No
PathogenFInder	N/A	N/A	N/A	N/A

Query of these databases at the 80% identity/70% coverage threshold identified three unique hits in the *S. dextrinosolvens* ASCUSBF53 genome. Full results can be found in Tables 2.12-2.18. The three unique hits were as follows:

- One hit was identified in each Victors, Victors (PATRIC), PATRIC_VF, and PhiBase to the same gene, hfq. The hfq gene is found in approximately 50% of all bacteria where it acts as a post transcriptional regulator of various metabolic processes (Sun 2002; Tsui, Leung, and Winkler 1994). It has been implicated as a growth promoter and virulence factor regulator in some pathogens including *Listeria monocytogenes* (Sun, Zhulin, and Wartell 2002), *Yersinia pseudotuberculosis* (Schiano, Bellows, and Lathem 2010), *Shigella sonnei* (Mitobe et al. 2009) and *Salmonella typhimurium* (Sittka et al. 2007) but is not solely responsible for pathogenicity or virulence. There were no other pathogenic or virulent genes detected in the *S. dextrinosolvens* ASCUSBF53 genome that would be regulated by hfq. Additionally, the match to the hfq gene from the PhiBase database was implicated with reduced virulence in *Yersinia pestis* suggesting the variant encoded by the *S. dextrinosolvens* ASCUSBF53 genome would not confer virulence or pathogenicity even if other virulence or pathogenicity genes were present.

- Two hits were identified in VFDB. The first being an (b) (4) from pathogenic *Francisella noatunensis* that shares 80.7% identity with a protein from the *S. dextrinosolvens* ASCUSBF53 genome. (b) (4) is a ubiquitous translational protein that catalyzes the binding of aminoacyl-tRNA to the A-site of the ribosome (Harvey et al. 2019). In some species, it is known to comprise up to 10% of the total expressed protein (Harvey et al. 2019; Dallo et al. 2002). Due to the proteins ubiquitous nature some studies have used it for phylogenetic reconstruction (Caamaño-Antelo et al. 2015). In some pathogenic species (b) (4) is a multifunctional "moonlighting" protein that can perform essential function in the cytosol and secondary functions on the cell surface (Ebner and Götz 2019). Some of these functions may act to complement virulence or pathogenicity but are not singularly responsible for the pathogenic or virulent nature of a given species (Harvey et al. 2019; Ebner and Götz 2019).
- The second feature from VFDB providing a match is (b) (4). The feature in question from *Aeromonas veronii* shares 88.8% identity with a protein from the *S. dextrinosolvens* ASCUSBF53 genome. This protein is known to interact with the flagellar motor and assist with the switch between clockwise and counterclockwise rotation of flagella (Sarkar et al. 2010; Nesper et al. 2017). This protein Is found in many pathogenic and non-pathogenic species containing flagella (Manson 2010).

The lower threshold alignment at E-value 1E-4 to the Gammaproteobacteria specific toxin database returned no matches to the *S. dextrinosolvens* ASCUSBF53 genome.

Nine genomic islands were identified by IslandViewer4, however none 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.

PathogenFinder deemed that *S. dextrinosolvens* ASCUSBF53 was not likely to be a human pathogen.

Table 2.12: Significant Alignments Between Virulence Databases and *S. dextrinosolvens* ASCUSBF53

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
S. dextrinosolvens ASCUSBF53	1	0	1	1	0	1

Table 2.13: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in Victors

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	identity	E-Value

Victors **(PATRIC)**	*Yersinia pseudotuberculosis IP 31758*	hfq	RNA-binding protein Hfq	Translational regulation	67	80	85	3.00E-25
Victors	***Neisseria meningitidis***	**hfq**	**RNA binding protein**	**Translational regulation**	**72.2**	**82.0**	**84.3**	**4.00 E-37**

Table 2.14: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in VFDB

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	Identity	E-value
VFDB	*Aeromonas veronii*	cheY	Response regulator	flagella	98.4	98	88.8	6.00E-76
VFDB	*Francisella noatunensis*	EF-TU	Elongation factor TU	translation	100	100	80.7	0

Table 2.15: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in PATRIC_VF

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	identity	E-Value
PATRIC_VF	*Shigella flexneri 2a*	hfq	RNA-binding protein Hfq	Translational Regulation	66	80	83	4.00E-25

Table 2.16: PathogenFinder Results *S. dextrinosolvens* ASCUSBF53

Gene Matches	Proteins from Pathogens Matched	Proteins from Non-Pathogens Matched	Predicted as Human Pathogen?
4	1	3	No

Table 2.17: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in PathogenFinder

Gene	Genbank Accession Number	Source Organism	Percent Identity
LSU ribosomal protein L14P	CP000378	*Burkholderia cenocepacia* AU 1054	79.5

Table 2.18: *S. dextrinosolvens* ASCUSBF53 Hits to Pathogenic Genes in PhiBase

Source	Source Organism	Gene	Product	Function	Query Coverage	Identity	E-Value	Phenotype

PhiBase	*Yersinia pestis*	hfq	RNA-binding protein Hfq	Translational Regulation	82	84.06	6E-38	Reduced Virulence

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 *S. dextrinosolvens* ASCUSBF53. In total, these databases encompass 138,461 known pathogen-related genes spanning all microbial taxonomies. Comprehensive alignment of the *S. dextrinosolvens* ASCUSBF53 genome to these databases yielded three unique hits above the 80% identity, 70% query coverage threshold across the databases. The hits were to proteins with general cellular function that can be present in the genomes of pathogens, but do not directly confer pathogenicity or virulence. A lower threshold alignment, at E-value 1E-4, to a Gammaproteobacteria specific toxin database returned no hits to the *S. dextrinosolvens* ASCUSBF53 genome. Pathogenicity island analysis with IslandViewer4 identified 9 genomic islands in the *S. dextrinosolvens* ASCUSBF53 genome, none of which were identified as pathogenicity islands. Analysis with PathogenFinder deemed that *S. dextrinosolvens* ASCUSBF53 was not likely to be a human pathogen.

2.1.9 Summary of Organism Safety Based on Genomics

S. dextrinosolvens ASCUSBF53 was identified as a strain of *S. dextrinosolvens* by 16S rRNA and whole genome analysis. Examination of the assembly graph of the *S. dextrinosolvens* ASCUSBF53 genome revealed two chromosomes. The smaller of the two chromosomes was analyzed to determine its standing as a chromid or plasmid. No plasmid based origin of replication, relaxases, or mate-pair formation genes were encoded. The chromosome was deemed non-mobile and was thus classified as a chromid. *In vitro* antimicrobial susceptibility testing revealed *S. dextrinosolvens* ASCUSBF53 was susceptible to a broad range of antimicrobial compounds. One antimicrobial resistance gene was identified in the genome, tufA, that in some cases contributes to resistance to the polyketides, kirromycin and pulvomycin. Phenotypic testing confirmed that no antimicrobials were produced by *S. dextrinosolvens* ASCUSBF53 during fermentation. Comparison of the *S. dextrinosolvens* ASCUSBF53 genome to several databases containing known pathogenic-related genes revealed three protein hits. The hits were to proteins with general cellular function that can be present in the genomes of pathogens, but do not directly confer pathogenicity or virulence. Based on these analyses, *S. dextrinosolvens* ASCUSBF53 is safe for use as a direct fed microbial.

[Revised]

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 *S. dextrinosolvens* ASCUSBF53 is to support normal rumen digestion. As described below, Native Microbials has determined that the technical effect of *S. dextrinosolvens* ASCUSBF53 when fed to beef 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 *S. dextrinosolvens* ASCUSBF53 in the feed of beef cattle are not required as part of this GRAS notice.

S. dextrinosolvens is a prominent member of the rumen of both bovine and ovine and is higher in animals on high grain diets where it acts to degrade starch and produce volatile fatty acids (VFAs) (Bryant and Small 1956; Wozny et al. 1977a; Hespell 1992; Hippe et al. 1999). Higher abundance of *S. dextrinosolvens* has been associated with more efficient dairy and beef cattle (Elolimy et al. 2018; Hailemariam, Zhao, and Wang 2020; Hernandez-Sanabria et al. 2012).

Within the rumen this organism facilitates the digestion of various carbohydrates of animal feed within the rumen to volatile fatty acids such as succinic acid, lactic acid and acetic acid (see table 2.3). *S. dextrinosolvens* has been found in the rumen in a variety of animals globally (Bryant and Small 1956; Bryant 1959; Wozny et al. 1977a; Wang et al. 2017; Hailemariam et al. 2020; Henderson et al. 2015) and has been assessed as a DFM in both cattle and sheep (Rigobelo et al. 2016; Bello et al. 2019). The contribution of the rumen microbiome is to support 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).

The species has been reported to ferment xylan and starch derived from plant material (Hespell et al. 1987; Kozakai et al. 2007). As a commensal microorganism, feeding *S. dextrinosolvens* would have no impact on animal health. Should *S. dextrinosolvens* not act to ferment xylan and starch, 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 less soluble carbohydrates.

2.5.1 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 diet offered to the animal would be formulated to meet the existing nutritional needs of the animal (NRC, 2016). Should *S. dextrinosolvens* ASCUSBF53 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.

