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Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
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August 9, 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 *Clostridium beijerinckii* ASCUSBR67-
for Poultry
Notifier: Native Microbials, Inc.
10255 Science Center
Dr., Suite C2
San Diego, California 92121

Dear Dr. Edwards:

On behalf of Native Microbials, Inc., I am providing an Animal General Recognized as Safe Notice for the use of *Clostridium beijerinckii* ASCUSBR67 as a direct fed microorganism for use in Poultry. The submission is compliant with 21CFR 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=ksmedley@cfr services.com, c=US
Date: 2021.08.09 20:43:02 -04'00'

Consultant to Native Microbials, Inc.

RECEIVED
SEP 02 2021
By

cc: Mallory Embree, Native Microbials, Inc.

ATTACHMENTS:

Letter of Smedley Authorization to Represent
GRAS Notice *Clostridium beijerinckii* ASCUSBR67 (Narrative Hard Copy, Full Submission on DVD)

August 9, 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 *Clostridium beijerinckii* ASCUSBR67 for use as a Direct Fed Microbial in Poultry.

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)

Kevin G Korth
Sr Manager, Regulatory Affairs
Native Microbials, Inc.
10255 Science Center Dr, Suite C2
San Diego, CA 92121

GRAS Notice for *Clostridium beijerinckii* ASCUSBR67 for Use as a Direct Fed Microbial in Poultry

August 5, 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 *Clostridium beijerinckii* ASCUSBR67 for Use as a Direct Fed Microbial in Poultry

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APPENDICES

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
BUSCO	Benchmarking Universal Single-Copy Orthologs
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
GIT	Gastrointestinal Tract
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 *Clostridium beijerinckii* ASCUSBR67. The spray dried powder comprises *C. beijerinckii* ASCUSBR67 cell concentrate on a starch carrier. The microbial strain is deposited in the NRRL culture collection as B-67689.

The microbial strain *Clostridium beijerinckii* ASCUSBR67 is often referred to in some appended reports as 'Broiler-67' or 'BR67', which is the internal research name for *Clostridium beijerinckii* ASCUSBR67.

GRAS Notice for *Clostridium beijerinckii* ASCUSBR67 for Use as a Direct Fed Microbial in Poultry

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 *Clostridium beijerinckii* ASCUSBR67.

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 *Clostridium beijerinckii* ASCUSBR67 (microbial strain). The product comprises *C. beijerinckii* ASCUSBR67 cell concentrate on a starch carrier. This product is referred to as '*Clostridium beijerinckii* ASCUSBR67 spray dried powder' or '*Clostridium beijerinckii* ASCUSBR67 SDP'. In addition, a number of the appended reports refer to *Clostridium beijerinckii* ASCUSBR67 or the spray dried powder product under the internal research name, Broiler-67 or BR67.

1.3 Intended Conditions of Use

C. beijerinckii ASCUSBR67 spray dried powder is intended for use as a supplemental source of viable microorganisms in the feed of poultry. The intended purpose of supplementation of the microorganism is to augment the digestion of various carbohydrates of animal feed to volatile fatty acids such as butyrate. Specifically, *C. beijerinckii* ASCUSBR67 is known to utilize simple carbohydrates, such as glucose and xylose, as well as ferment complex carbohydrates like starch. The spray dried powder will be delivered to poultry either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing *C. beijerinckii* ASCUSBR67 spray dried powder may be incorporated into the diet of poultry as part of the mash, as a feed supplement blended into the mash, or as pellets made from the mash. *C. beijerinckii* ASCUSBR67 spray dried powder will be incorporated into feed at a recommended level of $1\text{x}10^4$ CFU/bird/day.

1.4 Statutory Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §570.30(a) and (b), *C. beijerinckii* ASCUSBR67 spray dried powder manufactured by Native Microbials, has been concluded to have GRAS status for use as a direct fed microbial in poultry, 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 *C. beijerinckii* ASCUSBR67 spray dried powder 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 Appendix 10 and 15, which is 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 *Clostridium beijerinckii* ASCUSBR67.

Signed,

(b)(6)

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, *C. beijerinckii* ASCUSBR67, is provided in Table 2.1. *C. beijerinckii* occurs extensively in nature. *C. beijerinckii* ASCUSBR67 was isolated from the small intestine content of a healthy broiler chicken. *C. beijerinckii* is ubiquitous in nature and routinely isolated from soil samples as well as feces and gastrointestinal contents of livestock, including cattle and chickens (Sankar *et al.* 2003; Pan *et al.* 2008; Hussain and El Sanousi, 2011; Brändle *et al.* 2016; Driehuis *et al.* 2016; Barrios *et al.* 2013; Crippen *et al.* 2008; Barrios *et al.* 2013). Vanbelle *et al.* (1990) reported *C. beijerinckii* as one of the common bacteria in the digestive tract of chickens. Proietti *et al.* (2006) also noted that *C. beijerinckii* was the predominant Clostridial isolate from the ileum of 40 and 80 day old broilers and one of the predominant Clostridial isolates in the cecum of 80 day old broilers. In addition, it was one of the major populations of butyric acid bacteria isolated in 296 samples of soil, maize and grass silage, dairy cow feces and farm tank milk collected from dairy farms in the Netherlands (Driehuis *et al.* 2016). *C. beijerinckii* is an industrially relevant species for the production of butanol from lignocellulosic substrates (Guo *et al.* 2013; Qureshi *et al.* 2008; Zhang and Jia 2018). To a lesser extent, the species has also been studied for the ability to produce hydrogen, isopropanol, ethanol, and acetone (Driehuis *et al.* 2016; Máté de Gérando *et al.* 2018; Fonseca *et al.* 2019; Bellido *et al.* 2015).

The first *C. beijerinckii* strains isolated and developed for large scale production of these solvents were identified as *Clostridium acetobutylicum* but were later assigned to four species, *C. acetobutylicum, C. beijerinckii, Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum* (Johnson *et al.* 1997; Keis *et al.* 1995; Wilkinson and Young, 1995; Keis *et al.* 2001). Similarly, strains historically assigned to the species *Clostridium butylicum* have been subsequently shown to be members of the species *C. beijerinckii* (George and Chen, 1983).

Table 2.1: Taxonomic Classification of *C. beijerinckii*

Kingdom	Bacteria
Phylum	Firmicutes
Class	Clostridia
Order	Clostridiales
Family	*Clostridiaceae*
Genus	*Clostridium*
Species	*beijerinckii*

2.1.2 Source of the Microorganism

C. beijerinckii ASCUSBR67 was identified and isolated to axenicity from a small-intestine content of a healthy broiler chicken. The strain was isolated by Native Microbials (Native Microbials, 10255 Science Center Dr, Suite C2, San Diego, CA 92121). The isolate was deposited in the NRRL, Agricultural Research Service Culture Collection, and referenced as NRRL B-67689. A copy of the Certificate of Deposition is provided in Appendix 001.

2.1.3 Description of the Microorganism

C. beijerinckii ASCUSBR67 is an anaerobic, catalase and oxidase negative bacterium that readily sporulates. It is gram-positive and forms short chains of small rods when cultured in liquid medium. Images of *C. beijerinckii* ASCUSBR67 in vegetative form and gram stain are shown in Figures 2.1 and 2.2, respectively.

Figure 2.1: ***C. beijerinckii* ASCUSBR67 Colonies on YPD Agar (Magnification 100x)**

Figure 2.2: ***C. beijerinckii* ASCUSBR67 Gram Stain (Magnification 40x)**

In vitro assays demonstrate that *C. beijerinckii* ASCUSBR67 grows on a variety of soluble and insoluble carbon sources and the results are summarized in Table 2.2. The strain was found to utilize various carbon sources, particularly monosaccharides such as mannose, arabinose, galactose, glucose, fructose and xylose. The strain also demonstrated the ability to ferment a collection of disaccharides, glucosides, and starch. The study report is provided in Appendix 002. Similar phenotypes are reported for other *C. beijerinckii* strains in the published literature (Keis *et al.* 2001).

Table 2.2: Growth of *C. beijerinckii* ASCUSBR67 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	No Growth
D-Arabinose	No Growth	Methyl-aD-Mannopyranoside	No Growth
L-Arabinose	Growth	Methyl-aD-Glucopyranoside	Growth
D-Ribose	No Growth	N-AcetylGlucosamine	Growth
D-Xylose	Growth	Amygdalin	Growth
L-Xylose	No Growth	Arbutin	Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	Growth
Methyl-BD-xylopyranoside	Growth	Salicin	Growth
D-Galactose	Growth	D-Cellobiose	Growth
D-Glucose	Growth	D-Maltose	Growth
D-Fructose	Growth	D-Lactose	Growth
D-Mannose	Growth	D-Melibiose	No Growth
L-Sorbose	No Growth	D-Saccharose	Growth
L-Rhamnose	No Growth	D-Trehalose	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	Growth	D-Arabitol	No Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	Growth	Potassium Gluconate	No Growth
D-Turanose	Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth	Potassium 5-KetoGluconate	No Growth

Metabolite production of *C. beijerinckii* ASCUSBR67 was measured at 60 hours elapsed fermentation time using an (b) (4) with refractive index (RI) detector. The results are summarized in Table 2.3 and Appendix 002. Major fermentation products include acetate, butyrate, and 1-butanol when grown on complex media with glucose.

Table 2.3: Metabolite Production of *C. beijerinckii* ASCUSBR67 on Complex Media with Glucose

Metabolite	**Production (g/L)**
Pyruvic acid	0.0
Succinic acid	0.07
Lactic acid	0.0
Glycerol	0.09
Acetic acid	2.77
Propionic acid	0.0
Butyric acid	3.32
Ethanol	0.16
1-Butanol	1.83

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 *C. beijerinckii* ASCUSBR67 was most closely related to *Clostridium* *beijerinckii* (99.7%), *Clostridium diolis* (99.7%), *Clostridium acetobutylicum* (99.7%), and *Clostridium butyricum* (98.7%). Although the closest match is split between the top three species, all four species fall within the minimum 98.7% sequence identity threshold typically used to define a species (Yarza *et al.* 2014). 16S rRNA sequence analysis is unable to discriminate between these closely related species on the basis that the interspecies percent similarity ranges are too high to apply the 97%, 98.7% or 99% limits classically proposed for species delineation within a genus (Bouvet *et al.* 2014). Thus, further genetic analyses including whole genome Average Nucleotide Identity (ANI) and sequence analysis of additional housekeeping genes were conducted to obtain species-level taxonomic resolution.

2.1.4.2 *Whole Genome Sequence Assembly and Annotation*

Genomic DNA was isolated from a pure culture of *C. beijerinckii* ASCUSBR67 and sequencing libraries were prepared using the (b) (4) kit (b) (4)). The resulting libraries were sequenced (1x300bp) on an (b) (4) and in parallel, long-read libraries were prepared from the same extracted DNA using (b) (4) kit (b) (4)) following the protocol outlined by Jain *et al.* (2018b) and 1D sequenced on the (b) (4) (b) (4); (b) (4). The genome was assembled through hybrid methods utilizing both short and long reads. Read quality and genome coverage was evaluated using (b) (4) data and (b) (4) for the (b) (4) data. The complete genome sequence was assembled into one chromosome with a length of 5,966,546 bp and a GC content of 29.9%. 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 GLIMMER2 (Delcher *et al.* 1999) and through an iterative process of annotating putative genes using the FIGfams database (Meyer *et al.* 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).

C. beijerinckii ASCUSBR67 contains 7,271 coding sequences which were subsequently built into a metabolic reconstruction describing 307 functional subsystems using PATRIC and the RASTtk pipeline (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. Of the identified coding regions, 5,361 have functional assignments. Antimicrobial resistance and virulence genes identified by metabolic reconstruction are further discussed in Sections 2.1.6 and 2.1.8. The assembled genome has been deposited at NCBI under accession number CP070895.

Table 2.4: Assembly Statistics for *C. beijerinckii* ASCUSBR67	
# of Contigs	1
# of Contigs ≥ 5,000 bp	1
Longest Contig (bp)	5,966,546
Assembly Length	5,966,546
N50	5,966,546
N75	5,966,546
GC%	29.9

2.1.4.3 *Whole Genome Sequence Comparison*

To determine relatedness of *C. beijerinckii* ASCUSBR67 to other closely related species at a higher resolution, whole genomes were compared using ANI. All genomes used for the comparison were closed with no gaps, with the exception of *Clostridium diolis* for which no closed genome was available. MUMmer was used to generate the alignments for ANI on the basis that this software is adept at aligning highly similar sequences and is more stringent than most other aligners such as BLAST (Kurtz *et al.* 2004). The results indicated that *C. beijerinckii* (ANI 98%, coverage 88.6%) was the most closely related species (Table 2.5). *C. beijerinckii* ASCUSBR67 returned results above the 95% ANI species boundary established by Jain *et al.* (2018a) for both *C. beijerinckii* and *C. diolis*. Recent comparisons of *C. beijerinckii* and *C. diolis* at the genome level have revealed that these organisms are likely the same species (Kobayashi *et al.* 2020). It has been suggested that *C. diolis* be reclassified as *C. beijerinckii.* Thus, based on whole genome ANI, *C. beijerinckii* ASCUSBR67 has been unambiguously identified as *C. beijerinckii.*Full details of the analysis are provided in Appendix 003A and C.

Table 2.5: Average Nucleotide Identity (ANI) of Related *Clostridium* Species to *C. beijerinckii* ASCUSBR67

Genus species (assembly)	ANI (%)	Coverage (%)
Clostridium beijerinckii ATCC 35702 (GCA_000767745)	98.2	88.6
Clostridium diolis WST (GCA_003015255)	97.6	83.5
Clostridium diolis NJP7 (GCA_002176895)	97.5	85.4
Clostridium beijerinckii NRRL B-598 (GCA_000506785)	97.5	81.4
Clostridium butyricum TOA (GCA_001646605)	84.9	8.9
Clostridium butyricum KNUL09 (GCA_001456065)	84.8	8.8
Clostridium acetobutylicum JB200 (GCA_002216285)	84.6	1.6
Clostridium acetobutylicum ATCC 824 (GCA_000008765)	84.3	1.4

2.1.4.4 *Housekeeping Gene Comparison*

The comparison of housekeeping genes has been used successfully to distinguish *Clostridium* species (Udaondo *et al.* 2017). To further elucidate the taxonomy of the *C. beijerinckii* ASCUSBR67, DNA sequence comparison of the four housekeeping genes, ribonuclease P RNA (rnpB), ATP synthase alpha subunit (atpA), RNA polymerase alpha subunit (rpoA) and phenylalanyl-tRNA synthetase (pheS) was conducted. Gene sequences from *C. beijerinckii, C. diolis,* and *C. butyricum* were compared to the same gene sequences from *C. beijerinckii* ASCUSBR67. The closest match to *C. beijerinckii* ASCUSBR67 for all four housekeeping genes was to *C. beijerinckii* at 99.8% for atpA, 98.6% for pheS, 100% for rnpB, and

100% for rpoA. *C. diolis* provided close matches as well but not as close as *C. beijerinckii*. Full details of the analysis are provided in Appendix 003D.

2.1.4.5 Summary and Conclusions

16S rRNA analysis, whole genome sequence ANI analysis, and housekeeping gene comparisons have unambiguously confirmed the taxonomic identity of this commensal microorganism to be *C. beijerinckii*.

2.1.5 Plasmid Analysis

The assembly was contained in 1 contig with no unincorporated fragments. The existence of a singular chromosome eliminates the possibility of any plasmids or other extrachromosomal elements.

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

Phenotypic testing was conducted on *C. beijerinckii* ASCUSBR67 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials of relevance to human and veterinary medicine. The full study report is provided in Appendix 004. The results were evaluated against the microbiological cut-off values reported by the European Food Safety Authority for "other gram positive bacteria" (EFSA, 2018), as well as the resistant breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram positive anaerobes" and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). The MIC values reported for *C. beijerinckii* ASCUSBR67 were equal, or lower than, the cut-off values and break-points established by EFSA, for all antimicrobials except chloramphenicol and streptomycin. The MIC values reported for *C. beijerinckii* ASCUSBR67 were equal, or lower than, the cut-off values and break-points established by EUCAST and/or CLSI breakpoints for all antimicrobials other than streptomycin. Results can be found in Table 2.6.

MIC testing revealed values above one or more breakpoints for two antimicrobial compounds: chloramphenicol and streptomycin. For chloramphenicol, the MIC of 8 µg/mL exhibited by *C. beijerinckii* ASCUSBR67 is higher than the EFSA microbiological cut-off value (4 µg/mL) but equal to the EUCAST resistant breakpoint value (>8 µg/mL) and lower than the CLSI resistant breakpoint value (32 µg/mL). Resistance to aminoglycosides (gentamicin and streptomycin) is recognized to be intrinsic in anaerobic bacteria because the mechanism of uptake requires respiration (Bryan *et al.* 1979). Consistent with this expectation, the MIC of 16 µg/mL for streptomycin exhibited by *C. beijerinckii* ASCUSBR67 is not considered a concern.

Table 2.6: *C. beijerinckii* ASCUSBR67 Antimicrobial Susceptibility in Relation to EFSA, EUCAST, and CLSI Breakpoints

Antimicrobial	Tested Range (ug/mL)	MIC (ug/mL) of *C. beijerinckii* ASCUSBR67	2018 EFSA Microbiological Cut-off Values (ug/mL) for Other Gram +	EUCAST Resistant Breakpoints (ug/mL) Gram + Anaerobes	CLSI Resistant Breakpoints (ug/mL) Anaerobes
Ampicillin	0.5-64	≤ 0.5	1	8	≥2
Chloramphenicol	0.5-64	8	4	>8	≥32
Clindamycin	0.12-32	2	4	4	≥8
Erythromycin	0.12-16	1	1	Not Available	Not Available
Gentamicin	0.12-32	4	4	Not Available	Not Available
Kanamycin	0.5-64	4	16	Not Available	Not Available
Streptomycin	0.5-64	16	8	Not Available	Not Available
Tetracycline	0.06-64	2	2	Not Available	≥16
Vancomycin	0.12-16	2	4	2	Not Available

To evaluate the presence of antimicrobial resistance genes in the *C. beijerinckii* ASCUSBR67 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, 773 sequences from Clostridia, 2 of which are from *C. beijerinckii*. Characteristics of each database can be found in Table 2.7.

Table 2.7: Characteristics of Databases Used to Assess Antimicrobial Resistance

Database Name	Number of Entries	Number of Clostridial Entries	*C. beijerinckii* Entries	Contains Redundant Entries
CARD (PATRIC)	17,559 (2,227 non redundant proteins	463 (14 non redundant proteins)	1, Chloramphenicol O-acetyltransferase (EC 2.3.1.28) => CatA15/A16 family	Yes
NDARO (PATRIC)	5,138 (4,004 non redundant proteins)	141 (20 non redundant proteins)	1, Chloramphenicol O-acetyltransferase (EC 2.3.1.28) => CatA15/A16 family	Yes
ResFinder	3,105	38	No	No
AMRFinder Plus	6,946	28	No	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 virulence factors and toxins at the whole genome level, the 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 (Liang *et al.* 2020; Hu *et al.* 2013; Abril *et al.* 2020; Deng *et al.* 2021; Rojas-Estevez *et al.* 2020; 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 and antimicrobial resistance genes when detecting protein homology at the whole genome level. Therefore, for antimicrobial resistance and the following virulence gene alignments in section 2.1.7 it was prudent to use a 80% identity and 70% query coverage cutoff. Results for *C. beijerinckii* ASCUSBR67 can be found in Tables 2.8 to 2.10. Genetic analysis of *C. beijerinckii* ASCUSBR67 identified four potential resistance genes: tetA(P), tetB(P), catB, and RphC.

Two tetracycline resistance genes were of the identified proteins. One of the proteins identified exhibits 86% sequence similarity to tetA(P) in the NDARO database, 86.4% similarity to the same gene in the NCBI AMR database, and 86.8% similarity to the same gene in ResFinder. The other gene identified exhibits 85% sequence similarity to tetB(P) in the NDARO database, 85.43% similarity to the same protein in the NCBI AMR database, and 87.4% similarity to the same gene in ResFinder. These two genes fall under the same operon in other Clostridia, with tetA acting as an efflux pump and tetB conferring ribosomal protection (Sloan *et al.* 1994). These genes are chromosomally located (see Part 2.1.5) indicative of natural rather than acquired resistance. Additionally, tetracycline resistance was observed to be relatively common among 68 livestock derived *Clostridium* strains analyzed by Dutta *et al.* (1983) with 17/68 (25%) strains displaying MIC values above the EFSA microbiological cut-off value.

A chloramphenicol acetyltransferase (catB) protein homologue in the *C. beijerinckii* ASCUSBR67 genome that was 97%, 97.3%, and 97.6% identical to genes found in the NDARO, NCBI AMR, and ResFinder databases respectively. Sebald (1994) reviewed the resistance of gram positive anaerobes in *Clostridium* species and noted that chloramphenicol resistance in two strains of *C. butyricum* (ATCC 19398 and NCIB 7423) was due to chromosomally located genes for chloramphenicol acetyltransferase. Considering that *C. beijerinckii* and *C. butyricum* are closely related and no plasmids were identified in *C. beijerinckii* ASCUSBR67 (see Part 2.1.5), potential chloramphenicol resistance is likely to be the result of the chromosomally located chloramphenicol acetyltransferase gene identified by genetic analysis and chance of horizontal transfer should be extremely low.

The final gene identified is a rifamycin-inactivating phosphotransferase (RphC) protein homologue. The identified protein in the *C. beijerinckii* ASCUSBR67 exhibited an 80.6% similarity to the entry in NCBI AMR database. No match to the protein was identified by PATRIC or ResFinder. Rph genes encode for phosphotransferases that in some cases has been known to target, phosphorylate, and in turn inactivate rifamycin (Stogios *et al.* 2016; Spanogiannopoulos *et al.* 2014; Pawlowski *et al.* 2018). Rph genes are widely distributed in environmental microbes, pathogens, and non-pathogens alike (Spanogiannopoulos *et al.* 2014). The exact role of the protein encoded by RphC is still unclear. RphC and other Rph genes do not confer rifamycin resistance in all organisms possessing the genes and the protein is believed to have alternate function in metabolism and metabolite biosynthesis (Pawlowski *et al.* 2018; Spanogiannopoulos *et al.* 2014).

Table 2.8: *C. beijerinckii* ASCUSBR67 Antimicrobial Resistance by PATRIC

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	identity	E-Value
NDARO	*Clostridium perfringens*	tetB(P)	tetracycline resistance, ribosomal protection protein => TetB(P)	tetracycline resistance, ribosomal protection protein TetB(P); antibiotic target protection protein, tetracycline resistance gene	40	100	85	1e-132
NDARO	*Clostridium butyricum*	catB	Chloramphenicol O-acetyltransferase (E.C. 2.3.1.28) => CatA15/A16 family	MULTISPECIES: class A beta-lactamase TEM-156; antibiotic inactivation enzyme, chloramphenicol resistance gene	100	100	97	1e-128
NDARO	Firmicutes	tetA(P)	tetracycline resistance,MFS efflux pump => TetA(P)	efflux pump conferring antibiotic resistance	100	100	86	1e-207

Table 2.9: *C. beijerinckii* ASCUSBR67 Antimicrobial Resistance by ResFinder				
Gene	**Identity**	**Query Coverage**	**Function**	**Accession number**
catB	97.6	660/660 (100%)	phenicol resistance	M93113
tetA	86.8	1264/1263 (99.9%)	tetracycline resistance	AB001076
tetB	87.4	1960/1959 (99.8%)	tetracycline resistance	NC_010937

Table 2.10: *C. beijerinckii* ASCUSBR67 Antimicrobial Resistance by NCBI AMR BLASTp				
Gene	**e-value**	**Percent Identity**	**Query Coverage**	**Subject Coverage**
type A-15 chloramphenicol acetyltransferase	2.00E-158	97.26	100	100
tetracycline efflux MFS transporter TetA(P)	0.0	86.43	100	100
tetracycline resistance ribosomal protection protein TetB(P)	5.00E-59	93.1	99	15
rifamycin-inactivating phosphotransferase RphC	0.0	80.7	100	39

For comparative purposes, and to gauge the global pool of antimicrobial genes in *C. beijerinckii* the whole genome sequence for *C. beijerinckii* (accession #GCA_000833105) was downloaded from NCBI Genbank and analyzed for antimicrobial resistance by the same methods as *C. beijerinckii* ASCUSBR67.

Comparative analysis of *C. beijerinckii* (GCA_000833105) revealed no antimicrobial resistance genes.

2.1.6.1 Section Summary

MIC testing for *C. beijerinckii* ASCUSBR67 reported values below or at established EFSA, EUCAST and/or CLSI cutoffs for all antibiotics tested except for chloramphenicol and streptomycin. Genomic analysis indicates that *C. beijerinckii* ASCUSBR67 is potentially resistant to chloramphenicol, tetracycline, and rifamycin. However, only chloramphenicol resistance could be confirmed through *in vitro* testing. Together, these analyses suggest that should *C. beijerinckii* ASCUSBR67 cause an opportunistic infection in a human or animal, it can be readily treated using standard antibiotics.

2.1.7 Antimicrobial Production

C. beijerinckii ASCUSBR67 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 Pathogenicity and Virulence

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 virulence factor 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. Both the VFDB and Victors databases were downloaded and queried independently of PATRIC to ensure features in these databases that were excluded by PATRIC were represented. PathogenFInder and (b) (4) web servers (Cosentino *et al.* 2013; Bertelli *et al.* 2017) as well as BLASTp alignment to the (b) (4) (Urban *et al.* 2015) were also utilized to assess the pathogenicity and virulence of *C. beijerinckii* ASCUSBR67. The total number of sequences in the PATRIC and (b) (4) databases is 134,396 and includes 376 Clostridial sequences. Independently, Victors and VFDB contain 33,947 sequences including 284 Clostridial sequences, 4 of which originate in *C. beijerinckii.* (b) (4) contains 4,065 pathogenicity islands including 47 from Clostridia, and 3 pathogenicity islands from *C. beijerinckii.* The analysis in (b) (4) is database independent and uses a model trained with protein sequences from 886 whole genome sequences. Twenty-six of these whole genome sequences are from pathogenic *Clostridium*. The (b) (4) 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 (b) (4) 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.

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

Database Name	Number of Entries	Number of Clostridial Entries	*C. beijerinckii* Entries	Contains Redundant Protein ID entries
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	218 (23 non-redundant proteins)	No	Yes
Victors	4,965	23	No	No
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	124 (28 non redundant proteins)	No	Yes
VFDB	28,982	251	Yes, 4 proteins	No
PATRIC_VF	38,791(1,570 non-redundant proteins)	2 (0 non-redundant proteins)	No	Yes
(b) (4)	6,780	32	No	No
(b) (4)	4,065 pathogenicity islands	47 pathogenicity islands	Yes, 3 genomic islands	No
(b) (4)	N/A	N/A	N/A	N/A

The alignment process compares all identified *C. beijerinckii* ASCUSBR67 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 pathogenicity or virulence were identified by (b) (4), or by the BLAST search to the (b) (4) database. (b) (4), was utilized to identify potential pathogenicity islands in the *C. beijerinckii* ASCUSBR67 genome. Any genomic island that contained any antimicrobial resistance or virulence genes was considered a potential pathogenicity island. (b) (4) identified 24 genomic islands in the *C. beijerinckii* ASCUSBR67 genome none of which contained any antimicrobial resistance of virulence genes. Additionally, PathogenFinder deemed *C. beijerinckii* ASCUSBR67 to "not be predicted as a human pathogen". Results for these analyses can be found in Table 2.12 through Table 2.16.

Two potentially pathogenic genes were identified by PATRIC. Both genes were identified as being essential for lung infection by pathogenic *Streptococcus pneumoniae* in mutagenesis study by Hava and Camilli (2002). In this study pathogenic *Streptococcus pneumoniae* was randomly mutated and the mutant strains were assayed for their ability to cause lung infection. 337 genes were determined to be essential for *Streptococcus pneumoniae* to cause infection. Many of the genes identified by this study, while essential to cause lung infection in pathogenic species, are not sufficient to impart pathogenicity or virulence by themselves. The 337 genes identified included genes for essential amino acid biosynthesis, genes essential for nucleotide biosynthesis, genes involved in critical cellular processes, and key metabolic genes.

The first gene identified is an idnO homologue which encodes for 2-dehydro-3-deoxy-D-gluconate-5-dehydrogenase. This enzyme is predominantly known for its role in polygalacturonic acid metabolism and pectin degradation (Preiss and Ashwell 1963; Condemine, Hugouvieux-Cotte-Pattat, and Robert-Baudouy 1986). Though the exact functional role of protein in question is unknown in regards to infection, it is not uncommon for oxidoreductases to play an essential role in maintaining virulence of pathogens (Fuller *et al.* 2011; Reardon-Robinson *et al.* 2015; McMahon *et al.* 2018; Yu *et al.* 2020; Spahich *et al.* 2016). It should be noted that in these cases the oxidoreductases, while essential to maintain virulence, also catalyze redox reactions that are essential for cell function.

The second gene identified is a smmF homologue which encodes for pyruvate formate lyase (also known as formate C-acetyltransferase). This enzyme plays a critical role in anaerobic glucose metabolism and catalyzes the conversion pyruvate to formate and acetyl-CoA (mixed acid fermentation) (Thauer *et al.* 1972; Knappe and Sawers 1990; Hasona *et al.* 2004; Crain and Broderick 2014). In addition to the work done by Hava and Camilli (2002), Yesilkaya *et al.* (2009) proposed that this protein aids in the progression of pneumonia as the infection moves from oxygen rich lung mucosal surfaces to deeper tissue (Yesilkaya *et al.* 2009). While pyruvate formate lyase might help pathogens adapt to environmental changes, it is also a critical enzyme for non-pathogenic facultative anaerobes and central metabolism, and has been found to be constitutively active in non-pathogenic *E. coli* (Crain and Broderick 2014; Knappe and Sawers 1990).

Four hits were identified to the VFDB database. Of the four, two encode for hemolysins and will be discussed in Section 2.1.8.2. The remaining two hits, GroEL and a fibronectin binding protein similar to fbpA, correspond to genes that have been associated with adhesion.

GroEL is a heat shock protein that is upregulated in response to stress and can aid in cell-cell adherence (C. Hennequin *et al.* 2001; C. Hennequin, Collignon, and Karjalainen 2001; Jain *et al.* 2011). GroEL has been demonstrated to aid in colonization of pathogenic species of *Clostridium* such as *C. difficile* (Péchiné *et al.* 2013; Janoir 2016). While this protein contributes to the pathogenicity of some clostridium species, other protein toxins not encoded by the *C. beijerinckii* ASCUSBR67 genome are necessary to achieve a pathogenic phenotype (Péchiné *et al.* 2013; Janoir 2016). The protein is commonly found in non-pathogenic industrial *Clostridium* such as *C. diolis* and *C. acetobutylicum* (Narberhaus and Bahl 1992; Yu and Liu 2014).

Similar to GroEL, fibronectin binding proteins can aid in cell-cell adhesion. Fibronectin binding proteins have been demonstrated to aid in adherence of of pathogenic species of *Clostridium* such as *C. difficile* and *C. perfringens* (Hennequin *et al.* 2003; Barketi-Klai *et al.* 2011; Katayama *et al.* 2009; Denève *et al.* 2009). Though fibronectin binding proteins are found in pathogenic species they are found in a wide range of host associated commensals, probiotic strains, and non-pathogenic *Clostridium* (Henderson *et al.* 2011; Hymes and Klaenhammer 2016; Muñoz-Provencio, Pérez-Martínez, and Monedero 2010). Adherence and fibronectin binding proteins are involved in pathogenicity, but do not directly impart pathogenicity alone (Janoir 2016).

One hit was identified in the Victors database, gluconate-5-dehydrogenase. This gene is the same feature, 2-dehydro-3-deoxy-D-gluconate-5-dehydrogenase, identified by Victors in PATRIC.

Table 2.12: **Significant Alignments Between the Victors Virulence Database and *C. beijerinckii* ASCUSBR67**

Organism	Protein Hits to Victors	Protein Hits to VFDB	Protein Hits to PATRIC	Protein Hits to (b) (4)	Pathogenicity Island Hits in (b) (4)	Hits to Proteins from Pathogens in (b) (4)
C. beijerinckii ASCUSBR67	1	4	2	0	0	0

Table 2.13: **(b) (4) Results *C. beijerinckii* ASCUSBR67 Virulence/Pathogenicity by (b) (4)**

Gene_Matches	Proteins from Pathogens Matched	Proteins from Non-Pathogens Matched	Predicted as Human Pathogen?
144	0	144	No

Table 2.14: ***C. beijerinckii* ASCUSBR67 Hits to Pathogenic Genes in PATRIC**

Source	Source Organism	Gene	Product	Function	Subject Coverage	Query Coverage	identity	E-Value
Victors	*Streptococcus pneumoniae TIGR4*	idnO (SP_0320)	2-dehydro-3-deoxy-D-gluconate -5dehydrogenase (EC 1.1.1.127)	oxioreductase	43	99	91	3e-58
Victors	*Streptococcus pneumoniae TIGR4*	smmF (SP_0251)	Pyruvate formate-lyase (EC 2.3.1.54)	metabolism/ fermentation	10	95	80	4e-33

Table 2.15: ***C. beijerinckii* ASCUSBR67 Hits to Pathogenic Genes in VFDB**

ASCUSBR67 protein ID	VFDB ID	VFDB Product	Source Organism	Subject Coverage	Query Coverage	identity	E-Value
peg.5990	VFG012102	chaperonin GroEL	*C. beijerinckii* NCIMB 8052	100	100	100	0
peg.6429	VFG012173	hemolysin III	*C. beijerinckii* NCIMB 8052	100	100	99.5	5.00E-152
peg.4304	VFG012112	Fibronectin-binding A domain protein	*C. beijerinckii* NCIMB 8052	100	100	99.5	0
peg.4066	VFG012183	hemolysin A	*C. beijerinckii* NCIMB 8052	49.8	100	99.3	1.00E-92

Table 2.16: ***C. beijerinckii* ASCUSBR67 Hits to Pathogenic Genes in Victors**

ASCUSBR67 protein ID	Victors ID	Victors Product	Source Organism	Subject Coverage	Query Coverage	identity	E-Value
peg.1700	gi|15900252|ref|NP_344856.1|	gluconate 5-dehydrogenase	Streptococcus pneumoniae TIGR4	44.3	100	90.8	1.00E-77

For comparative purposes, and to better gauge the global pool of virulence factors in *C. beijerinckii* and related species, amino acid sequences for published genomes of *C. beijerinckii* (GCA_000833105) (Little *et al.* 2016) (NBRC Whole Genome Sequencing Project) were downloaded from NCBI Genbank and analyzed using the same method as *C. beijerinckii* ASCUSBR67.

Comparative analysis of *C. beijerinckii* (GCA_000833105) revealed no proteins involved in virulence or pathogenicity by PATRIC, [redacted] (b) (4) (Table 2.17 and 2.18). The same four hits that were identified in *C. beijerinckii* ASCUSBR67 from the VFDB were observed in *C. beijerinckii* (GCA_000833105). Similarly, one protein from the Victors database was identified in *C. beijerinckii* (GCA_000833105). This protein encodes for the same gluconate-5-dehydrogenase found in *C. beijerinckii* ASCUSBR67.