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 *et al.*, 2007, Abu-Tarboush *et al.*, 1996, Higginbotham *and Bath.*, 1993, McGilliard and Stallings, 1997). 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.*, 2009, Zebeli *et al.*, 2012; Hagg , 2008, 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 *S. dextrinosolvens* ASCUSBF53, if the DFM failed to provide any benefit, rumen fermentation of treated cattle would be identical to rumen fermentation of untreated cattle. Animals would be fed rations that meet established nutrient requirements as recommended by the NRC for beef cattle (NRC, 2016). Any inactive *S. dextrinosolvens* ASCUSBF53 or deceased *S. dextrinosolvens* ASCUSBF53 would pass through the GI tract with the normal flow of digesta.

Based on the results of published comparative studies, *S. dextrinosolvens* ASCUSBF53 will act only to support normal ruminal function of digestion of animal feed. The absence of the anticipated effect of *S. dextrinosolvens* ASCUSBF53 on feed in beef cattle would have no impact on safety. Native Microbials product labeling does not suggest a change in normal feeding regime. Animals would continue to be fed rations that meet established nutrient requirements as recommended by the NRC for beef cattle (NRC, 2016).

2.5.2 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 *S. dextrinosolvens* ASCUSBF53, that is "failure" of the intended use will not raise a safety concern, as the intended use is to support fermentation of nutritionally adequate feeds in the rumen. 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 *S. dextrinosolvens* ASCUSBF53 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.

Citations to be added

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ATTACHMENT 6

Botulinum Test Method 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)(6), Controller
Director of Operations
Corporate

(b) (4)

(b) (4)

ATTACHMENT 7

[Revised] Dossier Section 3.1.2

[Revised]

3.1.2 Exposure to the Other Components of the Fat Encapsulated Product

At the intended intake of $1x10^8$ CFU *S. dextrinosolvens* ASCUSBF53/hd/day, the animal will be exposed to up to 5 g of the notified substance. As the encapsulated organism is comprised of approximately (b) (4) *S. dextrinosolvens* ASCUSBF53 (see Appendix 010), Table 3.1 shows per head per day contribution of each of the components making up the encapsulated organism.

Table 3.1 Component Exposure of Fat Encapsulated *S. dextrinosolvens* ASCUSBF53

(b) (4)

As shown in Table 3.1, the animal will be exposed up to a maximum of (b) (4) and (b) (4). Considering that the typical dry matter intake by the beef cattle will be about 20 kg/head/day, the contribution of hydrogenated glycerides to the beef ration is expected to be no more than 0.0125% DM (Dry Matter). While the fat concentration of a typical beef cattle diet is reported to be relatively low (approximately 2.5% DM), supplemental fats can be added to achieve a total ration content of around 6% DM (MSD Veterinary Manual, 2019). On this basis, the use of hydrogenated glycerides or similar acceptable fat source as an encapsulating aid in the manufacture of fat encapsulated S. *dextrinosolvens* ASCUSBF53 will have a negligible impact on the total fat intake by beef cattle under the conditions of use. Similarly, an intake of 1 g/hd/day of sodium sulfate will provide beef cattle with approximately 0.48 g of sodium/hd/day, representing no more than 0.005% of the DM intake. The maximum tolerable levels of sodium chloride set by the National Research Council (NRC) for beef cattle is 3% of DM intake, equivalent to around 1% DM of sodium. Thus, the use of sodium sulfate as an encapsulating agent in the manufacture of fat encapsulated S. *dextrinosolvens* ASCUSBF53 is not expected to have any significant impact on the overall sodium intake by beef cattle under the intended conditions of use. Another element of interest is sulfur. The use of S. *dextrinosolvens* ASCUSBF53 would provide approximately 1 g of sodium sulfate or 0.34 g of sulfur per day. The NRC (2005) has suggested that Total Mixed Rations (grain based) of cattle diets should be at a maximum tolerable level of 0.3% sulfur (60 g/hd/day), as such this ingredient would provide an insignificant amount of the total sulfur in the diet of the beef cattle.

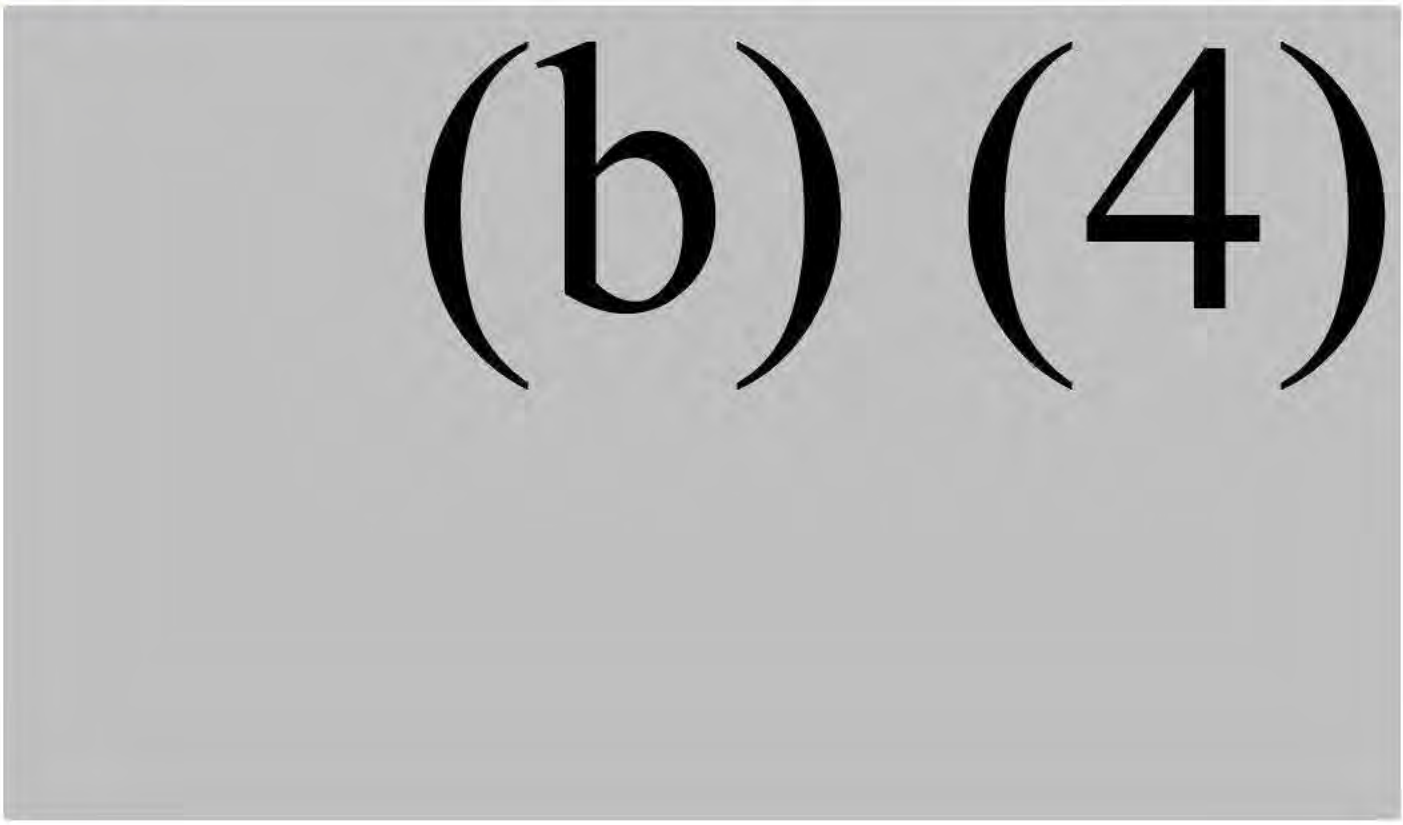
ATTACHMENT 8

BF53 Solid Intermediate Microbe Enumeration Method Validation Report

BF53 Solid Intermediate Microbe Enumeration Validation Summary Report

(b) (4)

(b) (4)



ATTACHMENT 9

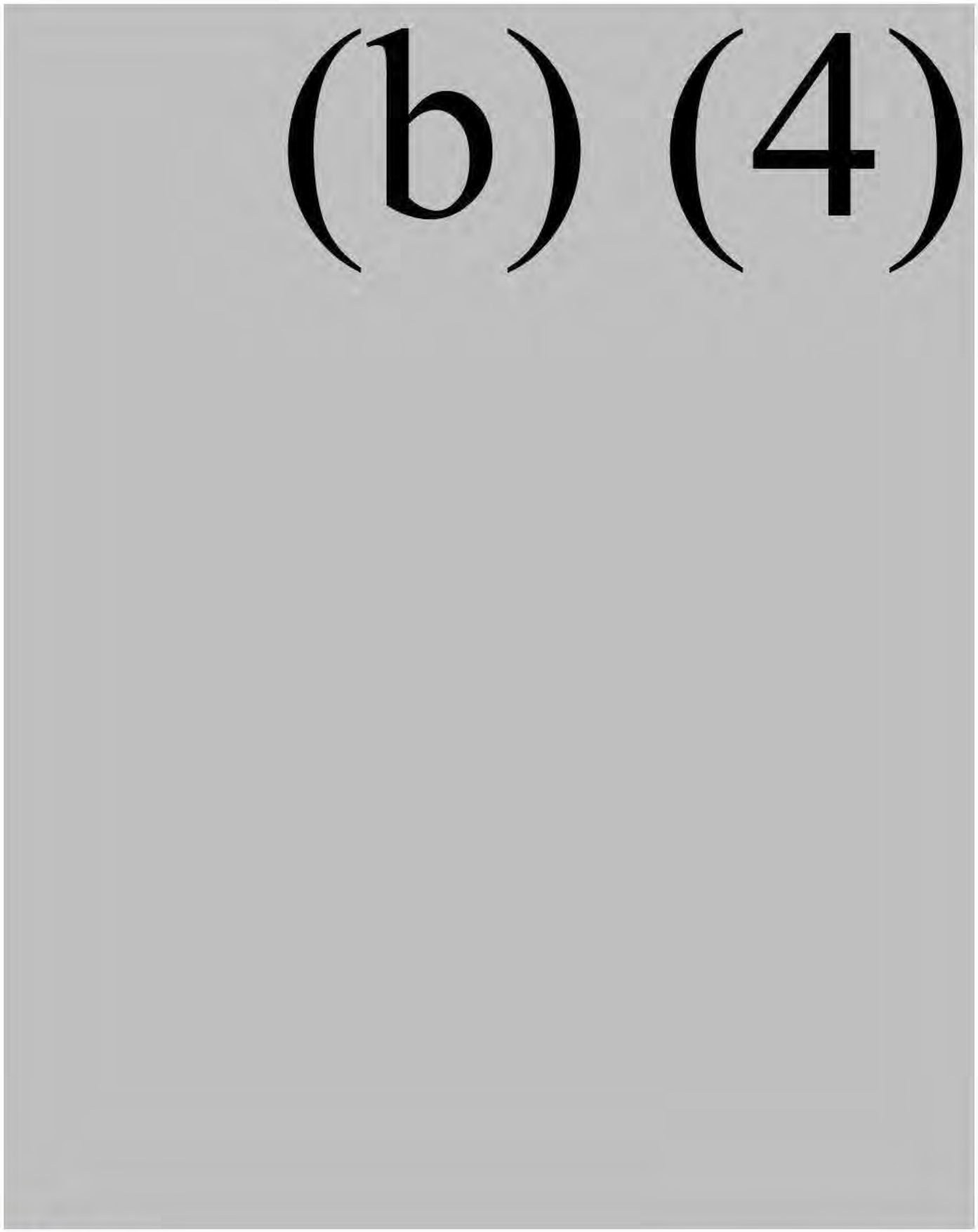
BF53 Liquid Intermediate Microbe Enumeration Validation Summary Report

BF53 Liquid Intermediate Microbe Enumeration Validation Summary Report

Methods

Beef-53 Liquid Intermediate Microbe Enumeration, V1

(b) (4)



Conclusion

The protocol was executed as written with no deviations or changes during execution. Repeatability, robustness, and linearity of the assay were demonstrated.

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

Approval

Name & Title	Signature & Date	
Chris Hartnett Vice President Manufacturing & Supply Chain	(b) (6)	7/27/2022
Kelly Mercier Quality Manager	(b) (6)	7/26/2022

ATTACHMENT 10

native
MICROBIALS

Ambient (b) (4) Stability Data summary for *S. dextrinosolvens* ASCUSBF53

BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate (b)(4) Stability Report

Purpose

The purpose of this report is to present the results and analysis of the real time stability study of BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate lots 1801.2041, 1801.2042, and 1801.2044 stored at (b)(4) to support the prediction of product stability at 2-10°C.

Results

Samples were placed at (b)(4) and analyzed monthly for viable cell count according to the approved Stability Protocol for BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate. See Table 1 below for test timepoints.

Table 1 – Tests and timepoints.

Assay	**T_0**	**1 Month**	**2 Months**	**3 Months**	**6 Months**	**9 Months**	**12 Months**
BF53 Solid Intermediate Microbe Enumeration method	X	X	X	X	X	X	X

The CFU/g for each lot are displayed in Table 2 below and graphed in Figure 1.

Table 2 – Test Results

Month	1801.2041 CFU/g	1801.2042 CFU/g	1801.2044 CFU/g
0	9.02E+07	8.18E+07	7.30E+07
1	(b) (4)		
2			
3			
6			
9			
12			

Figure 1 – CFU/g by month

(b) (4)

Conclusion

Real time stability data collected for 12 months at (b) (4) demonstrates that all 3 lots of BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate remain above the minimum specification for the duration tested.

Data Availability

All data is retained and available on the company drive:

(b) (4)/

Stability Protocol

The BF53 Long Term Stability Protocol (BF53_Long_Term_Stability_Protocol_V1.docx.pdf) can be found on the company drive:

(b) (4)

Approval

Name & Title	Signature & Date	
Chris Hartnett Vice President Manufacturing & Supply Chain	(b) (6)	7/28/2022
Kelly Mercier Quality Manager	(b)(6) 7BD513E026E94C0...	7/27/2022

ATTACHMENT 11

native
MICROBIALS

Accelerated (b) (4) Stability Data summary
for *S. dextrinosolvens* ASCUSBF53

BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate (b) (4) Stability Report

Purpose

The purpose of this report is to present the results and analysis of the real time stability study of BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate lots 1801.2041, 1801.2042, and 1801.2044 stored at (b) (4) to support the prediction of product stability at 2-10°C.

Results

Samples were placed at (b) (4) and analyzed monthly for viable cell count according to the approved Stability Protocol for BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate. See Table 1 below for test timepoints.

Table 1 – Tests and timepoints.

Assay	**T_0**	**1 Month**	**2 Months**	**3 Months**	**6 Months**	**9 Months**	**12 Months**
BF53 Solid Intermediate Microbe Enumeration method	X	X	X	X	X	X	X

The CFU/g for each lot are displayed in Table 2 below and graphed in Figure 1.

Table 2 – Test Results

Month	1801.2041 CFU/g	1801.2042 CFU/g	1801.2044 CFU/g
0	9.02E+07	8.18E+07	7.30E+07
1	(b) (4)		
2			
3			
6			
9			
12			

Figure 1 – CFU/g by month

(b) (4)

Conclusion

Real time stability data collected for 12 months at (b) (4) demonstrates that all 3 lots of BF53 *Succinivibrio dextrinosolvens* ASCUSBF53 Fat Encapsulate remain above the minimum specification for 9 months, which helps to justify the stability claim at 2-10°C for 12 months, allowing for normal excursions during transport and storage.

Data Availability

All data is retained and available on the company drive:
Lab/Process Development/Stability Documents/BF53_Stability/LongTerm_Stability/

Stability Protocol

The BF53 Long Term Stability Protocol (BF53_Long_Term_Stability_Protocol_V1.docx.pdf) can be found on the company drive:

(b) (4)

Approval

Name & Title	Signature & Date	
Chris Hartnett Vice President Manufacturing & Supply Chain	(b) (6)	7/28/2022
Kelly Mercier Quality Manager	DocuSigned by: (b)(6)	7/27/2022

ATTACHMENT 12

[Revised] Dossier Section 6

[Revised]

PART 6 – NARRATIVE

The conclusion that *S. dextrinosolvens* ASCUSBF53 fat encapsulated powder, as described herein, is GRAS under the conditions of intended use as a direct fed microbial in feed for 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 *Succinivibrio* species.

As mentioned in Part 1.3, *S. dextrinosolvens* ASCUSBF53 will be provided to cattle either alone or in combination with other direct fed microbials. The strain was isolated from the rumen content of a healthy Angus steer and is intended as a source of commensal microorganisms. In this respect, *S. dextrinosolvens* ASCUSBF53 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 a microorganism without an extensive history of use in food or feed is primarily addressed by evaluating the 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 *S. dextrinosolvens* ASCUSBF53 are detailed in Part 2. The microbial has been unambiguously characterized as *S. dextrinosolvens* (see Part 2.1.4). Whole genome sequence analysis using publicly available virulence factor databases revealed no protein toxins or virulent factors encoded by the *S. dextrinosolvens* ASCUSBF53 genome (see Part 2.1.8). Whole genome sequence analysis together with phenotypic testing indicate that *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* (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 are pivotal to the safety evaluation of *S. dextrinosolvens* ASCUSBF53 and are summarized below.

6.1 Functionality

The microbial population of the rumen plays an important role in the utilization of feed by cattle. As mentioned in Part 2, *S. dextrinosolvens* ASCUSBF53 was isolated from the rumen content of a healthy steer and can utilize various carbon sources including simple carbohydrates (e.g., glucose and fructose) as well as carbohydrates derived from plant materials such as xylose and arabinose, sugar alcohols such as (b) (4) (see Part 2.1). Similar phenotypes are reported in the published literature for other *S. dextrinosolvens* strains (Bryant 2015; Bryant and Small 1956, Hespell, Wolf, and Bothast 1987). The species has a demonstrated ability to colonize and aid in the digestion of corn silage in vivo (Kozakai et al. 2007). Thus, the microorganism has the potential to support digestion by fermentation of forages and partially degraded digesta in the rumen.

S. dextrinosolvens is a succinate producer (Bryant and Small 1956; Bryant 2015; Hespell 1992; O'Herrin and Kenealy 1993). Succinate is a precursor for propionate, which is an important VFA for rumen digestion and animal growth (Castillo et al. 2004; O'Herrin and Kenealy 1993; Clemmons et al. 2020). Clemmons (2020) found a significantly higher succinate concentration in rumen fluid of more efficient cattle than the less efficient cattle, suggesting that succinate may be an important metabolite in nutrient conversion. It has also been reported that a significantly greater abundance of succinate- and propionate- producing bacteria were observed in more efficient cattle (Myer et al. 2015). Therefore, supplementing *S. dextrinosolvens* ASCUSBF53 as a DFM has the potential to improve ruminal succinate and propionate production.