Table 2.17: Number of Alignments Between Pathogenicity and Virulence Databases and *C. beijerinckii* (GCA_000833105)

Organism	Victors	VFDB	PATRIC	[redacted]	[redacted]	(b) (4)
C. beijerinckii (GCA_000833105)	1	4	0	0	0	0

Table 2.18: PathogenFinder Results *C. beijerinckii* (GCA_000833105)

Gene_Matches	Proteins from Pathogens Matched	Proteins from Non-Pathogens Matched	The organisms is predicted as human pathogenic
49	0	49	No

2.1.8.1 Toxigenicity

As stated in Part 2.1.6, there are no widely accepted cutoffs for detecting virulence factors and toxins at the whole genome level. While a 80% identity and 70% query coverage is prudent for antimicrobial resistance and virulence gene alignments, a more conservative approach has been widely adopted in regards to protein toxins, particularly for known toxins. Typically, published studies have established toxin protein identity cutoffs between 30-50% or e-value cutoffs ranging from 1E-04 to 1E-05 (Wei *et al.* 2016; Surachat *et al.* 2017; Negi *et al.* 2017; Liang *et al.* 2018). 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.

A cutoff of e-value of 1E-04 was applied to alignments between *C. beijerinckii* ASCUSBR67 and all Clostridial toxins from the VFDB (117 proteins) and DBETH (29 proteins). The total number of matches between the databases is 17 features with 3 overlapping matches between the databases, for a total of 14 unique features identified in the *C. beijerinckii* ASCUSBR67 genome. Full results can be found in Tables 2.19 and 2.20. Each putative protein toxin identified by the database search was then subjected to a BLASTp search to the NCBI database as means to compare annotations and assess the distribution of the protein globally. The results from the BLAST search can be found in Table 2.21.

- Two genes matched toxin database entries above 99% identity. These features are entries from *C. beijerinckii* NCIMB 8052 in VFDB for hemolysin A and hemolysin III. Despite the identification of these hemolysins, there is no literature to support hemolytic activity in the species. To further investigate potential hemolytic activity of *C. beijerinckii* ASCUSBR67, an in vitro hemolytic assay was performed. Full details of the assay can be found in Appendix 019. The results of the assay showed no hemolytic activity by *C. beijerinckii* ASCUSBR67. This suggests that while hemolysin A and hemolysin III homologs were identified with a high protein sequence homology to known hemolysins in the *C. beijerinckii* ASCUSBR67 genome, the presence of these proteins did not result in a hemolytic phenotype.

- 11 of the 12 other potential toxins identified more closely matched proteins from non-pathogenic *C. beijerinckii* and *C. diolis* than the toxin sequences in the VFDB and DBETH databases. Upon further examination, with the exception of a HlyC/CorC family transporter and rRNA methylase identified in VFDB and the insulinase family peptidase identified in DBETH, none of proteins share similar annotations to their match in the toxin databases. Additionally, the proteins in question, with the exception of the HlyC/CorC family transporter and the C2 toxin Component I, only cover 19% or less of the toxin sequence.

    1. Published literature detailing the structure and function of the protein toxins in question (TcdA, TcdB, alpha toxin and cytotoxin L) confirmed that the proteins encoded by the *C. beijerinckii* ASCUSBR67 genome have too few residues to comprise a functional toxin (Reinert *et al.* 2005; Pruitt and Lacy 2012; G. A. Green, Schué, and Monteil 1995; Sakurai, Nagahama, and Oda 2004).
    2. The C2 toxin Component I homolog identified in *C. beijerinckii* ASCUSBR67 shares 19% identity with the toxin in pathogenic *Clostridium botulinum.* This protein comprises one subunit of the binary C2 toxin and acts to ADP-ribosylate actin (Schleberger *et al.* 2006). The second component of this toxin is not encoded by the *C. beijerinckii* ASCUSBR67, and therefore would be non functional (Barth *et al.* 2000). Additionally, the protein is low identity (19%) and the best BLAST match is not to a known toxin.
    3. The entA, entB, and entD proteins represent putative enterotoxins which no function of structure has yet been described. Despite a lack of literature on these two toxins the subject coverage of the proteins by the proteins encoded by the *C. beijerinckii* ASCUSBR67 genome is low (<16%) and the proteins are prevalent in non-pathogenic *Clostridium.*
    4. The HlyC/CorC family transporter in question shares 55% identity with a hemolysis protein in pathogenic *C. botulinum.* HlyC/CorC domain (pfam PF03471) proteins play an essential role in magnesium and cobalt transport as well as potentially playing a role in modulating the transport of other ions (Harris, Odzer, and Breaker 2019; Huang *et al.* 2021). The domain is widely distributed across proteins of differing function throughout the phylum *Firmicutes.* The pfam database has 2,145 entries for HlyC/CorC domain proteins in 999 species in the phylum *Firmicutes* with 99 of the species from the genus *Clostridium.* While the protein identified in the *C. beijerinckii* ASCUSBR67 contains this domain there is no evidence to suggest it engages in hemolytic activity. This conclusion is supported by the results of the in vitro hemolysis assay (Appendix 019). More

importantly, the protein is 99.7% identical with a 94% coverage to proteins in non pathogenic *C. beijerinckii.*

5. The rRNA methylase/TlyA methyltransferase homolog identified in *C. beijerinckii* ASCUSBR67 shares 79% identity to the protein in *Clostridium acetobutylicum.* Methylation of rRNA is a ubiquitous bacterial cellular process and in some cases differing patterns of methylation between clades can be used for phylogenetic reconstruction (Khaitovich and Mankin 2000; Green and Noller 1996; Liu and Douthwaite 2002). In some cases strain specific patterns of rRNA methylation has been demonstrated to impart antimicrobial resistance and enhance virulence and pathogenicity (Doi and Arakawa 2007; Sałamaszyńska-Guz *et al.* 2020; Rahman *et al.* 2015; Monshupanee 2013; Lata, Paul, and Chattopadhyay 2014). The rRNA methylase homolog in question more closely matches rRNA methylases from non-pathogenic *Clostridium beijerinckii* than any feature from pathogenic species.
6. The exo-alpha-sialidase (*nanJ*) homolog identified in *C. beijerinckii* ASCUSBR67 shares 40% identity to the protein in pathogenic *Clostridium perfringens.* This protein along with two other sialidases, nanI and nanH, act to cleave sialic acids in pathogenic *C. perfringens* (Newstead *et al.* 2008). While the nan group of sialidases are known as pathogenicity factors in *C. perfringens,* they do not impart virulence or pathogenicity, rather they act to enhance virulence through synergy with other toxins encoded by the species (Flores-Díaz *et al.* 2005). Chiarezza *et al.* (2009) conducted mutagenesis studies to demonstrate that nanJ is not essential for virulence in *C. perfringens.* In the study, nanJ mutants showed limited loss of sialic acid cleavage and that virulence was not attenuated. Furthermore, the protein identified in *C. beijerinckii* ASCUSBR67 had low coverage of the subject sequence at 4% and was found to align highly to proteins in non pathogenic *C. beijerinckii.*
7. The Zn-dependent peptidase homolog, identified in *C. beijerinckii* ASCUSBR67 shares 27% identity to the protein in pathogenic *Clostridium tetani.* Tetanus toxin is known to possess Zn-dependent protease activity in the light chain of the toxin that inhibits neurotransmitter transport and release (Schiavo *et al.* 1992; Inserte *et al.* 1999; Najib *et al.* 1999).While this peptidase motif is present in tetanus toxin it only comprises part of the light chain and is not sufficient for virulence (Behrensdorf-Nicol *et al.* 2008). Additionally, zinc dependent peptidases are common in pathogenic and non pathogenic bacteria alike and the peptidase in question was identified at <99% identity in non-pathogenic *C. beijerinckii* (Andreini *et al.* 2006; Häse and Finkelstein 1993).

- One protein closely matched a hypothetical protein from *C. botulinum* rather than a known toxin sequence.

Table 2.19: ***C. beijerinckii*** **ASCUSBR67 Significant Alignments to VFDB Toxin Sequences**

ASCUSBR67 protein ID	VFDB ID	VFDB Product	Source Organism	Subject Coverage	Query Coverage	identity	E-Value
peg.6429	VFG012173	hemolysin III	Clostridium beijerinckii NCIMB 8052	100	100	99.5	3.00E-154
peg.4066	VFG012183	hemolysin A	Clostridium beijerinckii NCIMB 8052	50	100	99.3	8.00E-95
peg.4065	VFG012182	rRNA methylase	Clostridium acetobutylicum ATCC 824	13	100	79.4	2.00E-15
peg.1748	VFG019155	transporter, HlyC/CorC family	Clostridium botulinum A3	81	89	55.3	5.00E-113
peg.2282	VFG012149	entB, enterotoxin	Clostridium perfringens	16	60	52.8	3.00E-27
peg.6984	VFG002288	Tcd, toxin B	Clostridium difficile 630	3	29	46.2	7.00E-09
peg.7060	VFG002287	TcdA, toxin A	Clostridium difficile 630	3	37	43.8	1.00E-09
peg.1553	VFG012162	nanJ, exo-alpha-sialidase	Clostridium perfringens ATCC 13124	4	51	40.0	2.00E-05
peg.3910	VFG012143	entA, enterotoxin	Clostridium perfringens ATCC 13124	15	83	36.3	1.00E-18
peg.4231	VFG012147	entD, enterotoxin	Clostridium perfringens str. 13	8	100	35.9	6.00E-06

Table 2.20:	***C. beijerinckii* ASCUSBR67 Significant Alignments to DBETH Toxin Sequences**						
ASCUSBR67 protein ID	**DBETH ID**	**DBETH Product**	**Source Organism**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
peg.4969	tr|Q46342|Q46342_CLOSO	cytotoxin L	Clostridium sordellii	1	100	57.69	9.00E-07
peg.7117	tr|Q46034|Q46034_CLODI	toxin B	Clostridium difficile	2	78	48.78	2.00E-05
peg.1830	tr|Q46149|Q46149_CLONO	alpha toxin	Clostridium novyi	2	75	45.95	6.00E-05
peg.3911	sp|P16154|TOXA_CLODI	toxin A	Clostridium difficile	2	96	43.55	1.00E-09
peg.6429	tr|Q897Y4|Q897Y4_CLOTE	Hemolysin III	Clostridium tetani	99	97	36.36	8.00E-37
peg.4799	tr|Q897D0|Q897D0_CLOTE	Zn-dependent peptidase, insulinase family	Clostridium tetani	19	36	27.42	2.00E-06
peg.1498	tr|O69275|O69275_CLOBO	C2 toxin Component I	Clostridium botulinum	48	79	19.71	7.00E-05

Table 2.21: Best BLAST Matches to Potential Toxin Sequences in the *C. beijerinckii* ASCUSBR67 Genome				
ASCUSBR67 protein ID	Organisms providing best match by BLAST	BLAST Annotation	identity %	Query Coverage
peg.4065	Clostridium beijerinckii NCIMB 8052, Clostridium beijerinckii ATCC 35702	TlyA family RNA methyltransferase	94.1	100
peg.1748	Clostridium beijerinckii NCIMB 8052, Clostridium beijerinckii ATCC 35702	HlyC/CorC family transporter	99.7	94
peg.2282	Clostridium beijerinckii ATCC 39058, Clostridium diolis DSM 15410	C40 family peptidase	99.3	100
peg.6984	Clostridium beijerinckii NCIMB 8052, Clostridium beijerinckii ATCC 35702	InlB B-repeat-containing protein	100	100
peg.7060	Clostridium beijerinckii	glycoside hydrolase	99.2	100
peg.1553	Clostridium beijerinckii ATCC 39058	cell wall-binding protein/ YHS domain-containing protein	96.6	100
peg.3910	Clostridium beijerinckii BGS1	cell wall-binding protein	99.4	100
peg.4231	Clostridium beijerinckii ATCC 39058	SH3 domain-containing protein	98.1	100
peg.4969	Clostridium beijerinckii NCIMB 8052, Clostridium beijerinckii ATCC 35702	glucan-binding YG repeat protein/glycoside hydrolase	100	100
peg.1830	Clostridium beijerinckii NRRL B-598, Clostridium diolis NJP7	cell wall-binding protein	99.0	97
peg.4799	Clostridium beijerinckii NCIMB 8052	insulinase family protein	99.8	100
peg.1498	Clostridium botulinum CB-K-34E	hypothetical protein	63.4	96

2.1.8.2 Section Summary

All publicly available pathogen and virulence-related databases were queried to determine the pathogenic potential of *C. beijerinckii* ASCUSBR67. In total, these databases encompass 172,408 known pathogen-related genes spanning all microbial taxonomies, of which 650 originated from *Clostridium* species. Comprehensive alignment of the *C. beijerinckii* ASCUSBR67 genome to these databases yielded eight hits at 80% identity, 70% query coverage. The eight hits encompassed 2-dehydro-3-deoxy-D-gluconate-5-dehydrogenase, GroEL, fibronectin binding proteins, and smmF, which are found in non-pathogens as well as pathogens and do not directly impart pathogenicity. Additionally, two hemolysin homologs were identified, hemolysin III and hemolysin A. To ensure that *C. beijerinckii* ASCUSBR67 did not possess hemolytic activity an in vitro hemolysis assay was performed (Appendix 19). Results of the assay concluded that *C. beijerinckii* ASCUSBR67 was not hemolytic. The analysis also included a search of 4,065 pathogenicity islands, 47 of which originated from *Clostridium* species, and 3 pathogenicity islands from *C. beijerinckii* by the IsandViewer web interface. Additionally, database independent analysis using the PathogenFinder web interface was conducted. IslandViewer did not identify any pathogenicity islands and *C. beijerinckii* ASCUSBR67 was deemed non-pathogenic by PathogenFinder.

To better assess the completeness of the analysis, genomes of publicly available *C. beijerinckii* (GCA_000833105) were analyzed using the same methods to assess completeness of the analysis: 5 non redundant genes aligned for *C. beijerinckii* (GCA_000833105). The same features identified in *C. beijerinckii* ASCUSBR67 were identified in *C. beijerinckii* (GCA_000833105). No genes directly involved in pathogenesis or toxin production were identified in either genome.

Potential toxigenicity of *C. beijerinckii* ASCUSBR67 was explored through alignment of *C. beijerinckii* ASCUSBR67 amino acid sequences against a curated list of Clostridial toxin sequences from the DBETH and VFDB databases. This search included 146 toxin sequences and was completed with a more conservative alignment cutoff using e-value of 1E-04. A total of 14 unique potential toxin sequences aligned above the cutoff to proteins in the *C. beijerinckii* ASCUSBR67 genome. Of these alignments, two were high identity matches (>99%) to hemolysin A and hemolysin III. These hemolysins were the same features identified in the initial database search with the 80% identity and 70% coverage cutoff. In vitro hemolysis assay of the strain revealed the lack of hemolytic activity. Eleven of the 12 other potential toxins identified more closely matched proteins from non-pathogenic *C. beijerinckii* and *C. diolis* than the toxin sequences in the VFDB and DBETH databases. One protein closely matched a hypothetical protein from *C. botulinum* rather than a known toxin sequence. Upon further examination of these features, they represent proteins in non-pathogenic species with different annotated function, or match catalytic domains that are also found in proteins with functions not related to toxigenicity.

2.1.9 Summary of Organism Safety Based on Genomics

C. beijerinckii ASCUSBR67 was unambiguously identified using 16S rRNA analysis and whole genome sequence ANI analysis. *In silico* analysis revealed the presence of four potential antimicrobial genes encoding for tetracycline, chloramphenicol, and rifamycin. *In vitro* testing demonstrated that *C. beijerinckii* ASCUSBR67 is resistant to chloramphenicol and streptomycin but is susceptible to a wide group of readily available antibiotics suggesting that should *C. beijerinckii* ASCUSBR67 cause an opportunistic infection in a human or animal, it can be readily treated. The isolate is susceptible to tetracycline despite the discovery of tetracycline resistance genes. Additionally, phenotypic testing confirmed that no antimicrobials were produced during fermentation. Comparison of the *C. beijerinckii* ASCUSBR67 genome to several databases containing known pathogenic-related genes yielded eight hits. However, these genes catalyze reactions critical for cellular function and metabolism and do not confer pathogenicity by themselves. Two of the identified proteins matched closely to hemolysins, however, an in vitro hemolysis assay confirmed that *C. beijerinckii* ASCUSBR67 is not hemolytic. To better assess the completeness of the analysis, genomes of publicly available *C. beijerinckii* (GCA_000833105) were analyzed using the same methods to assess completeness of the analysis. The same hemolysis genes found in *C. beijerinckii* ASCUSBR67 were found in *C. beijerinckii* (GCA_000833105). No other genes directly involved in pathogenesis or toxin production were identified in either genome Potential toxigenicity of *C. beijerinckii* ASCUSBR67 was explored through alignment of *C. beijerinckii* ASCUSBR67 amino acid sequences against a curated list of Clostridial toxin sequences. This alignment utilized less stringent cutoffs than the pathogenicity and virulence alignment. Alignment to the curated toxins revealed 14 potential homologs in the *C. beijerinckii* ASCUSBR67 genome. Of the 14, two represented the same hemolysins identified in the pathogenicity and virulence search. The remaining 12 proteins that provided significant alignments either more closely match proteins from non-pathogenic *C. beijerinckii* and *C. diolis* than the toxin sequences, or match catalytic domains that are also found in proteins with functions not related to toxigenicity. Based on these analyses, *C. beijerinckii* ASCUSBR67 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 starch encapsulated *C. beijerinckii* ASCUSBR67 are listed in Appendix 009. All raw materials used in the manufacture of *C. beijerinckii* ASCUSBR67 spray dried powder 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 *C. beijerinckii* ASCUSBR67 spray dried powder is provided in Figure 2.3. *C. beijerinckii* ASCUSBR67 is produced through a [redacted] (b) (4) The resultant *C. beijerinckii* ASCUSBR67 spray dried powder is suitable for use as a direct fed microbial in feed. Further details on the manufacturing process are provided in Appendix 010.

Figure 2.3: **Schematic Overview of the Manufacturing Process**

(b) (4)

2.2.3 Production Controls

Commercial manufacture of *C. beijerinckii* ASCUSBR67 spray dried powder 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 Specifications for the Cell Concentrate

An appropriate feed-grade specification has been established for the *C. beijerinckii* ASCUSBR67 cell concentrate and is presented in Table 2.22. The methods of analysis is provided in Appendix 007.