These examples of the potential functionality of *S. dextrinosolvens* in the rumen support the proposed role of *S. dextrinosolvens* ASCUSBF53 as a source of viable microorganisms in the diet to support the fermentation of feed in the rumen.

6.2 Identity

The family *Succinivibrionaceae* includes the genera *Anerobiospirillum, Ruminobacter, Succinatimonas, Succinimonas,* and *Succinivibrio.* The family clusters within the gamma-subclass of the class Proteobacteria, also known as the Gammaproteobacteria (Stackebrandt and Hespell 2006; Hippe et al. 1999). *Succinivibrionaceae* as a family shares 84.6-88.5% 16S rRNA similarity to species in neighboring families within Gammaproteobacteria. Amongst type strains of species within *Succinivibrionaceae,* a maximum of 93% 16S rRNA similarity is observed and the species can be identified unambiguously by 16S rRNA sequencing (Stackebrandt and Hespell 2006). *S. dextrinosolvens* is currently the only species with standing nomenclature in the genus *Succinivibrio* (Bryant 2015).

6.3 Literature Search

A comprehensive literature search was conducted in order to identify all publicly available information pertaining to the safety of *S. dextrinosolvens* for use as a direct fed microorganisms to support digestion in beef cattle feed. Details of the search strategy are provided in Appendix 017. Results from the literature search form the basis of the safety assessment found in Parts 6.4, 6.5, 6.6.

6.4 Natural Occurrence

6.4.1 Prevalence in Animals

S. dextrinosolvens is routinely isolated from rumen of cattle and sheep (Bryant and Small 1956; Bryant 1959; Wozny et al. 1977b; Wang et al. 2017; Hailemariam, Zhao, and Wang 2020) and has been found in swine colons (Li et al. 2012). A single isolate of a *Succinivibrio* species has been found in a human during an astronaut dietary study (Holdeman, Good, and Moore 1976). A *Succinivibrio* species was also identified in a study regarding advanced periodontitis and oral bone loss, though no connection between the health condition and the isolate was drawn (Tanner et al. 1979). A total of 4 different strains of *S. dextrinosolvens* have been isolated, sequenced, and analyzed in the JGI genome portal to date (https://genome.jgi.doe.gov/portal/), and 7 strains (excluding *S. dextrinosolvens* ASCUSBF53) were found in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Succinivibrio is a core genus of rumen microbiome (Petri et al. 2013; Seshadri et al. 2018) and *S. dextrinosolvens* is the only identified species in this genus. Published studies found the abundance of *Succinivibrio* in rumen ranged from 0.1% to 15% (Petri et al. 2013; Petri et al. 2013b; Myer et al. 2016; Seshadri et al. 2018; Stewart et al. 2019). Consistent with the literature, internal studies have shown that this species comprises 8.83% on average (ranging from 1.3% - 19.99%) of the rumen population. It is also important to note that *Succinivibrio* is often found in greater abundance in animals on a high-starch diet (Bryant 1959; Bryant 1970; Henderson et al. 2015).

6.4.2 Microbiome Safety

The rumen microbiome is crucial for the digestion of feed in ruminants (Faichney 1996; Huws et al. 2018). Members of *Succinivibrio* have been identified as core rumen microorganisms (Petri et al. 2013) in cattle. The Hungate1000 project isolated 3 *S. dextrinosolvens* strains representing 0.7% of the global ruminant rumen microbial community (Seshadri et al. 2018). However, *S. dextrinosolvens* is particularly enriched in rumen fluid of animals on diets with high levels of starch or rapidly fermentable carbohydrates (Hespell 1992).

Native Microbials conducted a series of experiments in order to better understand the rumen composition of beef cattle as well as the impacts of administering native rumen microorganisms, including *S. dextrinosolvens* ASCUSBF53. In one survey experiment (Clemmons et al. 2019a, Clemmons et al. 2019b), cattle fed a high-grain diet were followed over 10 weeks to determine how the microbiome changed and adapted in response to shifting to a step-up and high concentrate finishing diet. Two follow-up studies were conducted to determine the impacts of daily supplementation of *S. dextrinosolvens* ASCUSBF53 in conjunction with other native rumen microorganisms on rumen microbiome composition. In both experiments, the average abundance of *S. dextrinosolvens* in the rumen of cattle fed a high grain diet was 8.84% (ranging from 1.3%-19.99%) of the bacterial population. General observations indicated that all animals were in good health. Furthermore, *S. dextrinosolvens* ASCUSBF53 inoculation was not observed to significantly change the existing ruminal microbial community. Taken together, these studies provide corroborative experimental evidence that *S. dextrinosolvens* is naturally abundant in the rumen of feedlot cattle and not associated with any health concerns.

6.4.3 Environmental Occurrence

S. dextrinosolvens occurs extensively in the in the rumens of cattle and sheep (Bryant and Small 1956; Bryant 1959; Wozny et al. 1977b; Wang et al. 2017; Hailemariam, Zhao, and Wang 2020), as well as the colons of pigs (Li et al. 2012). Bacteria in the *Succinivibrio* genus have also been isolated from manure sludge from a Korean swine farm (Han et al. 2011).

Occurrences of *S. dextrinosolvens* being isolated in environmental samples that are not associated with animals are not well documented. Furthermore, the entry regarding *Succinivibrio* in Bergey's Manual of Systematics of Archaea and Bacteria only reported isolations from mammalian samples (Bryant 2015).

6.4.4 Section Summary

S. dextrinosolvens is readily found in the rumens of cattle and sheep. It is a common, ruminant commensal organism. Supplementation of the diet with *S. dextrinosolvens* ASCUSBF53 will not negatively impact the function of the rumen nor negatively impact the well-being of the animal.

6.5 History of Use in Manufacture of Food and Feed Ingredients

S. dextrinosolvens has been previously used as an additive in feed. *S. dextrinosolvens*, in conjunction with several other microbes, was administered to dairy cattle as a DFM (Bello et al. 2019). While no improvements to milk production were reported, no ill effects of the microbial supplementation were found in this study. It has also been used as a feed supplement for sheep, in efforts to reduce the amount of *E. coli* present in their gastrointestinal tracts (Rigobelo et al. 2016). No adverse effects were reported.

6.6 Toxigenicity and Pathogenicity

The family *Succinivibrionaceae* consists of 5 distinct genera, including *Succinivivibrio*. Members of the *Anaerobiospirillum* genus of the *Succinivibrionaceae* family have been identified in a number of clinical reports, however, these bacteria are considered to be uncommon causative agents and nearly all cases involving *Anaerobiospirillum* species were reported in people with additional health problems, suggesting that these infections are opportunistic (Tee et al. 1998; Kelesidis et al. 2010; Decroix et al. 2016; Epstein et al. 2017; Schaumburg et al. 2017; Madden et al. 2019). There have been no reported infections confirmed to be caused by other members of the *Succinivibrionaceae* family in the genera *Ruminobacter*, *Succinatimonas,* and *Succinimonas*. Furthermore, no published data was found regarding common pathogenic elements in the genomes across the genera in the *Succinivibrionaceae* family.

S. dextrinosolvens was isolated from two cases of bacteremia to date (Southern 1975; Porschen and Chan 1977). In both cases, the patients were suffering from other serious health conditions and the organism identification was made using morphology data, as well as metabolic and antimicrobial susceptibility profiles. No infections of *S. dextrinosolvens* have been confirmed using molecular or genetic sequencing-based methods.

1) The first suspected case was in a man that arrived at a hospital in a non-responsive state and suffering from heat stroke, numerous external injuries, and gastrointestinal hemorrhaging (Southern 1975). The patient's blood was diagnosed with bacteremia and an organism identified as *S. dextrinosolvens* was cultured from the blood. The patient died 60 hours after being admitted to the hospital, and the role of bacteremia in the death of the patient was uncertain, as there was little evidence of residual infection in the blood.

2) The second case reported was in a man suffering from gastrointestinal bleeding and an esophageal hernia (Porschen and Chan 1977). He underwent surgery, and then an organism later identified as *S. dextrinosolvens* was cultured from blood samples using agar plates. The authors speculated that the bacteria found in the blood originated in the gastrointestinal tract, and no signs of sepsis were observed after the bacteremia was identified.

6.6.1 Section Summary

Overall, the available information indicates that *S. dextrinosolvens* is an organism abundant in the rumens of cattle and sheep. Two clinical cases were associated with *S. dextrinosolvens* via cultivation-based methods, but none were confirmed by unambiguous genetic methods. As

indicated in Part 2.1.8, interrogation of the whole genome sequence of *S. dextrinosolvens* ASCUSBF53 did not reveal the presence of any significant genes that encode for virulence factors or protein toxins.

6.7 Studies in Target Animals

The determination that *S. dextrinosolvens* ASCUSBF53 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. However, two unpublished studies corroborate this safety assessment.

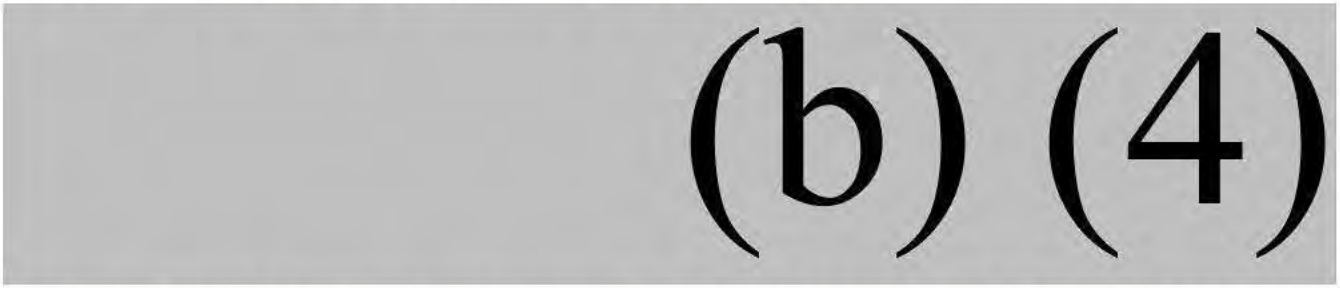
Two investigative studies in which cattle were provided with *S. dextrinosolvens* ASCUSBF53 were conducted by Native Microbials to corroborate the target animal safety determination. These unpublished studies, summarized in turn below, were designed primarily to assess the potential value of *S. dextrinosolvens* ASCUSBF53 and other microorganisms as potential direct fed microbials. In these studies, *S. dextrinosolvens* ASCUSBF53 was administered via ruminal cannulation or in feed in combination with at least one other microorganism. Overall, the study findings provide corroborative evidence that *S. dextrinosolvens* ASCUSBF53 is well-tolerated and without adverse effects but are of limited relevance to the assessment of safety.

6.7.1 Study BUS1801 (Unpublished Study Report – Appendix 019)

(b) (4)

No adverse effects were reported for any of the variables measured over the duration of the study. Overall, the findings of the study corroborate the safety of *S. dextrinosolvens* ASCUSBF53 for cattle.

6.7.2 Study BUS1901 (Unpublished Study Report – Appendix 020)



Overall, general health was within normal limits across all treatment groups. There were no adverse events attributable to or consistent with a specific test article. Health events were not outside of normal limits for cattle transitioning to high concentrate diets in the feed yard. Overall, the findings of the study corroborate the safety of *S. dextrinosolvens* ASCUSBF53 for cattle.

6.7.3 Published Articles Specific to Acidosis

Because *S. dextrinosolvens* is higher in abundance in the rumen of cattle on a high concentrate diet (Petri 2013; Petri 2013b), studies have suggested that members of *S. dextrinosolvens* may be contributing to the development of rumen acidosis or bloat (Azad et al. 2019; Dai et al. 2020). However, these studies are based on correlation and neither directly proves that *S. dextrinosolvens* can induce ruminal acidosis. Dai et al. (2020) examined the growth of *S. dextrinosolvens in vitro* and found that *S. dextrinosolvens* can use *Escherichia coli* lipopolysaccharide (LPS) as carbon source, which is abundant in rumen fluid under the rumen acidosis condition (Khafipour et al. 2011). The authors argued that the growth of *S. dextrinosolvens* in the presence of *E. coli* LPS can lead to the production of lactate and lead to rumen acidosis. Similarly, Azad et al. (2019) observed an increased relative abundance of *S. dextrinosolvens* in cattle experiencing diet induced frothy bloat. The authors concluded that Proteobacteria, including *S. dextrinosolvens* contribute to rapid fermentation of complex polysaccharides. Further, Hu et al. (2022) found the abundance of two Proteobacteria, *Stenotrophomonas* and *Succinivibrio,* increased significantly in the rumen of dairy cows experiencing high grain diet induced subacute rumen acidosis (SARA). However, data correlation and oral inoculation revealed that *Stenotrophomonas* induced the host inflammatory responses and was the causative microorganism. This suggests that correlation alone is insufficient to identify the causative microorganism.

The elevated abundance of *S. dextrinosolvens* observed under acidotic conditions is likely due to the changes in rumen environment created by a high concentrate diet, rather than direct causality. The highly variable abundances of *S. dextrinosolvens* (1.3%-19.99% of the rumen microbiome) observed in the microbiome of animals without any indication of acidosis would suggest *S. dextrinosolvens* is not the causative agent of acidosis, but its presence is more likely due to the abrupt change in diets (Samuelov et al. 1991; Petri et al. 2013b; Laporte-Uribe 2016).

6.8 Summary and Critical Evaluation of Target Animal Safety

S. dextrinosolvens is a commensal bacteria that occurs widely in the rumen of various ruminant species. No reports of toxigenicity or pathogenicity associated with *S. dextrinosolvens* were identified in the published literature. Native Microbials has conducted an assessment of *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 should not be associated with any safety concerns for 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 *S. dextrinosolvens* ASCUSBF53 from the rumen to edible tissues is anticipated under the conditions of intended use as a direct fed microbial in the feed of cattle. Furthermore, the strain has been unambiguously characterized as *S. dextrinosolvens* 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). Although testing is done for botulinum toxins with each fermentation run, it is done as a general cautionary practice for all anaerobic fermentation runs, as a way to rule out the contamination of the run, and not because *S. dextrinosolvens* ASCUSBF53 produces or even is capable of producing such toxins. The absence of pathogenicity or toxigenicity is supported by the ubiquitous nature of *S. dextrinosolvens* and its natural occurrence in the rumen of animals that are regularly consumed by humans. Taken together, these data indicate that *S. dextrinosolvens* ASCUSBF53 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 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.

Additional Citations Added

Azad, E., H. Derakhshani, R. J. Forster, R. J. Gruninger, S. Acharya, T. A. McAllister, and E. Khafipour. 2019. "Characterization of the Rumen and Fecal Microbiome in Bloated and Non-Bloated Cattle Grazing Alfalfa Pastures and Subjected to Bloat Prevention Strategies." Scientific Reports 9 (1): 4272.

Dai, Xiaoxia, Timothy J. Hackmann, Richard R. Lobo, and Antonio P. Faciola. 2020. "Lipopolysaccharide Stimulates the Growth of Bacteria That Contribute to Ruminal Acidosis." Applied and Environmental Microbiology 86 (4). https://doi.org/10.1128/AEM.02193-19.

Dehority, B. A. 1971. "Carbon Dioxide Requirement of Various Species of Rumen Bacteria." Journal of Bacteriology. https://doi.org/10.1128/jb.105.1.70-76.1971.

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ATTACHMENT 13

[Revised] Dossier Appendix 18 Microbiome Safety

Microbiome Safety for *Succinivibrio dextrinosolvens* ASCUSBF53

Objectives

The objective of this review is to:

a) Demonstrate that the typical microbial composition and diversity of the rumen microbial community of beef cattle is robust and stable across various diets and regions. We will demonstrate this by:

   i) Showing internal datasets (e.g. data and analyses created by Native Microbials)

   ii) Presenting data via external datasets (e.g. data published in peer reviewed manuscripts).

b) Present data that shows the feeding of native microorganisms does not negatively alter the microbiome composition. Specifically, that daily administration of *Succinivibrio dextrinosolvens* ASCUSBF53 does not increase its own abundance nor the overall composition of the microbiome beyond typically observed ranges.

Robust Nature of the Dairy Rumen Microbiome

Native Microbials Animal Experiments: A series of experiments were conducted 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.

Microbiome Survey : A survey experiment was conducted to identify the rumen composition of 50 Angus steers over a period of 70 days in (b) (4). The animals were fed a typical local diet for measuring feed efficiency (see Attachment 1). Rumen samples were taken every 7 days throughout the study to analyze and characterize the rumen microbiome. The study has been peer reviewed and published (Clemmons, Martino, Powers, et al. 2019; Clemmons, Martino, Schneider, et al. 2019).

Findings: The results of the survey experiment are summarized in Table 1, showing the average rumen bacterial phyla abundances. In all of these experiments, the abundances of the most predominant phyla were comparable to the ranges observed in the independent literature studies (presented below). The typical abundance of *S. dextrinosolvens*, specifically, in the rumen of an Angus steer based on Native Microbials survey was found to be 8.84% on average (ranged from 1.3% to 19.99%) of the rumen bacterial population.