Table 2.22: ***C. beijerinckii* ASCUSBR67 Concentrate Specifications**

Parameter	Specification Limits	Analytical Method
Botulinum toxins	Negative/2 g	FDA BAM

Abbreviations: BAM = Bacteriological Analytical Manual

2.3.2 Batch Analyses for the Cell Concentrate

Three batches of *C. beijerinckii* ASCUSBR67 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 2.23 and the Certificates of Analysis are provided in Appendix 013. Consistent with the findings of the WGS analysis (see Part 2.1), *C. beijerinckii* ASCUSBR67 does not harbor any genes encoding for toxin synthesis, and no botulinum toxins were identified in any of the batches (Appendix 008).

Table 2.23: Analytical Results for 3 Batches of *C. beijerinckii* ASCUSBR67 Concentrate

Parameter	Unit	Specification	Analytical Results		
			Lot 1801.2046	Lot 1801.2047	Lot1801.2048
Botulinum toxins*	Per 2 g	Negative	[redacted]	[redacted]	[redacted] (b) (4)

* Testing done at end of fermentation process after centrifugation

2.3.3 Proposed Product Specifications for the *C. beijerinckii* ASCUSBR67 Spray Dried Powder

As mentioned in Part 2.2, *C. beijerinckii* ASCUSBR67 spore concentrate is stabilized by spray drying with starch (approximately 70% starch by weight) to yield a product suitable for use in feed (the notified substance). Appropriate feed-grade specifications have been established for *C. beijerinckii* ASCUSBR67 spray dried powder and are presented in Table 2.24. Copies of the methods of analysis are provided in Appendices 007 and 012.

Table 2.24: *C. beijerinckii* ASCUSBR67 Spray Dried Powder Product Specifications

Parameter	Specification Limits	Analytical Method
Viable cells count	≥1x10^8 CFU/g	Internal Method
Coliform	<10 CFU/g	BAM-SPC
E. coli	<10 CFU/g	BAM-SPC
Salmonella	Negative/25 g	AOAC 2013.01
Listeria	Negative/25 g	AOAC 2013.10

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

2.3.4 Batch Analyses for *C. beijerinckii* ASCUSBR67 Spray Dried Powder

Three batches of *C. beijerinckii* ASCUSBR67 spray dried powder 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 2.25 and the Certificates of Analysis are provided in Appendix 013.

Table 2.25: Analytical Results for 3 Batches of *C. beijerinckii* ASCUSBR67 Spray Dried Powder

Parameter	Unit	Specification	Analytical Results Lot 20-0202-049-P6	 Lot 20-0202-049-P7	 Lot 20-0202-049-P12A
Viable cells count	CFU/g	≥1x10^8	(b) (4)		
Coliforms	CFU/g	<10			
E. coli	CFU/g	<10			
Salmonella	Per 25 g	Negative			
Listeria	Per 25 g	Negative			

Abbreviations: CFU = colony forming units.

2.3.5 Additional Analytical Data

The levels of heavy metals are also routinely monitored in batches of *C. beijerinckii* ASCUSBR67 spray dried powder. Three batches of *C. beijerinckii* ASCUSBR67 spray dried powder 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 2.26 and the Certificates of Analysis from analytical laboratories are provided in Appendix 14. 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 2.26: Further Analytical Results for 3 Batches of *C. beijerinckii* ASCUSBR67 Spray Dried Powder

Parameter	Unit	Analytical Results Lot 20-0202-049-P6	 Lot 20-0202-049-P7	 Lot 20-0202-049-P12A	Analytical Method
Arsenic	ppm	(b) (4)			AOAC 2015.01
Cadmium	ppm				AOAC 2015.01
Lead	ppm				AOAC 2015.01
Mercury	ppm				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 *C. beijerinckii* ASCUSBR67 spray dried powder to the product specification (see Table 2.22) for a minimum of 12 months when stored in the original, unopened packaging at room temperature (25°C ± 2°C). The proposed shelf life is supported through accelerated stability studies in which 3 batches of *C. beijerinckii* ASCUSBR67 spray dried powder representative of the commercial material were stored at 50°C, 60°C, and 70°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 006.

2.4.1.1 Accelerated Stability Study at 50°C

The results of the stability study conducted at 50°C for 28 days on *C. beijerinckii* ASCUSBR67 are summarized in Table 2.27 and the report is provided in Appendix 015. Over the period evaluated, changes in the viable spore count were observed representing a decay rate plotted in Figure 2.4 for the 3 batches of *C. beijerinckii* ASCUSBR67.

Table 2.27: Results of a Stability Study on 3 Batches of *C. beijerinckii* ASCUSBR67 Stored at 50°C

		Analytical Results					
		Lot 20-0202-049-P6		**Lot 20-0202-049-P7**		**Lot 20-0202-049-P12A**	
Time (Days)	**Unit**	**Viable Cells Count**	**SD**	**Viable Cells Count**	**SD**	**Viable Cells Count**	**SD**
0	**CFU/g**	(b)(4)					
1	**CFU/g**						
2	**CFU/g**						
7	**CFU/g**						
14	**CFU/g**						
21	**CFU/g**						
28	**CFU/g**						

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

2.4.1.2 Accelerated Stability Study at 60°C

The results of the stability study conducted at 50°C for 96 hours on *C. beijerinckii* ASCUSBR67 are summarized in Table 2.28 and the report is provided in Appendix 015. Over the period evaluated, changes in the viable spore count were observed representing a decay rate plotted in Figure 2.4 for the 3 batches of *C. beijerinckii* ASCUSBR67.

Table 2.28: Results of a Stability Study on 3 Batches of *C. beijerinckii* ASCUSBR67 Stored at 60°C

Time (Hours)	Unit	Analytical Results					
		Lot 20-0202-049-P6		Lot 20-0202-049-P7		Lot 20-0202-049-P12A	
		Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	(b)(4) [redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
1	CFU/g	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
2	CFU/g	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
7	CFU/g	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
14	CFU/g	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
21	CFU/g	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
28	CFU/g	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

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

2.4.1.3 Accelerated Stability Study at 70°C

The results of the stability study conducted at 70°C for 10 days on *C. beijerinckii* ASCUSBR67 are summarized in Table 2.29 and the report is provided in Appendix 015. Over the period evaluated, changes in the viable spore count were observed representing a decay rate plotted in Figure 2.4 for the 3 batches of *C. beijerinckii* ASCUSBR67.

Table 2.29:	**Results of a Stability Study on 3 Batches of *C. beijerinckii* ASCUSBR67 Stored at 70°C**						
		Analytical Results					
		Lot 20-0202-049-P6		**Lot 20-0202-049-P7**		**Lot 20-0202-049-P12A**	
Time (Days)	**Unit**	**Viable Cells Count**	**SD**	**Viable Cells Count**	**SD**	**Viable Cells Count**	**SD**
0	CFU/g	(b)(4)					
1	CFU/g						
2	CFU/g						
4	CFU/g						
5	CFU/g						
7	CFU/g						
10	CFU/g						

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

Figure 2.4: Rates of Decay at 50°C, 60°C, and 70°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

(b)(4)

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 batches at 60°C were not overlapping. Therefore, independent shelf-life analysis of each batch was required and the rate data from all 3 batches were demonstrated independently. The upper-tailed 95% confidence interval for a decay rate was calculated and used to define the shelf life of each lot at 25°C. Lot 20-0202-049-P7 had the highest extrapolated decay rate, which predicted a worst case of 2.08×10^{-4} log [CFU/day], resulting in a minimum shelf life of 4,516 days among the 3 batches of *C. beijerinckii* ASCUSBR67. 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, *C. beijerinckii* ASCUSBR67 spray dried powder (SDP) may be incorporated into the diet of poultry as part of the mash, as a feed supplement blended into the mash, or as pellets made from the mash. The strain is spray dried with starch to generate a stable product suitable for handling under practical commercial poultry conditions in the U.S for both direct feeding of mash or pellets. To demonstrate *C. beijerinckii* ASCUSBR67 spray dried powder is stable in common poultry feed configurations both mash stability and pellet stability studies were performed under ambient (real-time) conditions.

2.4.2.1 Mash Stability

As poultry feed mash is generally made and used within a very short time (normally less than a week), a study was conducted in which *C. beijerinckii* ASCUSBR67 spray dried powder (SDP) was added to poultry mash. Stability of *C. beijerinckii* ASCUSBR67 in mash was evaluated for two weeks at 25°C ± 2°C and >60% Relative Humidity (RH). The amount added was consistent with a use level that could be recommended for the addition of this organism, approximately 1×10^5 CFU/g of *C. beijerinckii* ASCUSBR67 SPD to gram of mash. Over two weeks, little degradation was observed and no sample fell below the protocol criteria of 1×10^2 CFU/gram (see Table 2.30, Figure 2.5) indicating *C. beijerinckii* ASCUSBR67 SDP is stable in poultry mash in typical use conditions. Stability protocols and report details can be found in Appendix 015.

Table 2.30: Results of a Stability Study on 3 Batches Poultry Mash containing *C. beijerinckii* ASCUSBR67

		Analytical Results					
		Mash 1102C		Mash 1102D		Mash 1102F	
Time (Days)	Unit	Ave Viable Cell Count	SD	Ave Viable Cell Count	SD	Ave Viable Cell Count	SD
0	CFU/g						
7	CFU/g					(b)(4)	
14	CFU/g						

Figure 2.5: *C. beijerinckii* ASCUSBR67 stability in poultry mash over two weeks in an open container held at 25°C ± 2°C and >60% RH.

(b)(4)

As seen in Table 2.30 and Figure 2.5, the *C. beijerinckii* ASCUSBR67 is stable in mash for up to two weeks at ambient temperature (25°C ± 2°C) with virtually no degradation. Therefore *C. beijerinckii* ASCUSBR67 is stable in poultry feed mash for up to two weeks.

2.4.2.2 Pellet Stability

In addition to mash, pellets made from mash were evaluated for stability. Like mash, pellets are typically made and used fairly quickly, with a single batch normally being held less than a week partly due to the prospects of mold formation as it ages. As such, the pellet stability study was conducted for two weeks.

The same three mash batches used for mash stability were subjected to pelleting at 185°F per the parameters described in the Pellet Stability protocols and Summary Report (Appendix 015). Containers were held at 25°C ± 2°C with >60% relative humidity open to the environment.

The results of the pellet stability are summarized in Table 2.31

Table 2.31: Results of a Stability Study on 3 Batches of Poultry Pellets containing *C. beijerinckii* ASCUSBR67 produced at 185°F

		Analytical Results					
		Pellet 1103C		Pellet 1103D		Pellet 1103F	
Time (Days)	Unit	Ave Viable Cell Count	SD	Ave Viable Cell Count	SD	Ave Viable Cell Count	SD
0	CFU/g						
7	CFU/g				(b) (4)		
14	CFU/g						

Figure 2.6: *C. beijerinckii* ASCUSBR67 stability in 185°F produced poultry feed pellets over two weeks in an open container held at ambient temperature (25°C ± 2°C and >60% RH).

(b) (4)

As seen in Table 2.31 and Figure 2.6, the pelleting process at 185°F did not significantly reduce the population of *C. beijerinckii* ASCUSBR67 viable count (less than one $\log_{10}$ reduction over that seen in mash) and the population remained stable over the two week test period. Therefore, *C. beijerinckii* ASCUSBR67 is stable in poultry feed pellets for up to two weeks.

2.4.3 Homogeneity Data

Although the ability of *C. beijerinckii* ASCUSBR67 spray dried powder to be mixed homogeneously into poultry feed mash was highly indicated by the results of the stability studies, a separate homogeneity study was conducted using the spray dried powder spores of *C. beijerinckii* ASCUSBR67 in poultry feed mash.

As poultry can be fed either mash or pellets and pellets are made from mash, only mash was evaluated for homogeneity. *C. beijerinckii* ASCUSBR67 spray dried powder was incorporated into a batch of poultry mash at ~1 x 10^5 CFU/gram which is representative of the conditions of use in poultry feed mash. Ten sub-samples of mash were collected across the blended mash and analyzed in triplicate for *C. beijerinckii* ASCUSBR67 viable cell count. The coefficients of variation (CV) in viable cells count for the 10 sub-sample replicates were determined for each mash sample, as well as the overall CV of the batch. The results are summarized in Table 2.32 and reported in Appendix 011. The overall CV reported for *C. beijerinckii* ASCUSBR67 viable cells count was 23% among the 10 mash samples indicating that under the conditions of intended use, *C. beijerinckii* ASCUSBR67 SPD is homogeneously distributed throughout the poultry feed mash.

Table 2.32: Results of a Homogeneity Study on 10 Samples from a Batch of Mash Containing *C. beijerinckii* ASCUSBR67 Spray Dried Powder

Mash Sample	Individual Samples			All Samples		
	Ave CFU/g	SD	CV	Ave CFU/g	SD	CV (%)
1	(b)(4)			2.26 x 10^5	5.21 x 10^4	23%
2						
3						
4						
5						
6						
7						
8						
9						
10						

Abbreviations: Ave = average; CFU = colony forming units; SD = standard deviation; CV = coefficient of variation.

2.4.4 Manufacturing Summary

Native Microbials will manufacture a safe stable product for poultry 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 *C. beijerinckii* ASCUSBR67 is to augment normal digestion. Specifically, *C. beijerinckii* ASCUSBR67 is known to ferment xylose and starch (see Part 2.1.3). As described below, Native Microbials has determined that the technical effect of *C. beijerinckii* ASCUSBR67 when fed to poultry 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 *C. beijerinckii* ASCUSBR67 in the feed of poultry are not required as part of this GRAS notice.

The use of this organism is to facilitate the digestion of various carbohydrates of poultry feed to volatile fatty acids such as butyrate and acetate. *C. beijerinckii* has been found in monogastric animals including chickens (Crippen *et al.* 2008; Barrios *et al.* 2013; see Part 2.5.1), and has also been assessed as a probiotic for monogastric animals (Vanbelle *et al.* 1990; Prosekov *et al.* 2015). The contribution of DFMs to the broiler GIT has been extensively evaluated (Heak *et al.* 2018), and is further described below in context of technical effect and animal safety (Part 6.4 of this notice).

As a commensal microorganism, feeding *C. beijerinckii* would have no impacts on animal health. Should *C. beijerinckii* not act to ferment carbohydrates, there would be no safety impact, as the other GIT microorganisms will continue to support digestion, and the feed was formulated to assure nutrient requirements were met without consideration of the potential for increased digestion.

2.5.1 Poultry Gastrointestinal Tract Microbiome

The poultry gastrointestinal tract (GIT) harbors a diverse and dynamic microbial consortia (Shang *et al.* 2018; Borda-Molina, Seifert, and Camarinha-Silva 2018; Zhu *et al.* 2002; Apajalahti, Kettunen, and Graham 2004; Perry 2006; Wei, Morrison, and Yu 2013). In broilers, colonization of the GIT begins in ovo when microbes are transferred to the developing chicken via the oviduct and from the environment through eggshell pores (Gantois *et al.* 2009; Roto, Kwon, and Ricke 2016). After hatching, diet, environment, host genetics, disease, bird age, farm management practices, and other factors play a role in shaping and shifting the microbial community (Shang *et al.* 2018; Knarreborg *et al.* 2002; Yadav and Jha 2019; Lu *et al.* 2003; Wang, Lilburn, and Yu 2016; Zhao *et al.* 2013; Kers *et al.* 2018; Feye *et al.* 2020).

Of the factors affecting succession in the chicken GIT microbiome, diet is among the most impactful (Pan and Yu 2014; Oakley *et al.* 2014; Shang *et al.* 2018). Microbiome adaptation in response to dietary changes is due in part to the consortia's ability to utilize specialized enzymes and enzyme complexes to convert feed components to end products of digestion (Mahmood and Guo 2020; Yadav and Jha 2019; Pan and Yu 2014). It is this specific understanding that Native Microbials uses in their identification of existing, commensal microorganisms in the GIT of high producing broilers. Particularly, understanding of their unique enzymatic properties and physiology support the selection and use of them as DFMs.

Numerous studies have linked broiler GIT microbial communities to animal performance and feed digestibility (Johnson *et al.* 2018; Stanley *et al.* 2016; Yadav and Jha 2019; Stanley *et al.* 2013; Rodríguez *et al.* 2012; Mahmood and Guo 2020; Lee, Kil, and Sul 2017). As previously noted, the poultry GIT microbiome is diverse and impacted by a wide variety of environmental, genetic, and management related variables. Microbial community composition also varies by organ and anatomical position along the GIT (Oakley and Kogut 2016; Yeoman *et al.* 2012; Rehman *et al.* 2007; Feye *et al.* 2020). To better study the microbiome in context of this variability, studies have focused on identifying and characterizing the core commercial poultry GIT microbiome (Ocejo, Oporto, and Hurtado 2019; Johnson *et al.* 2018; Oakley and Kogut 2016; Oakley *et al.* 2013, 2014; S. Wei, Morrison, and Yu 2013; Wei, Lilburn, and Yu 2016). The concept of core microbiome, a common assemblage of microorganism species that exists in or is associated broadly across a particular environment, 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).

The chicken microbiome consists of distinct and specialized groups of microorganisms across the GIT (Oakley *et al.* 2014, 2013). Despite this variability, a core microbiome for the ileum and cecum has been established (Ocejo, Oporto, and Hurtado 2019; Johnson *et al.* 2018). For the other regions of the GIT, research has identified several trends in microbiome composition. The crop is largely populated by *Lactobacillus* species at concentrations ranging from 10^8 to 10^9 cfu/g of content (Rehman *et al.* 2007; Shang *et al.* 2018) and *Bifidobacteria* species at concentrations of 10^7 cfu/g of content (Petr and Rada 2001; Oakley *et al.* 2014). The crop also harbors a wide range of transient bacteria that can be found under various conditions (Hinton *et al.* 2000). The stomach, which consists of the proventriculus and the gizzard/ventriculus (Yeoman *et al.* 2012), share similar microbial profiles (Oakley *et al.* 2014). This section is largely populated by *Lactobacillus* species, *Enterococcus* species, *E. coli* and other coliform bacteria (Bjerrum *et al.* 2006; Engberg *et al.* 2000; Engberg, Hedemann, and Jensen 2002; Gong *et al.* 2007). All referenced studies found greater abundance of *Lactobacillus* species than any other in this section of the GIT (Engberg *et al.* 2000; Engberg *et al.* 2002).