Table 1. Abundance of Rumen Bacterial Phyla from Native Microbials Survey Experiment, Reported as a Percent

Phylum	Average Abundance (%)	Abundance Range (%)
Bacteroidetes	48.02	29.91 - 60.71
Proteobacteria	26.86	8.70 - 46.13
Firmicutes	21.99	8.86 - 40.70
Cyanobacteria/Chloroplast	1.60	0.44 - 7.94
Actinobacteria	1.16	0.55 - 2.65
Spirochaetes	0.8871	0.1866 - 2.2603
Lentisphaerae	0.3829	0.0098 - 1.0838
Tenericutes	0.3032	0.0307 - 1.0240
Fibrobacteres	0.1736	0.0050 - 0.7579
TM7 (Candidatus Saccharibacteria)	0.1663	0.0034 - 2.2466
Verrucomicrobia	0.1382	0.0007 - 1.0695
Chloroflexi	0.1212	0.0061 - 0.3732
Acidobacteria	0.0920	0.0150 - 0.5587
Planctomycetes	0.0781	0.0039 - 0.5447
Synergistetes	0.0739	0.0109 - 0.3845
Elusimicrobia	0.0662	0.0001 - 1.7201
Armatimonadetes	0.0450	0.0007 - 0.2095
Fusobacteria	0.0426	0.0004 - 1.4855
Deinococcus-Thermus	0.0214	0.0008 - 0.1972
Thermotogae	0.0162	0.0014 - 0.0914
SR1	0.0058	0.0004 - 0.0258
Chrysiogenetes	0.0053	0.0005 - 0.0297

Cloacimonetes	0.0047	0.0012	-	0.0195
Aquificae	0.0044	0.0007	-	0.0216
Chlorobi	0.0030	0.0030	-	0.0030
Chlamydiae	0.0021	0.0005	-	0.0043
Parcubacteria	0.0007	0.0005	-	0.0008

Product Study:

Study 1: In this study, six native rumen microorganisms were administered directly to the rumen of 16 cannulated Angus heifers daily to determine the effect of microorganism supplementation on the native rumen microbial community. The animals were located in (b)(6) and were fed a typical feedlot diet (see Attachment 2) over 110 days. Eight control animals received a saline buffer solution, while eight experimental animals received the same buffer solution containing multiple rumen microorganisms. The microbial blend consisted of *S. dextrinosolvens* ASCUSBF53, *Chordacoccus ruminofurens* ASCUSBF65, *Prevotella albensis* ASCUSBF41, *Bacteroides xylanisolvens* ASCUSBF52, and *Clostridium sp.* ASCUSBF26. The animals were transitioned from a low-grain diet (<50% concentrate) to a high-grain diet (≥50% concentrate) (see Attachment 2) over 21 days following a standard step-up procedure. The animals were also challenged with a more fermentable diet to induce acidosis. Rumen samples were taken periodically throughout the study to analyze and characterize the rumen microbiome.

Findings: In this administration experiment, it can be seen that addition of *S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, *P. albensis* ASCUSBF41, *B. xylanisolvens* ASCUSBF52, *Clostridium sp.* ASCUSBF26 to Angus heifers did not significantly alter the rumen bacteria microbiome composition when compared to the control group within each diet condition (Table 2). Abundances of all bacterial phyla are within standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum tended to be similar across experimental groups.

Table 2. Abundance of Rumen Bacterial Phyla in the Rumen from Native Microbials Product Study 1, Reported as a Percent

	Low-grain Diet		High-grain Diet		Acidosis Challenge	
Phylum	No Microbes	Six Microbes	No Microbes	Six Microbes	No Microbes	Six Microbes
Bacteroidetes	32.90	38.15	40.69	42.38	37.86	36.50
Firmicutes	40.32	37.32	24.23	23.37	28.07	30.67
Proteobacteria	4.20	3.30	20.11	18.36	17.22	17.07
Actinobacteria	0.54	0.49	0.96	0.99	2.34	1.77
Synergistetes	0.10	0.08	1.52	1.55	1.04	0.92
Spirochaetes	0.75	0.54	0.27	0.22	0.11	0.11
Fibrobacteres	1.29	1.58	0.23	0.17	0.07	0.07
Tenericutes	0.30	0.34	0.08	0.06	0.03	0.00
TM7 (Candidatus Saccharibacteria)	0.21	0.20	0.08	0.07	0.02	0.05
Cyanobacteria/Chloroplast	0.00	0.00	0.03	0.01	0.02	0.02
Armatimonadetes	0.05	0.03	0.04	0.04	0.01	0.01
Lentisphaerae	0.00	0.00	0.00	0.02	0.01	0.01
Fusobacteria	0.03	0.01	0.61	0.18	0.00	0.00
SR1	0.33	0.26	0.04	0.03	0.00	0.00

Study 2: In the second experiment, three native rumen microorganisms were added to diet and fed to 75 Angus steers daily over 168 days to determine the effect of microorganism supplementation on the native rumen microbial community. The study took place in (b) (4). The animals were transitioned from a low-grain diet (<50% concentrate) to a high-grain diet (≥50% concentrate) (see Attachment 3). The study consisted of two study groups with 50 animals receiving a microbial blend (*S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41) and 25 animals receiving no microbes (control). Rumen samples were taken periodically throughout the study to analyze and characterize the rumen microbiome.

Findings: In this administration experiment, it can be seen that addition of *S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41 to Angus steers did not significantly alter the rumen bacteria composition when compared to the control group (Table 3). Abundances of all bacterial phyla are within standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum did not differ significantly across experimental groups.

Table 3. Abundance of Rumen Bacterial Phyla in the Rumen from Native Microbials Product Study 2, Reported as a Percent.

	Low-grain Diet		High-grain Diet	
Phylum	Control	Three Microbes	Control	Three Microbes
Bacteroidetes	71.43	66.30	39.52	44.34
Firmicutes	14.29	21.45	14.96	19.60
Proteobacteria	11.51	9.33	44.40	34.69
Cyanobacteria/Chloroplast	0.6596	0.4492	0.3146	0.3731
Synergistetes	0.4559	0.6250	0.2401	0.3010
Actinobacteria	0.3855	0.5046	0.1982	0.3043
Spirochaetes	0.3725	0.3126	0.1654	0.1729
TM7 (Candidatus Saccharibacteria)	0.3661	0.3612	0.0347	0.0375
Tenericutes	0.2262	0.2837	0.0670	0.0725
Lentisphaerae	0.0716	0.0681	0.0141	0.0143
Planctomycetes	0.0461	0.0610	0.0131	0.0145
Fibrobacteres	0.0395	0.0407	0.0573	0.0414
Chloroflexi	0.0368	0.0593	0.0102	0.0155
Verrucomicrobia	0.0298	0.0420	0.0130	0.0173
Elusimicrobia	0.0285	0.0209	0.0120	0.0119
Acidobacteria	0.0245	0.0282	0.0133	0.0105
Armatimonadetes	0.0190	0.0488	0.0167	0.0206

Fusobacteria	0.0190	0.0181	0.0136	0.0339
Deinococcus-Thermus	0.0188	0.0186	0.0093	0.0134
Candidate Division WPS-2	0.0134	0.0251	0.0046	0.0082
SR1	0.0092	0.0052	0.0066	0.0100

Study 3: In the third experiment, three native rumen microorganisms were added to the ration and fed to Angus steers daily over 109 days. This study took place in (b) (4). The animals were fed typical local farm diets and transitioned from a low-grain diet (<50% concentrate) to a high-grain diet (≥50% concentrate)(see Attachment 4). A blend of microbes (*S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41) were administered to 100 animals, while the other 100 animals received none and served as controls. Rumen samples were taken periodically from a subset of animals throughout the study to analyze and characterize the rumen microbiome.

Findings: In this administration experiment, it can be seen that addition of *S. dextrinosolvens* ASCUSBF53, *C. ruminofurens* ASCUSBF65, and *P. albensis* ASCUSBF41 to Angus steers did not significantly alter the rumen bacteria composition when compared to the control group (Table 4). Abundances of all bacterial phyla are within standard ranges observed in animals not fed native rumen microbes. The average abundance of each phylum tended to be similar across experimental groups.

Table 4. Abundance of Rumen Bacterial Phyla in the Rumen from Native Microbials Product Study 3, Reported as a Percent.

	Low-grain Diet		High-grain Diet	
Phylum	**Control**	**Three Microbes**	**Control**	**Three Microbes**
Firmicutes	48.05	51.02	37.67	38.12
Bacteroidetes	43.82	41.10	28.56	31.82
Proteobacteria	3.26	3.11	30.81	26.99
Actinobacteria	1.0844	1.3023	0.9479	0.8821
Spirochaetes	0.6173	0.5373	0.2481	0.4223
Lentisphaerae	0.4313	0.3514	0.0188	0.0590
Tenericutes	0.3916	0.2875	0.1768	0.2222
TM7 (Candidatus Saccharibacteria)	0.3542	0.4225	0.1278	0.1075

Chloroflexi	0.3519	0.3223	0.0211	0.1300
Fibrobacteres	0.3474	0.1589	0.1324	0.0575
Cyanobacteria/Chloroplast	0.2635	0.3141	0.7363	0.4122
Synergistetes	0.2120	0.2145	0.4497	0.6552
SR1	0.1686	0.2193	0.0184	0.0153
Elusimicrobia	0.1158	0.1042	0.0149	0.0247
Fusobacteria	0.1122	0.0215	0.0450	0.1385
Acidobacteria	0.1104	0.2117	0.0514	0.0529
Planctomycetes	0.0944	0.0597	0.0000	0.0000
Verrucomicrobia	0.0825	0.0688	0.1003	0.0760
Armatimonadetes	0.0484	0.0427	0.0220	0.0234
Deferribacteres	0.0374	0.0554	0.0019	0.1806
Candidate Division WPS-2	0.0316	0.0369	0.0000	0.0474
Dictyoglomi	0.0102	0.0021	0.0000	0.0000
Thermodesulfobacteria	0.0087	0.0062	0.0000	0.0000
Deinococcus-Thermus	0.0050	0.0096	0.0159	0.0134
Poribacteria	0.0049	0.0052	0.0000	0.0000
Aquificae	0.0045	0.0018	0.0245	0.0081
Thermotogae	0.0039	0.0055	0.0000	0.0000
Chrysiogenetes	0.0024	0.0066	0.1505	0.2137