As previously stated, the core microbiome for the ileum has been established (Johnson *et al.* 2018; Ocejo, Oporto, and Hurtado 2019). The major group of bacteria present in this organ are *Lactobacillus* species (Johnson *et al.* 2018; Rehman *et al.* 2007), which can represent greater than 50% of the microbiome (Lu *et al.* 2003; Wei, Lilburn, and Yu 2016) at concentrations up to 10^9 cfu/g of content (Bjerrum *et al.* 2006). *Clostridium* species also constitute a significant portion of the core microbiome in the ileum (Kumar *et al.* 2018; Johnson *et al.* 2018).

The cecum hosts the highest density of bacteria throughout the entire GIT (Rehman *et al.* 2007). Like many other portions of the GIT, the caecum microbiome changes as the chicken ages and matures (Rehman *et al.* 2007; Yadav and Jha 2019; Lu *et al.* 2003; Oakley *et al.* 2014). Despite these changes, a core microbiome is present (Johnson *et al.* 2018). In mature broilers, the bacteria in the caecum are primarily *Bacteroides* species, *Bifidobacteria* species, *Clostridium* species, *Lactobacillus* species, and *E. coli* (Rehman *et al.* 2007; Bjerrum *et al.* 2006; Johnson *et al.* 2018). Salanitro *et al.* (1974) isolated 325 bacterial strains from caecum content of mature chickens, and while there was variation between the birds that were examined, *Propionibacterium, Eubacterium, Bacteroides*, and *Clostridium* were the major genera that were identified. Several studies identified *Bacteroides fragilis* in the ceacum in nearly every bird tested (Salanitro, Blake, and Muirhead 1974; Bjerrum *et al.* 2006).

Diet is the major determinant of microbiome composition (Pan and Yu 2014; Oakley *et al.* 2014; Shang *et al.* 2018; Yadav and Jha 2019). If the abundances of core microbiome members are within typically observed ranges, it is likely that digestion and fermentation in the broiler GIT is also operating within normal ranges as well. 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 fermentation and digestion in the GIT are expected (Appendix 018). The intent of feeding DFMs, particularly *C. beijerinckii* ASCUSBR67, is to improve the nutrient availability from feed. Feeding *C. beijerinckii* ASCUSBR67 to broiler chickens supplements the existing populations of *C. beijerinckii* in the GIT, and ultimately provides additional nutrient availability to the animal. Should *C. beijerinckii* ASCUSBR67 fail, other members of the existing GIT microbiome will continue to ferment and digest 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 GIT, 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 GIT. The diet offered to the animal would be formulated to meet the existing nutritional needs of the animal (Perry 2006; National Research Council, Board on Agriculture, and Subcommittee on Poultry Nutrition 1994). Should *C. beijerinckii* ASCUSBR67 fail, other members of the existing broiler GIT 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 inactive or "dead" microbes under a variety of treatment conditions. Incharoen *et al.* (2019) compared 300 broilers being fed a basal diet to 300 broilers on the same diet treated with heat inactivated *Lactobacillus plantarum* L-137 at a concentration of 10 mg/kg of feed. The two groups were first administered treatment at 5 days old and were treated and observed until they were 42 days old. The treatment animals showed increased performance with higher body weight, larger average daily gain, and improved feed conversion ratio. No differences were observed between the treatment and control in general health related metrics such as mortality, carcass characteristics, visceral organ condition, and feed intake indicating the control animals' health did not suffer as a result of not receiving live *Lactobacillus plantarum* L-137 treatment.

Similarly, Huang *et al.* (2004) investigated the effects of administering high pressure inactivated/homogenized *Lactobacillus acidophilus* ATCC 43121, *Lactobacillus casei* CL96, or the fungus *Scytalidium acidopilum* to broilers. Birds treated with the inactivated DFM were compared to animals on the same basal diet with no DFM (negative control) or with an alternative, live commercial DFM (positive control). The 10 treatment groups (92 birds/group) consisted of a high and low dose of each inactivated DFM, a high and low dose live commercial DFM, and two negative controls on the basal diet. One of the negative control diets was supplemented with whey. The treatments were administered to 1 day old chicks and the treatment and observation continued until the broilers were 42 days old. Performance metrics including body weight, body weight gain, feed intake, and feed conversion ratio were measured on a weekly basis. Mortality rates were also recorded. When compared to the negative controls, body weight and body weight gain were significantly enhanced by both the live and inactivated DFM at either the high dose, low dose, or both. Feed intake was significantly increased in comparison to the negative control with the high dose *Scytalidium acidopilum* treatment. No significant difference in bird mortality was observed between any of the groups.

To study the effect of DFM administration on resident broiler microbiota, Heak *et al.* (2018) conducted a meta-analysis of 42 trials that enumerated culturable microbes pre- and post-DFM supplementation. Of the 42 studies, 17 used a commercial DFM and 28 administered a single species. Administration concentrations of the DFM varied from 10^5 - 10^{12} cells and 41 of the trials fed the DFM on a daily basis. These analyses measured 18 different bacterial groups. The groups included broad classifications such as aerobes, anaerobes, gram + cocci, coliforms, and mesophilic bacteria, as well as more specific classification such as *Bacteroides, Streptococcus, Enterococcus, Salmonella, Bifidobacterium, Lactobacillus, Bacillus, Enterobacteriaceae, Clostridium coccoides, Escherichia coli, Clostridium perfringens,* and *Clostridium butyricum*. Overall, the effect of DFM was highly heterogeneous across studies but the overall effect was that the 18 taxa did not significantly differ between the DFM supplemented group and the controls. Importantly, log-concentration of total bacteria was not significantly different in the DFM group when compared to the control, indicating that the DFM did not have a material impact on the number of culturable bacteria in the broiler GIT. The authors observed a general trend in the increase log-concentrations of "beneficial bacteria" (*Bifidobacterium, Lactobacillus, Bacillus)* and decrease in log-concentrations of "detrimental bacteria" (*Enterococcus, Salmonella, Clostridium perfringens)* in DFM treated animals when the group of 18 taxa were subdivided. However, the authors note that publication bias could have been a contributing factor to the trend.

Murugesan *et al.* (2014) investigated the effects of a *Bacillus sp.* on caecal and ileal digestion and fermentation. Broilers were assigned one of four treatment groups; all groups were administered the same basal diet. The groups were as follows: a control on a basal diet (CON), birds with a protease and phytase supplement added to their diet (PP), *Bacillus sp.* treated (DFM), or birds with a protease and phytase supplement added to their diet and treated with the *Bacillus sp.* (DFM+PP). Short chain fatty acid (SCFA) concentration was assayed to quantify fermentation in the caecum. Total SCFA concentration did not differ significantly between the DFM treated group or the control, though the proportion of butyrate was significantly increased in the DFM treated group. Digestibility of starch, amino acids , and crude protein were measured in the ileum. Additionally, illeal enzyme activity of sucrase, maltase, and L-alanine aminopeptidase were assayed. Digestibility of starch, amino acids, and crude protein were increased in the group supplemented with the DFM in comparison to the control. Digestive enzyme activity in the ileum was not significantly increased in the DFM group in comparison to the control group.

No negative changes in any of the parameters or health complications were reported in any of the treatment groups or the control. Similar results have been reported in regards to feeding *Bacillus* based DFMs though there is some heterogeneity in regards to SCFA concentration (Dersjant-Li *et al.* 2015; Teng *et al.* 2017; Grant *et al.* 2018; Lei *et al.* 2014). Croom *et al.* (2009) investigated the effect of the commercial DFM Primalac (*Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium thermophilum, and Enterococcus faecium*) on SCFA concentration. Animals received the DFM for 21 days and were compared to a control consuming the same basal diet. Feed intake by DFM treated birds was not significantly different from the control, however, the DFM treated animals exhibited lower SCFA concentration in the cecum. The results of this study were similar to the findings by Mountzouris *et al.* (2007) which evaluated a DFM of a similar composition (*Lactobacillus reuteri, Lactobacillus salivarius, Bifidobacterium animalis, Enterococcus faecium,* and *Pediococcus acidilactici).* In the study by Mountzouris and colleagues, a DFM treated group was compared to a control group on the same basal diet over the course of 6 weeks. In addition to measuring SCFA concentration in the cecum, glycolytic enzyme activity was measured, bacteria were enumerated using culture dependent methods, and performance metrics were collected. Broilers treated with the DFM showed no significant difference from the control in regards to total caecal SCFAs. DFM treated birds showed higher specific activities of α-galactosidase and β-galactosidase, lower feed intake, and more efficient feed conversion when compared to control. Bacterial enumeration revealed no significant differences in total aerobes, total anaerobes, or coliform counts when compared to control, though *Bifidobacterium spp., Lactobacillus spp.*, and gram-positive cocci were enriched in the DFM treated animals.

A collection of other organisms have also been evaluated as DFMs in broilers and administered as single strains or as part of a consortia including *Clostridium butyricum, Rhodopseudomonas palustris, Saccharomyces cerevisiae*, various *Lactobacillus* sp., and various *Bacillus* sp. (Simon *et al.* 2001; Patterson and Burkholder 2003; Griggs and Jacob 2005; Lutful Kabir 2009; Yeo and Kim 1997; Khan *et al.* 2007; Apata 2008; Nakphaichit *et al.* 2011; Salim *et al.* 2013; Mookiah *et al.* 2014; Lee *et al.* 2010; Lee *et al.* 2011; Liu *et al.* 2012; Park and Kim 2014; Zhang *et al.* 2014; Yang *et al.* 2012; Zhang *et al.* 2016; Liao *et al.* 2015; Xu *et al.* 2014; Mountzouris *et al.* 2010; Karimi Torshizi *et al.* 2010). While these studies were heterogeneous in regards to parameters measured and overall impact of the DFM, there were no negative changes in assessed parameters that would suggest a decrease in health. Several meta-analyses have been conducted on the effect of DFMs on broilers. Blajman *et al.* (2014) assessed 48 randomized controlled studies that investigated DFM administration and body weight gain and 46 randomized controlled studies that investigated DFM administration and feed efficiency. The meta-analysis concluded that DFMs had a positive effect on both body weight gain and feed efficiency. No negative health consequences were reported in either control or DFM treated groups. Faria Filho *et al.* (2006) conducted a meta-analysis of 27 studies examining DFM supplementation to broilers in Brazil. The analysis assessed the performance of 12 different probiotics and focused on performance parameters. Similarly to Blajman *et al.* improvements in body weight gain and feed efficiency were observed in the DFM treated animals. No negative health consequences were reported in either control or DFM treated groups.

In these examples, failure of DFM supplementation or the DFM itself did not cause any harm to digestion and normal GIT function in broilers or animal well-being. In the case of *C. beijerinckii* ASCUSBR67, if the DFM failed to provide improved digestibility, GIT function and feed fermentation would be identical to that of untreated broilers. 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 poultry (National Research Council, Board on Agriculture, and Subcommittee on Poultry Nutrition 1994). Any non-performing *C. beijerinckii* ASCUSBR67 or deceased *C. beijerinckii* ASCUSBR67 would pass through the GIT with the normal flow of digesta (National Research Council, Board on Agriculture, and Subcommittee on Poultry Nutrition 1994; Perry 2006).

In this respect, based on the results of published comparative studies, *C. beijerinckii* ASCUSBR67 will act only to support normal GIT function and digestion of animal feed. Like other DFMs, while *C. beijerinckii* ASCUSBR67 may aid the digestion of feed, the effect is not required for the general well-being and normal performance of chickens. Thus, the absence of the anticipated effect of *C. beijerinckii* ASCUSBR67 on feed digestion by chickens 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 broilers (National Research Council, Board on Agriculture, and Subcommittee on Poultry Nutrition 1994).

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 *C. beijerinckii* ASCUSBR67, 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 *C. beijerinckii* ASCUSBR67 fail, other members of the existing GIT microbiome will continue to aid in the digestion of 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, *C. beijerinckii* ASCUSBR67 spray dried powder is intended for use as a source of viable microorganisms in feed for poultry. The microbial strain will be delivered as a spray dried product with starch (*C. beijerinckii* ASCUSBR67 spray dried powder, min. 1×10^8 CFU/g) to poultry either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing *C. beijerinckii* ASCUSBR67 spray dried powder may be incorporated into the diet of poultry include as part of the mash or as the mash further pelleted. The spray dried powder will be incorporated into poultry feed at the recommended (minimum) use level of 1×10^4 CFU of *C. beijerinckii* ASCUSBR67/bird/day. Example is given for broilers, as broilers eat more per bird/day than breeders or layers. A 7 day old broiler will consume 36 grams of mash per day (Cobb-Vantress, 2018), whereas breeder (22g/day) and layers (14g/day) would consume less mash. Therefore a dose of 2.78 grams of Spray Dried Powder (at 1×10^8 CFU/g) per metric ton (1000 kilos) of mash will be needed to achieve a concentration of 2.78×10^2 CFU/gram, which delivers 1×10^4 CFU/bird/day on a 36 gram/day diet. By the time the broiler is 42 days old (market-ready), it will be consuming 208 grams per day (breeders 152g/day and layers 105 g/day at full maturity). At that same feed inclusion rate the resulting market-ready broiler consumption will be 5.78×10^4 CFU/bird/day of *C. beijerinckii* ASCUSBR67 Spray Dried Powder. No withdrawal period is considered necessary on the basis that *C. beijerinckii* ASCUSBR67 is native to the GIT of poultry and as detailed in Part 6, is not expected to be pathogenic or toxigenic.

3.1.2 Exposure to the Other Components of the Starch Encapsulated Product

The spray dried powder is comprised of approximately [redacted] (b) (4) (see Appendix 010). As such, using the calculations from above for a market-ready broiler (highest food intake per day) the bird will be exposed to up to 1.73×10^{-4} g of the spray dried powder. As mentioned in Part 2, the amount of food starch and spray-dried *C. beijerinckii* ASCUSBR67 is adjusted for each batch to standardize the viable cells count. The food starch complies with the specifications laid down in 21 CFR §172.892(d) and these ingredients are generally accepted for use as starches in animal feed (see Appendix 009). At the recommended rate of incorporation of *C. beijerinckii* ASCUSBR67 spray dried powder in broiler chicken feed, market-ready broilers (consuming 5.78×10^4 CFU *C. beijerinckii* ASCUSBR67/bird/day in 208 grams) will be exposed to approximately 4.04×10^{-4} g food starch and 1.73×10^{-4} g of *C. beijerinckii* ASCUSBR67 cell concentrate per bird per day. Considering the typical feed intake of a market-ready broiler (208 g/bird/day), the contribution of food starch to the daily ration of the animal will be negligible (<0.0002% DM). Thus, the use of food starch or similar acceptable carbohydrate-based carrier, is not expected to significantly impact the nutrient intake under the intended conditions of *C. beijerinckii* ASCUSBR67 spray dried powder as a source of viable microorganisms in feed.

3.1.3 Background Exposure to the Microorganism

As mentioned in Part 2, the strain was isolated from small-intestine content of a healthy broiler chicken and in this respect, *C. beijerinckii* ASCUSBR67 spray dried powder will contribute to the native population of *Clostridium* species in the gut of the animal (see Part 6.4). *C. beijerinckii* is part of the chicken GIT microflora and is routinely isolated from livestock feces, soil samples and silages (Vanbelle *et al.* 1990; Proietti *et al.* 2006; Barrios *et al.* 2013; Crippen *et al.* 2008; Laviad-Shitrit *et al.* 2019; Sankar *et al.* 2003; Pan *et al.* 2008; Hussain and El Sanousi, 2011; Brändle *et al.* 2016; Driehuis *et al.* 2016). Thus, while not present to a significant or intentional degree in feedstocks, background exposure to *C. beijerinckii* from the environment is likely to be significant.

3.2 Human Exposure

C. beijerinckii ASCUSBR67 spray dried powder is intended for use as a supplemental source of viable microorganisms in the feed of poultry. As mentioned in Part 2.1, the strain was isolated from the small-intestine content of a healthy broiler chicken and in this respect, *C. beijerinckii* ASCUSBR67 spray dried powder will contribute to the native GIT population of *Clostridium* species (see Part 6).

The strain has been unambiguously characterized as *C. beijerinckii* 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).

The absence of pathogenicity or toxigenicity, regardless of potential exposure, is further supported by the ubiquitous nature of *C. beijerinckii* and its natural occurrence in the GIT of animals. Based on the literature search conducted using Google Scholar, *C. beijerinckii* is part of the normal flora of healthy birds, including chickens, and its transference from GIT to meat and eggs has not been reported (Vanbelle *et al.* 1990; Proietti *et al.* 2006; Barrios *et al.* 2013; Crippen *et al.* 2008; Laviad-Shitrit *et al.* 2019). Although *C. beijerinckii* infection has been reported in patients with severe trauma or surgeries, none was from consumption. Literature review of food safety concerns revealed no reports of botulism or infection caused by *C. beijerinckii* despite its ubiquitous presence in the environment and bird GIT. Taken together, there is no reason to believe *C. beijerinckii* ASCUSBR67 would impact the quality or composition of any edible tissues or eggs were it to be present. Literature review also indicates that *C. beijerinckii* ASCUSBR67 spray dried powder has not been associated with any human food safety concerns under the intended conditions of use as a direct fed microbial in the feed of poultry and therefore would pose no risk for human consumption were it to somehow be found in meat or on eggs.

No withdrawal period is considered necessary on the basis that *C. beijerinckii* ASCUSBR67 should not impact the quality or composition of any edible tissues when used as a direct fed microbial in poultry feed.

PART 4 – SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with *C. beijerinckii* ASCUSBR67 spray dried powder.

PART 5 – EVIDENCE BASED ON COMMON USE BEFORE 1958

Not applicable.

PART 6 – NARRATIVE

The conclusion that *C. beijerinckii* ASCUSBR67 spray dried powder, as described herein, is GRAS under the conditions of intended use as a direct fed microbial in feed for poultry 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 *Clostridium* species.

As mentioned in Part 1.3, *C. beijerinckii* ASCUSBR67 will be provided to poultry as a spray dried powder with starch, either alone or in combination with other direct fed microbials. The strain was isolated from the gastrointestinal tract (GIT) content of a healthy broiler chicken and is intended as a source of commensal microorganisms. In this respect, *C. beijerinckii* ASCUSBR67 spray dried powder will contribute to the native microbial population in the GIT and the functionality of the direct fed microbial strain is considered in Part 6.1.

The safety of *C. beijerinckii* ASCUSBR67 for use as a direct fed microbial for poultry is evaluated using several different pieces of data regarding strain characterization and the evaluation of its pathogenic and toxigenic potential. In order to understand the pathogenic and toxigenic potential, the microbial strain must be fully characterized and the body of knowledge pertaining to safety based on its taxonomic unit considered. Full details of the characterization of *C. beijerinckii* ASCUSBR67 are detailed in Part 2. The microbial has been unambiguously characterized as *Clostridium beijerinckii* (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 *C. beijerinckii* ASCUSBR67 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 *C. beijerinckii* ASCUSBR67 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 *C. beijerinckii* (see Part 6.2); (b) the history of exposure of the species by animals and humans (see Parts 6.4 and 6.5); and (c) the potential for toxigenicity and pathogenicity (see Part 6.6). These data represent another important component of the safety evaluation of *C. beijerinckii* ASCUSBR67 spray dried powder and are summarized below.

6.1 Functionality

The microbial population of the chicken GIT plays an important role in the digestion of feed. Support of the GIT microbiota by dietary supplementation with sources of viable microorganisms is common practice in the poultry industry in the U.S. in order to assist digestion and fermentation as well as contribute to the digestive processes of the animal (Apata 2008; Croom *et al.* 2009; Grant *et al.* 2018; Heak *et al.* 2018). The contribution of bacteria to the digestion processes and health in the chicken GIT has been widely studied with important functions reported to include the digestion of complex carbohydrates, fermentation of indigestible fibers into volatile fatty acids, and contribution to nitrogen metabolism (Perry 2006; Rehman *et al.* 2007; Svihus *et al.* 2013; Pan and Yu 2014; Borda-Molina *et al.*

2018). As mentioned in Part 2, *C. beijerinckii* ASCUSBR67 was isolated from the GIT of a healthy chicken and is expected to contribute in the same manner that other bacteria do to digestion and metabolism in the chicken GIT environment.

In particular, *C. beijerinckii* ASCUSBR67 was shown to utilize various carbon sources including simple carbohydrates (e.g., glucose and xylose) as well as complex carbohydrates (e.g., starch) (see Part 2.1). Similar phenotypes are reported in the published literature for other *C. beijerinckii* strains (*e.g.*, Zhang *et al.* 2012; Ezeji *et al.* 2007). Zhang et al (2012) found that *C. beijerinckii* NCIMB 8052 could ferment corncobs and produce butanol, acetone, ethanol, acetate, and butyrate. Thus, the microorganism has the potential to support digestion by aiding in fermentation and digestion of feed and partially degraded digesta in the GIT.

Similar to other *Clostridium* species, *C. beijerinckii* ASCUSBR67 has been shown to utilize a range of monosaccharides including glucose, fructose and xylose to produce relatively high levels of butyric acid and acetic acid (Ho *et al.* 2011). VFAs also serve as both intermediary products used by other members of the microbiome and as end products that are either utilized or excreted by the chicken (Józefiak *et al.* 2004). Volatile fatty acids (VFAs) provide 10%-30% of the total energy to the animal (Bergman 1990; Cummings *et al.* 1987; Svihus *et al.* 2013), and have other important roles in the chicken GIT.

Taken together, these examples of the potential functionality of anaerobic *Clostridium* species in the chicken GIT support the proposed role of *C. beijerinckii* ASCUSBR67 as a source of viable microorganisms in the diet to support the digestion of carbohydrates in feed and production of VFAs. While *C. beijerinckii* ASCUSBR67 may contribute to the native population of *Clostridium* 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 poultry. Should *C. beijerinckii* ASCUSBR67 fail, other members of the existing GIT microbiome will continue to ferment and digest feed, thus supplying the animal with sufficient nutrients. On this basis, no further demonstration of the technical effect (utility) of *C. beijerinckii* ASCUSBR67 was required for the safety evaluation (see Part 2.5).

6.2 Identity

The genus *Clostridium* consists of a heterogeneous set of species which are not phylogenetically coherent. Many species were assigned to the genus based only on their being gram positive anaerobic rods which are capable of endospore formation. Phylogenetic and comparative analyses indicate that of the >150 *Clostridium* species identified, fewer than half are part of cluster I, the distinct cluster in the 16S rRNA tree which are generally regarded as the true representatives of the genus *Clostridium* and which includes *C. beijerinckii* (Johnson & Francis, 1975; Collins *et al.* 1994; Stackebrandt *et al.* 1999; Gupta & Gao, 2009; Lawson & Rainey, 2016; Udaondo *et al.* 2017). *Clostridium* cluster I is recognized as *Clostridium sensu stricto* and includes the medically important *Clostridium botulinum* but not *Clostridium difficile*. The species assigned to *Clostridium* cluster I are metabolically and physiologically diverse species capable of utilizing carbohydrates and peptones to produce organic acids and alcohols. The G+C content of cluster I species varies from between 22 and 37 mol%, and the 16S rRNA gene sequence similarities range from 92 to 99% (Wiegel *et al.* 2006; Rainey *et al.* 1993).

In the past, *Clostridium* species were extensively used for the industrial production of butanol and acetone from starch-based substrates (Jones and Woods, 1986; Jones and Keis, 1995; Keis *et al.* 2001). The first strains isolated and developed for large scale production of these solvents were identified as *Clostridium acetobutylicum* but were later assigned to four species, *C. acetobutylicum, C. beijerinckii, Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum* (Johnson *et al.* 1997; Keis *et al.* 1995; Wilkinson and Young, 1995; Keis *et al.* 2001). Similarly, strains historically assigned to the species *Clostridium butylicum* have been subsequently shown to members of *C. beijerinckii* (George and Chen, 1983).

Additionally, some strains originally designated as *C. butyricum* have been renamed *C. beijerinckii.* While challenging to distinguish phenotypically, these species can largely be (Cummins & Johnson, 1971; Ikeda *et al.* 1988) differentiated based on electrophoretic patterns of cellular proteins or by testing nutritional requirements (Brändle *et al.* 2016).

From the perspective of the safety assessment, it is therefore acknowledged that some strains of *C. beijerinckii* were formerly classified as *C. acetobutylicum, C. butylicum* and *C. butyricum* and all species were included in the literature search and subsequent review of published information.

6.3 Literature Search

A comprehensive literature search was conducted in order to identify all publicly available information pertaining to the safety of *C. beijerinckii* for the intended use as a source of viable microorganisms for poultry. As mentioned in Part 6.2, all potential nomenclature was included in the literature search (*i.e., Clostridium acetobutylicum, Clostridium butylicum, Clostridium butyricum* and *Clostridium beijerinckii*) and details of the search strategy are provided in Appendix 017. Results from the literature search form the basis of the safety assessment as found in Part 6.4, 6.5, and 6.6.

6.4 Natural Occurrence

6.4.1 Prevalence in Animals

C. beijerinckii is ubiquitous in nature and routinely isolated from soil samples as well as feces and gastrointestinal contents of livestock, including cattle and chickens (Sankar *et al.* 2003; Pan *et al.* 2008; Hussain and El Sanousi, 2011; Brändle *et al.* 2016; Driehuis *et al.* 2016, Barrios *et al.* 2013). It was one of the major populations of butyric acid bacteria isolated in 296 samples of soil, maize and grass silage, dairy cow feces and farm tank milk collected from dairy farms in the Netherlands (Driehuis *et al.* 2016). Similarly, in a study by Hussain *and El sanousi.,* 210 fecal samples were collected from 7 different animals (cattle, sheep, goats, camels, horses, donkeys, and chickens) to investigate the prevalence of *Clostridium*-like species. The prevalence of *C. beijerinckii* among these samples was 3.3% (Hussain and El Sanousi, 2011). Isolation and characterization of xylanolytic solventogenic bacteria from cow rumen fluid identified 5 strains of *C. beijerinckii* to be present, in combination with *C. acetobutylicum, C. butyricum* and *C. bifermentans* strains (Sankar *et al.* 2003). Dutta *et al.* (1983) isolated members of Clostridia, Lactobacilli, and Streptococci from pigs, cattle, and poultry to investigate their antibiotic susceptibility. The group was able to isolate 3 *C. beijerinckii* strains, 2 from poultry and 1 from cattle, and found that all three did not possess any acquired antimicrobial resistance.

A total of 207 different strains of *C. beijerinckii* have been isolated, sequenced, and analyzed in the JGI genome portal to date (https://genome.jgi.doe.gov/portal/) and 234 genome sequences can be found in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Many strains within this database represent industrial fermentation strains, however, several of the isolates were obtained from cows, pig feces, goats, and humans. While no poultry derived isolates exist in these databases, *C. beijerinckii* has been isolated from chickens (Crippen *et al.* 2008; Barrios *et al.* 2013). Furthermore, Proietti *et al.* (2006) noted that *C. beijerinckii* was the predominant Clostridial isolate from the ileum of 40 and 80 day old broilers and one of the predominant Clostridial isolates in the cecum of 80 day old broilers. Laviad-Shitrit *et al.* 2019 has also reported the presence of *C. beijerinckii* in intestinal sections of wild waterbird species.

6.4.2 Microbiome Safety

The poultry GIT microbiome is crucial for the digestion of feed (Yeoman *et al.* 2012; Oakley *et al.* 2014). It hosts a diverse group of microorganisms that work closely to metabolize proteins and degrade dietary carbohydrates. The deconstruction of dietary carbohydrates releases volatile fatty acids (VFA), predominantly butyrate, acetate, and propionate. These VFA are absorbed transepithelial as important sources of energy (Yeoman *et al.* 2012). Studies have found that VFAs comprise 10% to 30% of host daily energy requirements in monogastric animals (Bergman 1990; Cummings *et al.* 1987; Svihus *et al.* 2013). Among the three dominant VFA, butyrate has been widely recognized as beneficial for GI health in monogastric animals, including poultry (Bedford and Gong 2018; Yeoman *et al.* 2012).

The poultry GIT microbiome is dynamic. Studies have shown that microbiome species composition and abundance can fluctuate due to spatial (ie., GIT sample location) and temporal (ie., age) variability throughout the life of the bird (Oakley *et al.* 2014; Oakley and Kogut 2016; Awad *et al.* 2016). These variations reflect the different niches in the poultry GIT, where multiple microbial community states can exist depending on the physical characteristics of the environment and host metabolic needs (Mahmood and Guo 2020). Identification of the optimal microbiome state and important microbial interactions could drive improved digestibility (Mahmood and Guo 2020; Oakley *et al.* 2014; Yeoman *et al.* 2012). The contribution of DFMs to the chicken GIT has been extensively evaluated. Heak *et al.* (2018) conducted a meta-analysis of 42 trials that enumerated culturable microbes pre- and post-DFM supplementation. Of the 42 studies, 17 used a commercial DFM and 28 administered a single species. Administration concentrations of the DFM varied from 10^5 - 10^{12} cells and 41 of the trials fed the DFM on a daily basis. These analyses measured 18 different bacterial groups. The groups included broad classifications such as aerobes, anaerobes, gram + cocci, coliforms, and mesophilic bacteria, as well as more specific classification such as *Bacteroides, Streptococcus, Enterococcus, Salmonella, Bifidobacterium, Lactobacillus, Bacillus, Enterobacteriaceae, Clostridium coccoides, Escherichia coli, Clostridium perfringens,* and *Clostridium butyricum*. Overall, the effect of DFM was highly heterogeneous across studies but the overall effect was that the 18 taxa did not significantly differ between the DFM supplemented group and the controls. Importantly, log-concentration of total bacteria was not significantly different in the DFM group when compared to the control, indicating that the DFM did not have a material impact on the number of culturable bacteria in the broiler GIT. The authors observed a general trend in the increase log-concentrations of "beneficial bacteria" (*Bifidobacterium, Lactobacillus, Bacillus)* and decrease in log-concentrations of "detrimental bacteria" (*Enterococcus, Salmonella, Clostridium perfringens)* in DFM treated animals when the group of 18 taxa were subdivided. However, the authors note that publication bias could have been a contributing factor to the trend.

A collection of other organisms have also been evaluated as DFMs in broilers and administered as single strains or as part of a consortia including *Clostridium butyricum, Rhodopseudomonas palustris, Saccharomyces cerevisiae*, various *Lactobacillus* sp., and various *Bacillus* sp. (Simon *et al.* 2001; Patterson and Burkholder 2003; Griggs and Jacob 2005; Lutful Kabir 2009; Yeo and Kim 1997; Khan *et al.* 2007; Apata 2008; Nakphaichit *et al.* 2011; Salim *et al.* 2013; Mookiah *et al.* 2014; Lee *et al.* 2010; Lee *et al.* 2011; Liu *et al.* 2012; Park and Kim 2014; Zhang *et al.* 2014; Yang *et al.* 2012; Zhang *et al.* 2016; Liao *et al.* 2015; Xu *et al.* 2014; Mountzouris *et al.* 2010; Karimi Torshizi *et al.* 2010). While these studies were heterogeneous in regards to parameters measured and overall impact of the DFM, there were no negative changes in assessed parameters that would suggest a decrease in health. Several meta-analyses have been conducted on the effect of DFMs on broilers. Blajman *et al.* (2014) assessed 48 randomized controlled studies that investigated DFM administration and body weight gain and 46 randomized controlled studies that investigated DFM administration and feed efficiency. The meta-analysis concluded that DFMs had a positive effect on both body weight gain and feed efficiency. No negative health consequences were reported in either control or DFM treated groups. Faria Filho *et al.* (2006) conducted a meta-analysis of 27 studies examining DFM supplementation to broilers in Brazil. The analysis assessed the performance of 12 different probiotics and focused on performance parameters. Similarly to Blajman *et al.* improvements in body weight gain and feed efficiency were observed in the DFM treated animals. No negative health consequences were reported in either control or DFM treated groups. Collectively, these studies demonstrate that DFMs can promote better microbial interactions and improve the overall digestibility of feed without significantly changing microbial community structures.

C. beijerinckii has been frequently isolated from dietary fiber rich environments (Jones and Woods, 1986; Trudeau *et al.* 1992; Noparat *et al.* 2011; Hoang *et al.* 2018). Studies conducted on different *C. beijerinckii* strains revealed a high level of amylase production when grown on starch (Qureshi and Blaschek, 2001; Taguchi *et al.* 1993). *C. beijerinckii* is a common member of poultry GIT microbiomes. Vanbelle *et al.* (1990) reported *C. beijerinckii* as one of the common bacteria in the digestive tract of poultry. Proietti *et al.* (2006) found that *C. beijerinckii* was one of the dominant bacteria isolated from the 40-80 days old healthy organic chicken intestinal samples. Barrios *et al.* (2013) isolated *C. beijerinckii* from the stomach content of healthy 21-day old chickens. In addition, Crippen *et al.* (2008) used bioreactors to simulate the development of chicken GI microbiomes by using a single 14-day old chicken cecal microflora. The authors isolated *C. beijerinckii* from 2 out of 3 bioreactors, suggesting that *C. beijerinckii* is a common member of chicken GI microflora throughout various growth stages.Laviad-Shitrit *et al.* 2019 has also reported the detection of *C. beijerinckii* from the intestinal sections of wild waterbird species. Furthermore, *C. beijerinckii* is a member of the phylum Firmicutes, which is one of the most abundant (24%-99%) bacterial phyla in chicken GIT across breeds, ages, and GI locations (Paul *et al.* 2021; Fidler *et al.* 2020; Oakley and Kogut 2016; Awad et al. 2016; Wang *et al.* 2018; van der Hoeven-Hangoor *et al.* 2013).

Native Microbials conducted a series of experiments in order to obtain a representative sampling of the chicken GIT microbial community under farm-like conditions in the U.S. The full study report is provided in Appendix 018. In two general survey experiments, birds were sacrificed and GI contents were collected over the growth period. In three other studies, *C. beijerinckii* ASCUSBR67 along with another native chicken GI microorganism was supplemented in feed to chickens daily throughout the growing period. In all of the experiments, the typical abundance of *C. beijerinckii* in the GI tract of chickens was found to comprise 0.033% of the bacterial population on average. General observations indicated that all birds were in good health. The administration of *C. beijerinckii* ASCUSBR67 did not have a significant impact on the chicken GI tract microbial community. Taken together, these studies provide corroborative experimental evidence that *C. beijerinckii* is naturally abundant in the GIT of chickens and not associated with any health concerns.