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 *S. dextrinosolvens*. Several bacterial analyses conducted by academic institutions were found for beef cattle including: R. M. Petri et al. 2013; Myer et al. 2016; Ribeiro et al. 2017; Khafipour et al. 2009; Stewart et al. 2019, 2018; Kocherginskaya, Aminov, and White 2001). 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:

a) Ribeiro et al. (2017)) 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 transfer microbiome composition are reported in the manuscript, only the pre-transfer results are presented here.

b) Petri et al. (2013 and 2013b) studied the rumen and epimural 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.

c) Seshadri et al. (2018) reported an effort on culturing rumen representative microorganisms from global ruminants. The collection represents ~75% of the rumen microbiome at genus level.

d) Myer et al. (2016) studied the rumen microbiome of 3 steers. The animals were cross-breeds of a variety of feedlot cattle on a high-grain diet. The animals were selected based on their similar feed efficiency phenotype and minimal deviation among each other.

e) Stewart et al. (2018) sequenced the rumen samples from 42 Scottish beef cattle and identified 913 representative microorganisms. The cattle were fed a high-concentrate diet. The microbiome was profiled on all samples based on the representative microorganisms.

f) Stewart et al. (2019) sequenced the rumen samples from 283 Scottish beef cattle. The animals were on a high-concentrate diet. The study identified 4,941 representative microorganisms. The microbiome was profiled on all samples based on the representative microorganisms.

g) Auffret et al. (2017) studied the rumen microbiome from 50 beef cattle. The animals were either on a high-concentrate diet or a forage-based diet. The microbiome was profiled for each diet.

h) Myer et al. (2016), R. M. Petri et al. (2013), Ribeiro et al. (2017), and Stewart et al. (2019) have also identified the abundance of *Succinivibrio* in the rumen microbiome. *Succinivibrio* was particularly abundant in animals fed with a high-grain diet, ranging from 0% to 1.6%,

i) Kocherginskaya, Aminov, and White (2001) and Khafipour et al. (2009) also evaluated the rumen microbiome of cattle fed with a high-grain diet. Although their microbiome analysis was not robust enough to include in the analysis here, their results are consistent with others' and Native Microbials' findings.

Findings:

i) 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, and season. Additionally, there are groups of microorganisms that are unique to particular breeds of cattle, 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 the 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 cattle (see Table 5).

Table 5. Abundance of bacterial phyla in the rumen from independent studies, reported as a percent. Empty cells indicate that data was not reported.

	Seshadri et al. 2018	Petri et al. 2013	Myer et al. 2016	Petri et al. 2013b				Ribeiro et al. 2017	Stewart et al. 2019	Stewart et al. 2018	Auffret et al. 2017	
Phylum	(Global Rumen Representatives)	(Rumen Core[a])	(High grain)	(Forage)	(High grain)	(Acidotic)	(Recovery)	(Barley straw)	(High grain)	(High grain)	(Forage)	(High-grain)
Bacteroidetes	12.78	32.8	68.64	25.7	40.3	40	31.5	20.29	49.85	36	31-61	46-61
Firmicutes	68.06	43.2	21.58	55.2	37	33.6	43.7	40.53	33.73	50	20-55	24-76
Proteobacteria	6.19	14.3	0.51	4.7	17.9	16.5	15.2	1.64	7.21	3.1	3-11	3-11
Fibrobacteres	0.4		< 1	7.1				25.04	0.59		0-7	0-2
Spirochaetes	1.2		< 1	2.8				6.13	0.43	1	0-2	0-2
Tenericutes			< 1						0.14			
Actinobacteria	6.59		< 1		1.6			1.78	1.8	3.5	2-21	2-12
Genus												
Succinivibrio	0.60[b]	0.1 to 15	0.26	0	0.39	0.56	0		1.6			

a. "Rumen core" values reported in Petri et al. (2013) were sourced from Jouany (1991).

b. The abundance of *S. dextrinosolvens*

ii) 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 and global ruminant microbiome effort were found for cattle fed a variety of diets (Seshadri et al. 2018; Ribeiro et al. 2017; Petri et al. 2013; Petri et al. 2013a; Myer et al. 2016; Stewart et al. 2019, 2018; Auffret et al. 2017). These manuscripts were further investigated to determine prevalence of the overall bacterial taxonomic composition of the typical rumen microbiome. These studies showed that diet formulation has the greatest impact on microbiome composition.

iii) Cumulatively, these independent studies investigated the microbial community across a variety of breeds, diets, and feed management regimes. Table 5 (above) summarizes the findings from Seshadri et al. (2018), Ribeiro et al. (2017), Petri et al. (2013), Petri et al. (2013a), Myer et al. (2016), Stewart et al. (2019), Stewart et al. (2018), and Auffret et al. (2017) 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. Proteobacteria is the third most

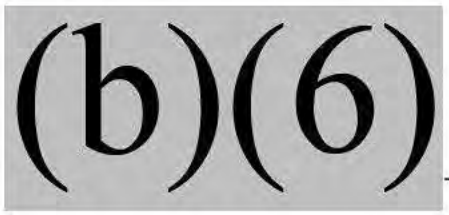
prevalent phylum in ruminants and its abundance is directly positively correlated with the amount of corn in diet (Kocherginskaya, Aminov, and White 2001). As can be seen from this data, there is a broad range of abundances. *S. dextrinosolvens* ASCUSBF53 falls into the Proteobacteria phylum, which was found to comprise 0.51% - 17.9% of the rumen microbial community. Kocherginskaya, Aminov, and White (2001) found the abundance of ruminal Proteobacteria is enriched in animals on a corn based high-grain diet. The study reported that Proteobacteria can comprise up to 27% of the rumen microbiome.

iv) Based on the global rumen microbiome effort, *S. dextrinosolvens* represents 0.6% of the rumen microbial populations. The abundance of genus *Succinivibrio* ranged from 0.26% to greater than 15% in rumen content of animals fed a high-grain diet but few were detected in animals on a forage based diet (Table 5). This is consistent with the findings of Khafipour et al. (2009) that the abundance of *S. dextrinosolvens* is associated with a high-grain diet.

v) 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 12.78%-68.64%, while the observed abundance of Firmicutes ranged from 20%-76%. Proteobacteria ranged from 3%-27% and could be higher, depending on the corn content in the diet. Other phyla did appear, but often represented less than 10% of the total bacterial population. These ranges were utilized to describe the "average" rumen in comparative analyses.

Conclusion

This summary covers the Native Microbial studies as well as published data to assess the potential microorganisms shift in microbiome that may raise safety concerns. Information presented demonstrated that the normal microbial community in the rumen is robust and not adversely affected by the addition of native external microbes, including *S. dextrinosolvens*. Hence, it is clear that the dietary addition of *S. dextrinosolvens* will not cause a safety concern based on changes in the microbiome.

Signed: 

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Attachment 1: (b) (4) **Survey details**

Diet: The survey took place in (b) (4) and utilized the following diet:

As-fed Ingredients	%
Corn Silage	80
Cracked Corn	10
Protein Supplement	10
DM	%
Crude Protein	11.57
Total Digestible Nutrients	76.93

Rumen samples were collected every 7 days via oro-gastric tubes. Steers were observed daily for overall clinical health throughout the study.

Attachment 2: (b) (4) **Product Study details**

Diet: The study took place in (b) (4) and utilized the following diet:

As-fed Ingredients (%)	Low-grain	High-grain	Acidosis Challenge
Hay	60.8	2.3-40.6	2.38-2.54
Corn Silage	17.6	14.8-17.1	7.02-7.87
Dry Rolled Corn	13	25.5-50.9	53.12-54.19
MDGS	7.2	14.1-25.3	--
Reconstituted DDGS	--	--	18.43-19.48
DDGS	--	10.99-11.04	11.01-11.06
Vitamin and Mineral Premix	1.4	2.7-5	5
water	--	14.38-15.85	11.28-21.16
Roughage Dry Matter (%)	**92.31**	**1.97-42.61**	**0.9-2.05**
Concentrate Dry Matter (%)	**7.69**	**57.39-98.03**	**97.95-99.1**

Animals were transitioned from a low-grain diet to a high-grain diet. Rumen acidosis was induced twice by increasing the amount of grain 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 every 3 to 4 days. Heifers were observed daily for overall clinical health throughout the study.

Attachment 3: (b) (4) **product study**

Diet: The survey took place in (b) (4), and utilized the following diet:

As-fed Ingredients (%)	Low-grain Diet	High-grain Diet
Alfalfa Hay	--	2.5-33.8
Corn (Rolled)	--	10.2-12
Corn Syrup	15	14.6-21
Distillers	--	0-7
Earlage	--	22-44.2
Grass Hay	77.5	0
Vitamin and Mineral Mix	2.5	3-4.6
Tallow	--	0-1.8
Wheat	--	10-27
Wheat Straw	--	0-4
Water	5	0
Roughage Dry Matter (%)	**91.42**	**10.23-44.63**
Concentrate Dry Matter (%)	**8.58**	**55.37-89.77**

Rumen samples were collected via oro-gastrictubing on days 0, 13, 28, 56, 88, 127, 153, and 168. Animals were observed daily for overall clinical health throughout the study.

Attachment 4: Native Microbial's third product study experiment

Diet: The survey took place in (b) (4) and utilized the following diet:

Ingredients (% DM)	Low-grain Diet	High-grain Diet
Alfalfa hay	100	6-35
Dry rolled corn	--	54-81
Molasses (cane 64)	--	6
CA23.00 Early Pel	--	5-9.04
Roughage Dry Matter (%)	**100**	**6-35**
Concentrate Dry Matter (%)	**0**	**65-94**

Rumen samples were collected via oro-gastrics tubing on days 7, 15, 29, 57, 83, and 109. Animals were observed daily for overall clinical health throughout the study.

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 ((b) (4) USA) and 5% TRI-Reagent ((b) (4), 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.

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 (b) (4) , USA). The 16S rRNA gene was amplified using 27F and 534R (LANE and J 1991; Muyzer, de Waal, and Uitterlinden 1993) primers modified for (b) (4) sequencing, following standard protocols (b) (4) , USA). Following amplification, PCR products were verified with a standard 2% agarose gel electrophoresis and purified using (b) (4), CA, USA). The purified amplicon library was quantified and sequenced on the (b) (4) USA) according to standard protocols using a 2x300 v3, 600-cycle kit. Raw fastq reads were de-multiplexed on the (b) (4), USA). All samples were sequenced at a depth such that each sample file contained at least 10,000 sequences after processing.

Analysis Method

All raw sequencing data was trimmed of adapter sequences and phred33 quality filtered at a cutoff of 20 using (b) (4) (Krueger and Others 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 (b) (4) (v10.0.240) (Edgar 2016; Edgar and Flyvbjerg 2015; Edgar, 2016a) 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.

ATTACHMENT 14

GRAS Safety Summary and Target Animal Safety for the Direct Fed Microbial *Succinivibrio dextrinosolvens* ASCUSBF53

Attachment 14

GRAS Safety Summary and Target Animal Safety for the Direct Fed Microbial *Succinivibrio dextrinosolvens* ASCUSBF53

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 *Succinivibrio dextrinosolvens* ASCUSBF53, 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 *S. dextrinosolvens* has been robustly studied and reported on by microbiologists studying the rumen microbiome)
2. Identify *S. dextrinosolvens* as a common member of the core rumen microbiome of feedlot cattle
3. Identification 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 *S. dextrinosolvens* or in Native Microbials studies in which feedlot cattle were fed *S. dextrinosolvens* ASCUSBF53.

Based on our detailed understanding of the impact of feeding *S. dextrinosolvens* ASCUSBF53 in feedlot 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."

Succinivibrio dextrinosolvens is a common member of the core rumen microbiome of feedlot cattle

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. *S. dextrinosolvens* 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, *S. dextrinosolvens* is found ubiquitously in feedlot 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 18 (Microbiome Safety for *Succinivibrio dextrinosolvens* ASCUSBF53). Both internal and external datasets were utilized to identify the prevalence and range of abundance of *S. dextrinosolvens* in feedlot cattle. *S. dextrinosolvens* is the only identified species of genus *Succinivibrio*. The genus *Succinivibrio* is considered a core member of the rumen microbiome (Petri et al. 2013). Six published studies reported the abundance of *Succinivibrio* (ranging from 0.1% to 15%, Attachment 13 Table 5). In internal datasets, *S. dextrinosolvens* ASCUSBF53 was detected in nearly all healthy beef cattle rumen microbiome datasets with an average abundance of 8.84% (ranging from 1.3%-19.99%) (Attachment 13). This evidence suggests that *S. dextrinosolvens* is a common and prevalent member of the rumen microbiome of beef cattle.

Isolation and Ecology

As presented in Section 2.1.1-2.1.3 of the main text of the dossier, *S. dextrinosolvens* ASCUSBF53 was isolated from the rumen content of a healthy steer obtained via orogastric tubing. *S. dextrinosolvens* 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. The species is widely understood to be a non-pathogenic commensal organism in published literature. As such, The American Type Culture Collection (ATCC) lists *S. dextrinosolvens* 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 *S. dextrinosolvens* as TRBA Risk Group 1, indicating that the organism is unlikely to cause disease. The source of isolation (a healthy steer) together with the species classification by experts in the field (BSL-1) suggests that *S. dextrinosolvens* 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 *S. dextrinosolvens* ASCUSBF53 most closely matched 16S rRNA sequences from other *S. dextrinosolvens* strains. The 16S rRNA alignment between *S. dextrinosolvens* ASCUSBF53 and other *S. dextrinosolvens* 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 *S. dextrinosolvens* ASCUSBF53. Matches between *S. dextrinosolvens* ASCUSBF53 and other strains of *S.*

dextrinosolvens provided whole genome alignment values above the 95% sequence identity threshold used to define a species using ANI. The assembly providing the best alignment values by ANI to *S. dextrinosolvens* ASCUSBF53 is the type strain of the species, DSM 3072. Together, the 16S rRNA and ANI analyses confirm that *S. dextrinosolvens* ASCUSBF53 has been identified correctly.

In Silico Safety Assessment

The genome assembly for *S. dextrinosolvens* ASCUSBF53 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 chromosome with a smaller secondary chromosome (chromid), and no unincorporated fragments. Comprehensive analysis of the secondary chromosome of *S. dextrinosolvens* ASCUSBF53 was conducted as detailed in Section 2.1.5, and the secondary chromosome was found to be a chromid rather than plasmid. 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 *S. dextrinosolvens* ASCUSBF53 genome sequence, suggesting that it has not acquired any pathogenicity or resistance genes via plasmid transfer from the environment or other microorganisms.

As detailed in Section 2.1.6, *S. dextrinosolvens* ASCUSBF53 was aligned to various databases containing antimicrobial resistance genes. A single gene for tetracycline resistance, tufA, was found to be encoded by the genome. Literature review of antimicrobial resistance in Section 2.1.6 revealed that the feature represents a ubiquitous translational elongation factor. Point mutations in the feature can impart resistance to kirromycin and pulvomycin.

To assess genome encoded toxins, pathogenicity, and virulence factors, *S. dextrinosolvens* ASCUSBF53 was aligned to a collection of databases as detailed in Section 2.1.8. Three features were identified by the database alignment: a post transcriptional regulator, a translational elongation factor, and a flagellar regulator. All of these features are widely distributed in pathogenic and non-pathogenic species. None of the identified features directly impart pathogenicity or virulence.

Thus, based on a thorough screening of the *S. dextrinosolvens* ASCUSBF53 genome using all applicable and relevant databases and the current state of the art, nothing of concern was identified suggesting that *S. dextrinosolvens* ASCUSBF53 is safe for humans and animals.

Safety Based on *In Vitro* Experiments

Phenotypic testing was conducted to evaluate antimicrobial resistance and antimicrobial production by *S. dextrinosolvens* ASCUSBF53 using methods described in Section 2.1.6 and 2.1.7 in the main text of the dossier.

S. dextrinosolvens ASCUSBF53, an anaerobic bacteria, was demonstrated to be resistant to aminoglycosides and macrolides. Resistance to aminoglycosides and macrolides is reflective of *S. dextrinosolvens* ASCUSBF53 being anaerobic rather than any specific resistance mechanism or genotype. *S. dextrinosolvens* ASCUSBF53 is susceptible to chloramphenicol, tetracycline, and ampicillin. *S. dextrinosolvens* ASCUSBF53 was not found to produce any antimicrobial compounds.

Feeding Trial Summary

As presented in Section 6.7 of the main text of the dossier, *S. dextrinosolvens* has been fed to cattle in two studies conducted by Native Microbials. Full descriptions of these studies have been included in Appendix 019 and Appendix 020 of the original dossier submission. Animals in each study were administered *S. dextrinosolvens* ASCUSBF53 in excess of 100 days. General performance and health measurements were recorded throughout the length of both studies. No negative health effects due to the feeding of *S. dextrinosolvens* were reported during either study.

Moreover, as *S. dextrinosolvens* ASCUSBF53 is a commensal organism and is naturally present in cattle rumens, low level cross contamination through animal interactions would have a negligible impact. These feeding studies, although not necessary for GRAS determination, corroborates the safety of feeding *S. dextrinosolvens* ASCUSBF53 as no adverse health impacts were observed.

Overall Summary of Safety

S. dextrinosolvens ASCUSBF53 belongs to species *S. dextrinosolvens* and is a well understood and studied commensal microorganism in the rumen. *S. dextrinosolvens* 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 *S. dextrinosolvens* ASCUSBF53 is susceptible to a wide variety of common antibiotics and does not produce any antimicrobial compounds. Studies that fed *S. dextrinosolvens* ASCUSBF53 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 *S. dextrinosolvens* ASCUSBF53 is generally recognized as safe as a direct fed microbial in dairy cattle at the intended rate of inclusion.

Cerrito, Chelsea

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Wednesday, August 10, 2022 6:00 PM
To: Animalfood-premarket
Cc: Kevin Korth
Subject: [EXTERNAL] RE: Status Update for GRAS Notice AGRN # 45 Succinivibrio dextrinosolvens ASCUSBF53
Attachments: (M-Z)_Section_2_References_Combined_AGRN45.pdf; (A-L)_Section_2 _Amendment_References_Combined_AGRN45.pdf

In response to the July 21, 2022 request for additional information to support the GRAS Notice 45, for the use of Succinivibrio dextrinosolvens ASCUSBF53 in beef cattle feed, we are providing the attached information.

This email has attached the reference material to support the narrative of the amendment of AGRN 45.

Please confirm that you have received this email.

Kristi O. Smedley, Ph.D.

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