6.4.3 Environmental Occurrence

C. beijerinckii occurs extensively in nature. It can be found in a variety of environments, including soil (Wu *et al.* 2019; Bhutada and Shrivastava, 2018; Dobbin *et al.* 1999; Montoya *et al.* 2000; Abd-Alla *et al.* 2015), insects (Taguchi *et al.* 1993; Cruden *et al.* 1987), wastewater (Fonseca *et al.* 2019; Rosewarne *et al.* 2013), manure (Ostling and Lindgren. 1991; Scott *et al.* 2018; An *et al.* 2014; Pandey *et al.* 2009; Islas-Espinoza *et al.* 2012; Lin *et al.* 2013; Scott and Murano 2007), as well as anaerobic sludge (Fonseca *et al.* 2016; Pan *et al.* 2008; Scott and Murano, 2007; Zhao *et al.* 2011). A strain of *C. beijerinckii* capable of coupling dechlorination of pentachlorophenol with Fe(III) reduction has been isolated from rice paddy fields in Zhejiang, China (Xu *et al.* 2014). Eighteen different strains of *C. beijerinckii* have been deposited in the open collection of the American Type Culture Collection (ATCC) to date. Similarly, a total of 207 different strains of *C. beijerinckii* have been isolated, sequenced, and analyzed in the JGI genome portal to date (https://genome.jgi.doe.gov/portal/) and 234 genome sequences can be found in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Many strains within these databases represent industrial fermentation strains, however a small subset are from environmental and agricultural sources.

Clostridium species are extensively used for the industrial production of butanol and acetone from starch-based substrates (Jones and Woods, 1986; Jones and Keis, 1995; Keis *et al.* 2001; Valdez-Vazquez *et al.* 2015). The first strains isolated and developed for large scale production of these solvents were identified as *Clostridium acetobutylicum* but were later assigned to four species, *C. acetobutylicum*, *C. beijerinckii*, *Clostridium saccharoperbutylacetonicum* and *Clostridium saccharobutylicum* (Johnson *et al.* 1997; Keis *et al.* 1995; Wilkinson and Young, 1995; Keis *et al.* 2001). New strains of these species (including *C. beijerinckii*) capable of more efficient industrial production of solvents, particularly acetone, butanol, and ethanol, are still being screened for in environmental samples for industrial applications (Abd-Alla *et al.* 2015). Abd-Alla *et al.* (2015) for example, isolated 107 *Clostridium* isolates from agriculture soil, several of which were *C. beijerinckii*.

6.4.4 Section Summary

C. beijerinckii occurs widely in the environment and is prevalent as a commensal member of the poultry GIT microbiome. Supplementation of the diet with *C. beijerinckii* ASCUSBR67 will not negatively impact the function of the GIT nor negatively impact the well-being of the animal.

6.5 History of Use in Manufacture of Food and Feed Ingredients

The application of *C. beijerinckii* is primarily in anaerobic acetone-butanol-ethanol (ABE) production, especially using food wastes as the fermentation substrate due to its ability in dietary fiber degradation (Ezeji, Qureshi, and Blaschek 2007; Qureshi and Blaschek 1999, 2001; Sabater *et al.* 2020). *C. beijerinckii* has been proposed as a downstream biorefinery process to follow fermentation of seaweed for animal feed (Bikker *et al.* 2016). *Ulva lactuca*, a type of green seaweed, can be fractionated and the subsequent liquid fraction can leverage *C. beijerinckii* for fermentative production of chemicals. *C. beijerinckii* has also been commonly detected and isolated from silage as well as fermented foods, including cheese, Chinese sufu, Ethiopian kocho, and Greek chickpea bread (Qiong *et al.* 2021; Ashenafi 2006; Cremonesi *et al.* 2012; Hatzikamari *et al.* 2007; Queiroz *et al.* 2018). These environments are inline with the dietary fiber degradation and fermentative nature of *C. beijerinckii* and also indicate that *C. beijerinckii* is prevalent in the agriculture related environments.

C. beijerinckii has been associated with food spoilage. Postollec *et al.* (2012) evaluated 15 types of foods and found that *C. beijerinckii* is a common contaminant in milk and dairy products (Postollec *et al.* 2012). André, Vallaeys, and Planchon (2017) reviewed that *C. beijerinckii* is the main bacteria causing spoilage in fermented milk. Although the presence of *C. beijerinckii* is not considered the main cause of late blown in cheese, Le Bourhis *et al.* (2007) found that *C. beijerinckii* can cause cheese late blown defects if its spore count is more than 4×10^3 spores/L in starter milk. Naturally, the abundance of *C. beijerinckii* in food is low. Specifically, there are 10-100 total Clostridial spores/ml of raw milk in general (Aureli, Franciosa, and Scalfaro 2011) and Komori *et al.* (2019) isolated 6-17 *C. beijerinckii* from 336 raw milk samples (200 ml each). The overall abundance of *Clostridium* in Chinese sufu ranged from < 3 - 4.12 CFU/g and the abundance of *C. beijerinckii* was a fraction of the total *Clostridium* (Qiong *et al.* 2021). Thus, despite its prevalence and the potential of continuous exposure to the low levels of spores, no deleterious impacts of *C. beijerinckii* on health have been observed or reported.

Bacteria that are similar to *C. beijerinckii*, including *C. acetobutylicum* and *C. butyricum*, have also been reported in various fermentation environments. *C. acetobutylicum* is primarily studied for ABE production (Pataková *et al.* 2009; Khedkar *et al.* 2017; Janssen, Wang, and Blaschek 2014) and reported as part of the microflora of fermented foods (Jiménez *et al.* 2018; Miambi, Guyot, and Ampe 2003; Bermúdez *et al.* 2016). While *C. butyricum* can convert food wastes to biohydrogen (Ortigueira *et al.* 2019), it has been used as feed additives and potential probiotics in animals (Liu *et al.* 2019; Duan *et al.* 2017; Yang *et al.* 2010; Tran *et al.* 2020; Song *et al.* 2006; Zhang *et al.* 2017). For example, Zhang *et al.* (2017) orally administered *C. butyricum* to mice with food allergies and found the microorganism reduced intestinal anaphylaxis. Liu *et al.* (2019) fed *C. butyricum* in-feed to rex rabbits and found the microorganism enhanced the rabbits' small intestine enzyme activity, improved intestinal health, and increased the animal weight gain. Song *et al.* (2006) isolated a strain of *C. butyricum* from a healthy chicken intestine and used it as a feed supplement for fish (*Miichthys miiuy*). The authors found that supplementing *C. butyricum* improved the fish humoral immune response and increased weight gain. A strain of *C. butyricum* is also approved to be used as feed additives for chicken and pigs in Europe (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) *et al.* 2021). Although the use of *C. butyricum* as feed additives has been documented, it is important to note that some strains of *C. butyricum* are toxigenic and have led to infant botulism (Aureli *et al.* 1986). This will be specifically discussed below in Part 6.6.

6.6 Toxigenicity and Pathogenicity

The potential pathogenicity of *Clostridium*, including *C. beijerinckii*, is widely studied and discussed in the published literature. The American Type Culture Collection (ATCC) lists *C. beijerinckii* as BSL-1, indicating that it is a low-risk microorganism that poses little to no threat of infection in healthy humans and animals. DSMZ also classifies *C. beijerinckii* as BSL-1. Unlike known pathogenic *Clostridium,* such as *C. difficile* and *C. perfringens, C. beijerinckii* is considered an opportunistic pathogen in humans and animals. Under rare circumstances, such as soil particle contamination during severe trauma and preexisting health conditions, *C. beijerinickii* has been isolated from infected tissues (Newton, *et al.* 1999; Gorbach and Thadepalli 1975; Ibnoulkhatib *et al.* 2012). These reports support that *C. beijerinckii* is an opportunistic pathogen as it is normally harmless and rarely causes disease.

Large scale genome analysis of both pathogenic and nonpathogenic *Clostridium* has revealed that there are only 169 orthologous gene groups shared by all *Clostridium* strains, and a pangenome of 22,668 genes (Zhou *et al.* 2014). This finding suggests that *Clostridium* contains a diverse gene pool that enables adaptation to different environments. Many of the Clostridia are normally harmless and ubiquitously found in soil and in the gastrointestinal tract of healthy animals, including humans. Clinical cases are only rarely seen (Moore, and Lacey, 2019). Disease generally occurs when there is some tissue damage to the skin or gastrointestinal tract that results in proliferation of *Clostridium* and opportunistic infection. This bimodal lifestyle, split between a free-living, otherwise harmless existence in the environment and host colonization and pathogenesis is reflected in the genomes of the Clostridia. The spore-forming machinery encoded Clostridial genomes increases resilience and survival in the environment, and the wide array of toxins and degradative enzymes that are encoded assist in nutrient scavenging. Many of the toxins produced by Clostridia are encoded on large plasmids (Moore,and Lacey, 2019). The genomes of normally harmless *Clostridium* tend to be larger, suggesting that they are generalists that can survive in a range of ecological niches and use a wider variety of substrates for growth (Suen, *et al.* 2007). Different levels of genomic plasticity have been noted amongst Clostridial species, with sequence variation being driven primarily by mobile elements, chromosomal rearrangement, deletions and insertions, and SNPs (Moore and Lacey, 2019). The genomes of true Clostridia (cluster I), including *C. beijerinckii,* were found to be more stable than other clusters of Clostridia, with plasmids playing a large role in determining strain variation. Large conjugative toxin gene carrying plasmids, in particular, often determine the virulence of *Clostridium* isolates (Moore and Lacey, 2019).

The Google Scholar literature search revealed 2,000+ hits when searching for "*Clostridium beijerinckii*" and various forms of the word "toxins" and "toxicity". Google Scholar provided 5,000+ hits when all safety related terms (various forms of the words "toxin", "pathogen", "safety", "infection", "disease", or "mortality") were included (Appendix 17, Table 2A). Further investigation of these results revealed that the vast majority of hits were related to butanol toxicity, a physiological by-product inhibition common in the industrial production of butanol with *C. beijerinckii.* Similarly, the term "infection" was frequently referring to bacterial phage infection of *C. beijerinckii.*

Published reports of *C. beijerinckii* being isolated from infected tissues, especially in patients who have injuries due to traumatic events, were identified. The example reports of clinically isolated *C. beijerinckii* are provided below.

1. Gorbach and Thadepalli (1975) isolated 152 *Clostridium* strains from the clinic samples of 144 patients. These patients experienced trauma, underwent prior surgeries, had preexisting conditions, such as carcinoma, or were drug addicts. Without identifying the source, the authors isolated 1 *C. beijerinckii* strain from 1 infected soft tissue and none from blood. The study also noted the prevalence of pathogenic species, such as *C. perfringens*, isolated from both tissue and blood samples.
2. Newton *et al.* (1999) reported a case of *C. beijerinckii* secondary infection in an abattoir worker who suffered a penetrating ocular trauma. A foreign body was retained in the eye after the injury and led to *C. beijerinckii* endophthalmitis. The identity of *C. beijerinckii* was confirmed based on physiological reactions.
3. Ibnoulkhatib *et al.* (2012) specifically studied the presence of *Clostridium* species in post-traffic-related-trauma infections. The authors suggested that soil is a common contamination source of *Clostridium* species, including *C. beijerinckii* found in the wound. Patients involved in traffic accidents are particularly prone to *Clostridium* infections due to the close contact of wound and soil particles. The study identified *C. beijerinckii* in 3 patients out of 12. Multiple bacterial species were found in infected tissues from all patients. The identity of *C. beijerinckii* was confirmed based on physiological reactions and marker gene sequences.

These studies suggest that *C. beijerinckii* is normally harmless, however it can cause secondary infections due to wound contamination, especially in conjunction with severe trauma.

To further address the safety of *C. beijerinckii*, neurotoxins and other known *Clostridium*-related toxins were investigated directly by performing an additional literature search based on the Clostridial virulence genes identified using the virulence factor database (VFDB; http://www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Clostridium; 42 genes) cross referenced with Victor's database. Victor's database includes animal pathogens as well as human pathogens, making it the most appropriate and relevant database to assess animal-related pathogenicity and toxigenicity of *C. beijerinckii* ASCUSBR67. However, VFDB contains more *Clostridium*-specific genes. Thus, both databases were leveraged to identify gene names to use in the literature search. A total of 42 Clostridial virulence genes and the terms "hemolysin" and "neurotoxin" were used in combination with *C. beijerinckii, C. acetobutylicum, C. butylicum,* and *C. butyricum* in Google Scholar. These results are reported in Appendix 17, Table 3. Thirty-nine results were identified across all four organisms searched, with the bulk of the results (35) arising for *C. butyricum*. Two relevant results were identified for *C. beijerinckii* and are summarized below:

1. "Clostridial genetics: Genetic manipulation of the pathogenic Clostridia" (Kuehne *et al.* 2019)

> "If used without the Gram positive replicon, they act as suicide vectors in the Clostridia and have been effectively used in this capacity in studies involving the nonpathogenic species *C. beijerinckii* and *Clostridium autoethanogenum*.
>
> Most recently, CRISPR-gene editing technologies were successfully used in the clostridia. Initially developed in nonpathogenic Clostridia, such as *C. pasteurianum*, *C. beijerinckii*, and *C. cellulolyticum*, this emerging technology was used in 2017 to generate deletion mutants in *C. difficile*."

Although *C. beijerinckii* is mentioned in this article about pathogenic Clostridia, it is only discussed as an example of nonpathogenic Clostridia (see excerpts above).

2. "Development of a reporter system for the study of gene expression for solvent production in *Clostridium beijerinckii* NRRL B592 and *Clostridium acetobutylicum* ATCC 824" (Li, 1998)
   This document is a dissertation discussing the development of a reporter system to control the expression of solvent-producing genes in *C. beijerinckii.* Although it appeared in the search results, it does not discuss pathogenicity of *C. beijerinckii.*

No hits for *C. butylicum* or *C. acetobutylicum* were relevant (the two relevant hits for *C. acetobutylicum* are the same as the two above from *C. beijerinckii* results). However, there were several relevant hits for *C. butyricum.* There are some rare instances of *C. butyricum* causing human disease, including necrotizing enterocolitis in premature infants when associated with very specific risk factors (Howard *et al.* 1977; Sturm *et al.* 1980; Caya and Truant, 2000; Gardner *et al.* 2008; Morowitz *et al.* 2010; EFSA, 2014; Cassir *et al.* 2016). For example, Caya and Truant (2000) reported the diagnosis of 53 infant pediatric patients with Clostridial bacteremia, of which 25.9% cases were identified as being associated with *C. butyricum.* Necrotizing enterocolitis-related lesions were also induced by *C. butyricum* in chicken and quail experimental models (Popoff and Dodin, 1985; Butel *et al.* 1998; Waligora-Dupriet *et al.* 2009). The toxigenic mechanism responsible for the pathogenesis of necrotizing enterocolitis is not fully understood but β-haemolysin production has been proposed as a primary virulence factor (Cassir *et al.* 2015). *C. butyricum* has also been shown to produce type E botulinum toxin (Aureli *et al.* 1986; Tsukamoto *et al.* 2002; Hill and Smith 2013; Fu and Wang 2008; de Santos *et al.* 2011). The ability of the host to ferment lactose and the production of high levels of butyric acid by intestinal *C. butyricum* are also suggested by other researchers in the development of necrotic lesions (Waligora-Dupriet *et al.* 2009; Azcarate-Peril *et al.* 2011; Cassir *et al.* 2016). Recently, a systematic characterization of necrotizing enterocolitis and control strains was conducted by Schönherr-Hellec *et al.* (2018) suggested the existence of a specific signature associated with pathogenicity and that a unifying causative mechanism for development of the disease may be activation of an innate immune response. The presence of neuraminidase was investigated in several *Clostridium* strains belonging to *C. butyricum, C. difficile,* and *C. beijerinckii* (Popoff and Dodin, 1985). Neurominidase-production has been implicated in the pathogenesis of neonatal necrotizing enterocolitis. Of the 98 strains tested, including 16 *C. beijerinckii,* only a few *C. butyricum* strains were found to produce neuraminidase.

The potential presence of *C. beijerinckii* and relatives (*C. acetobutylicum, C. butylicum,* and *C. butyricum*) in chicken eggs has also been evaluated via Google Scholar literature search (Appendix 017 Table 4). While the initial search for all four species yielded hundreds of results, many studies used egg or egg ingredients as part of the growth media. Further evaluation, as described in Appendix 017 Table 4, revealed that no *C. beijerinckii* has been isolated from eggs. There are no reports of *C. beijerinckii* causing infections through eggs or related products. Notably, Tamime (2017) described *C. beijerinckii* as a butyric acid producing bacteria that may be found in milk and cheese due to their ubiquitous presence in the environment, while *Bacillus cereus, Staphylococcus* spp., and *Salmonella* spp. are the main disease causing microorganisms found in eggs and egg associated products.

Among *C. beijerinckii* relatives (ie., *C. acetobutylicum, C. butylicum,* and *C. butyricum*), only *C. butyricum* has been isolated from egg samples (Ghoddusi and Sherburn, 2010). However, a total of ten studies are relevant and discussed below:

1. Li *et al.* (2014) described some pathogenic bacteria, such as *Salmonella* spp. and *Campylobacter* spp., may enter a dormant state and cannot be cultured while still posing threats to animal and human health. These dormant pathogenic bacteria can be resuscitated under specific conditions. The study reviewed that *Campylobacter* spp., *Edwardsiella tarda*, and *Enterococcus faecalis* can resuscitate in embryonated chicken eggs. No growth of *Clostridium* spp. was mentioned. *C. acetobutylicum* was mentioned in the study for its mannose-specific phosphotransferase was used as a reference protein.

2. Ghoddusi and Sherburn (2010) described *C. butyricum* as a microorganism that may cause food spoilage. The authors screened 45 food materials, including eggs. Out of 25 egg shell and flesh samples, *C. butyricum* was isolated from 8 samples and none of which contains botulinum toxin type E. The study did not describe the condition of the egg samples. *C. beijerinckii* was used as a control bacteria during isolation.

3. McCaskey and Anthony (1979) isolated 2 *C. butyricum* isolates from 44 poultry litter samples. The presence of *C. butyricum* in chicken eggs was not evaluated. "Chicken egg" was mentioned in the study because poultry litter may provide an ideal environment for mold, which can be toxic for embryonated chicken eggs.

4. Crookshank (1887) described *C. butyricum* as a microorganism that is widely distributed in nature. The book stated:

   > "They convert the lactic acid in milk into butyric acid, and produce the ripening of cheese. They occur also in solutions of starch, dextrine, and sugar, and are the active agents in the fermentations of sauerkraut and sour gherkins."

   No isolation of *C. butyricum* from chicken eggs was mentioned. However, *Bacillus pullorum*, a bacteria that contributes to white diarrhea in adult chickens, was isolated from abnormal egg-yolks in the ovaries of hens, from freshly laid eggs, and from the yolk-sacks of fully developed chicks within the shell.

5. Several studies reported using *C. butyricum* as a DFM for chickens (Zhao *et al.* 2020; Abdelrahman 2020; Gupta, Srivastava, and Lall 2019; Sabry, Ammar, and Bakr 2019; Pepin 2018). All found that feeding *C. butyricum* improved chicken GIT health and none reported the presence of *C. butyricum* in eggs.

The findings from the above studies are in agreement with that *C. beijerinckii* is part of the normal bird GIT microflora and does not pose threat to bird or human health under normal conditions. *C. beijerinckii* has not been reported in normal chicken eggs. One of the relative species, *C. butyricum* has been mentioned in literature relating to foodborne diseases due to the presence of botulinum neurotoxin type E in some strains (see discussion below). However, the *C. butyricum* strains isolated from chicken egg samples do not contain neurotoxin genes, suggesting that these naturally occurring *C. butyricum* pose low risks (Ghoddusi and Sherburn, 2010). It is also important to note, while *C. butyricum* has been associated with fermented food spoilage, literature reports that gram-negative bacteria, including

Salmonella spp. and *Chlamydia* spp., are the main pathogens in bird eggs and egg products (Li *et al.* 2014; Labbé and García 2013; Parin, Kirkan, and Erbaş 2018; Dickx and Vanrompay 2011; Wareing and Fernandes 2009; Beuchat *et al.* 2013; Kornacki 2010).

The pathogenic Clostridial species with the greatest impacts on human and animal health are *C. difficile, C. perfringens, C. tetani, C. botulinum*, and other species that produce botulinum toxins and other neurotoxins (Moore and Lacey, 2019). Birds, particularly waterfowls, are primarily affected by botulinum toxin C (Poulain and Popoff, 2019). Although specific to the Great Lakes area of North America, diver duck botulism caused by toxin E has been reported and it is linked to the fish botulism in the area (Brand *et al.* 1988; Lafrancois *et al.* 2011; Yule *et al.* 2006). Humans tend to be impacted by botulinum toxins A, B, E, and F (Moeller and Puschner 2007). The Clostridial species that produce botulinum toxins are well studied. Botulism, caused by botulinum neurotoxin (BoNT) is most frequently associated with *C. botulinum* but can occasionally arise from *C. butyricum* (Peck, 2002; Cassir *et al.* 2016). The disease can occur when BoNT-producing *Clostridium* species colonize the intestine or wounds of animals or humans and subsequently produce the toxin, or alternatively, when contaminated foods are ingested in which the toxin has already been formed. No reports of BoNT-producing *C. beijerinckii* were identified in the published literature. However, as mentioned above, a minority of closely-related *C. butyricum* strains harbor the *BoNT/E* gene and are capable of producing botulinum neurotoxin E (Hauser *et al.* 1992; Peck, 2002; Dupuy *et al.* 2006; Cassir *et al.* 2015; EFSA, 2011; Ghoddusi and Sherburn, 2010). The operon harboring the *BoNT/E* gene in *C. butyricum* is reportedly similar to that carried by type II E toxin-producing *C. botulinum* strains consistent with horizontal transfer events and recombinant events mediated by mobile genetic elements. Toxigenic *C. butyricum* have been responsible for a number of cases of infantile and infantile-like botulism arising from the production of the toxin in the intestine after germination and colonization of *C. butyricum* (*e.g.*, Abe *et al.* 2008; Aureli *et al.* 1986; Fenicia *et al.* 1999 and Dykes *et al.* 2015). The reports of infantile-like botulism are generally isolated cases (single or few cases) in immunocompromised individuals (*e.g.*, following intestinal surgery). Likewise, cases of food-borne botulism from the ingestion of *C. butyricum*-derived toxin type E are rare (Anniballi *et al.* 2002; Peck, 2002). The first report of an outbreak of food-borne botulism caused by toxin type E produced by *C. butyricum* was in China in 1994, when 6 people became ill after consuming a home-made salted and fermented paste of soybeans and wax gourds (Meng *et al.* 1997 and 1999). Subsequently, *C. butyricum* has been implicated in two earlier outbreaks of botulism following the consumption of soybeans in China and an incident in which 34 students contracted botulism after eating gram-flour based crisps in India (Peck, 2002). Thus, although some *Clostridium* species can produce medically relevant toxins, neurotoxins are rarely found and have not been reported in *C. beijerinckii.* Tetanus toxin (*Clostridium tetani*) and botulinum toxin (*Clostridium botulinum*) have not been associated with *C. beijerinckii.* Type E botulinum toxin has been observed in rare cases in *C. butyricum,* but it has not been associated with *C. beijerinckii.*

C. beijerinckii is genotypically and phenotypically different from *C. butyricum.* Cato *et al.* (1982) showed that *C. beijerinckii* strains shared similar electrophoretic protein patterns with each other but the patterns are distinctively different from those of *C. butyricum.* The authors have also found that *C. beijerinckii* were slower at adapting to grow in the laboratory setting than *C. butyricum*. Furthermore, Popoff *et al.* (1985) observed pathogenicity differences between *C. butyricum* and *C. beijerinckii.* The authors fed two strains of *C. butyricum* (isolated from newborns with neonatal necrotizing enterocolitis) and a strain of *C. beijerinckii* (isolated from dairy products) to gnotoxenic chickens. It was found that both *C. butyricum* strains caused cecitis in the chickens, with significantly increased cecum weight to body weight ratios and significantly reduced cecum content pH. However, in the gnotoxenic chickens fed with *C. beijerinckii*, the cecum weight to body weight ratios and cecum content pH did not differ from the controls. Although a low level of antibody response was detected due to the monoassociation of *C. beijerinckii* in cecum, no pathological changes were observed in the *C. beijerinckii* fed birds.

Overall, the available data indicate that *C. beijerinckii* strains are not associated with botulinum toxin or other neurotoxins, and only rarely cause infection in humans and animals. Under normal circumstances, it is a harmless organism. These data are consistent with the whole genome analyses (see Part 2.1.8) which confirmed the absence of the botulinum toxin and tetanus toxin biosynthetic operons.

6.6.1 Summary

Overall, the available information indicates that *C. beijerinckii* is a prevalent organism in the environment and in the microbiome of animals. Although generally benign, it can be an opportunistic pathogen and has been associated with infections in immunocompromised humans and animals. Despite the wide prevalence and exposure to *C. beijerinckii* in the environment and food, the number of reported cases of invasive infection caused by *C. beijerinckii* is low (see Appendix 17). As indicated in Part 2.1.8, interrogation of the whole genome sequence of *C. beijerinckii* ASCUSBR67 did not reveal the presence of any genetic element sequences that code for virulence factors or protein toxins.

6.7 Studies in Target Animals

The determination that *C. beijerinckii* ASCUSBR67 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 GIT organism.

C. butyricum, a neighboring species of *C. beijerinckii,* has been used as a DFM in rodents, chickens, swine, ruminants, and aquatic animals (Peeters *et al.* 2019; Sato *et al.* 2019; Qiao *et al.* 2020; Cheng *et al.* 2019; Zhao *et al.* 2013; Cao *et al.* 2019; Liu *et al.* 2019; Duan *et al.* 2017; Yang *et al.* 2010; Tran *et al.* 2020; Song *et al.* 2006; Zhang *et al.* 2017; EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) *et al.* 2021). Zhao *et al.* (2013) fed *C. butyricum* at a level of $1x10^9$ cfu/kg of feed with or without *E. faecium* to 1 day old chickens for 42 days. In comparison to control, average daily weight gain (ADG) and feed intake (ADFI) increased in birds fed *C. butyricum.* Zhang *et al.* (2011) observed no performance differences when they fed *C. butyricum* at the same level of $1x10^9$ cfu/kg of feed. While no performance improvements were observed, no deleterious health effects were noted. The species has also been used as an anti-diarrheal in Japan and investigated as a potential treatment for a wide variety of health conditions in humans (Seki *et al.* 2003; Tian *et al.* 2019; Shinnoh *et al.* 2013).

Three investigative studies in which chickens were fed a collection of microbes, including *C. beijerinckii* ASCUSBR67, 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 *C. beijerinckii* ASCUSBR67 and other microorganisms as potential direct fed microbials. In all studies, *C. beijerinckii* ASCUSBR67 was administered via feed in combination with at least one other microorganism. Overall, the study findings provide corroborative evidence that *C. beijerinckii* ASCUSBR67 is well-tolerated and without adverse effects.

6.7.1 Study 1 (Unpublished Study Report – Appendix 020)

Ninety-six pens of Cobb 500 broiler chicken (26 birds/pen) were divided into 8 treatment groups . The birds were allocated at random to one of the 8 treatment groups (12 Pens/treatment, 1 pen/replicate) and fed with either a mash mixed with calcium carbonate (control) or a mash mixed with a selection of microorganisms including *C. beijerinckii* ASCUSBR67 at various doses for 42 days. The birds were monitored daily. Observations included feed intake, pen body weight, spleen and liver weight, and cecum and illeal microbial content analysis.

Abnormal clinical health observations were considered minimal, incidental and not related to treatment for the duration of the study. No adverse effects were reported in any of the other variables measured for the duration of the study. Overall, the findings of the study corroborate the safety of *C. beijerinckii* ASCUSBR67 for chickens.

6.7.2 Study 2 (Unpublished Study Report – Appendix 021)

Ninety-six pens of Cobb 500 broiler chicken (35 birds/pen) were divided into 8 treatment groups. The birds were allocated at random to one of the 8 treatment groups (12 Pens/treatment, 1 pen/replicate) and fed with either a ration pelleted with calcium carbonate (control) or a ration pelleted with a selection of microorganisms including *C. beijerinckii* ASCUSBR67 for 42 days. The birds were monitored daily. Observations included feed intake, pen body weight, spleen and liver weight, and cecum and illeal microbial content analysis.

Abnormal clinical health observations were considered minimal, incidental and not related to treatment for the duration of the study. No adverse effects were reported in any of the other variables measured for the duration of the study. Overall, the findings of the study corroborate the safety of *C. beijerinckii* ASCUSBR67 for chickens.

6.7.3 Study 3 (Unpublished Study Report – Appendix 022)

Ninety-six pens of Cobb 500 broiler chicken (28 birds/pen) were divided into 8 treatment groups . The birds were allocated at random to one of the 8 treatment groups (12 Pens/treatment, 1 pen/replicate) and fed with either a ration pelleted with calcium carbonate (control) or a ration pelleted with a selection of microorganisms including *C. beijerinckii* ASCUSBR67 for 42 days. The birds were monitored daily. Observations included feed intake, pen body weight, spleen and liver weight, and cecum and illeal microbial content analysis.

Abnormal clinical health observations were considered minimal, incidental and not related to treatment for the duration of the study. No adverse effects were reported in any of the other variables measured for the duration of the study. Overall, the findings of the study corroborate the safety of *C. beijerinckii* ASCUSBR67 for chickens.

6.8 Summary and Critical Evaluation of Target Animal Safety

C. beijerinckii is a commensal bacteria in the gut of humans and animals. It occurs widely in soil and is commonly found in food. No reports of toxigenicity or pathogenicity associated with *C. beijerinckii* were identified in the published literature. However, reports of *C. beijerinckii* acting as an opportunistic pathogen in immunocompromised patients or patients suffering from severe trauma have been reported. Native Microbials has conducted an assessment of *C. beijerinckii* ASCUSBR67 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 *C. beijerinckii* ASCUSBR67 strains to antibiotics of veterinary and pharmaceutical relevance, and the absence of antimicrobial production has been demonstrated (see Part 2.1.6 and 2.1.7, and Appendices 004 and 005). Collectively, these data indicate that *C. beijerinckii* ASCUSBR67 spray dried powder should not be associated with any safety concerns for poultry under the intended conditions of use as a direct fed microbial.

6.9 Summary and Critical Evaluation of Human Food Safety

C. beijerinckii ASCUSBR67 has been unambiguously characterized as *C. beijerinckii* 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 further supported by the ubiquitous nature of *C. beijerinckii* and its natural occurrence in the GIT of animals. Based on the literature search conducted using Google Scholar, *C. beijerinckii* is part of the normal flora of healthy birds, including chickens, and its transference from GIT to meat and eggs has not been reported (Vanbelle *et al.* 1990; Proietti *et al.* 2006; Barrios *et al.* 2013; Crippen *et al.* 2008; Laviad-Shitrit *et al.* 2019). Although *C. beijerinckii* infection has been reported in patients with severe trauma or surgeries, none was from consumption. Literature review of food safety concerns revealed no reports of botulism or infection caused by *C. beijerinckii* despite its ubiquitous presence in the environment and bird GIT. Taken together, there is no reason to believe *C. beijerinckii* ASCUSBR67 would impact the quality or composition of any edible tissues or eggs when used as a direct fed microbial in poultry feed. Literature review also indicates that *C. beijerinckii* ASCUSBR67 spray dried powder has not been associated with any human food safety concerns under the intended conditions of use as a direct fed microbial in the feed of poultry and therefore would pose no risk for human consumption were it to somehow be found in meat or on eggs.

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)) and addressed those items accordingly. Based on the preponderance of evidence, Native Microbials' conclusion of safety is scientifically justified.

PART 7 – LIST OF SUPPORTING DATA AND INFORMATION

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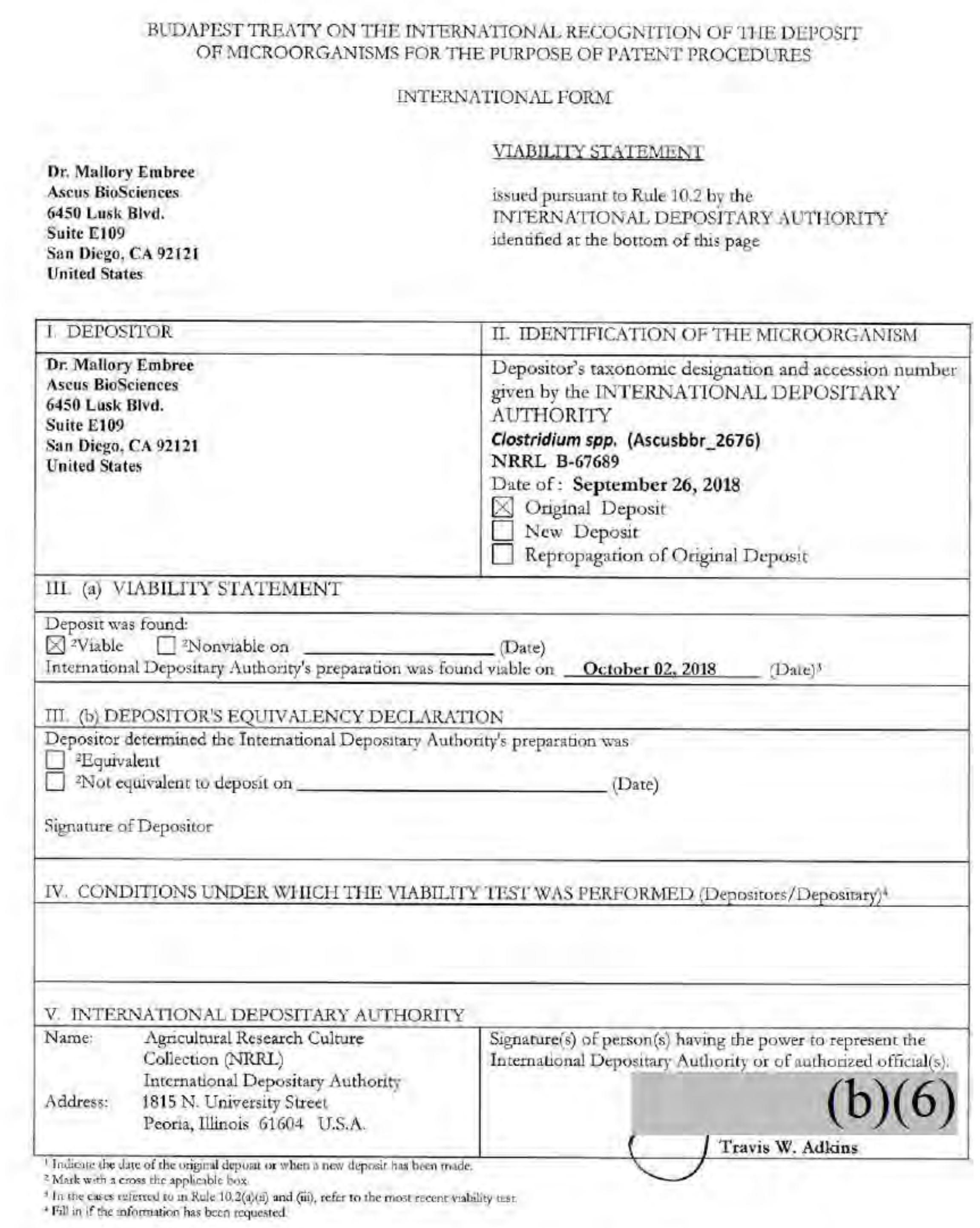
Appendix 001 Certificate of Deposition and Taxonomy Statement



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

INTERNATIONAL FORM

Dr. Mallory Embree
Ascus BioSciences
6450 Lusk Blvd.
Suite E109
San Diego, CA 92121
United States

VIABILITY STATEMENT

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

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Dr. Mallory Embree **Ascus BioSciences** **6450 Lusk Blvd.** **Suite E109** **San Diego, CA 92121** **United States**	Depositor's taxonomic designation and accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY ***Clostridium spp.*** **(Ascusbbr_2676)** **NRRL B-67689** Date of : **September 26, 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 **October 02, 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 ____________ (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): (b)(6) **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.

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

INTERNATIONAL FORM

Dr. Mallory Embree
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San Diego, CA 92121
United States

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: ***Clostridium spp.*** ***(Ascusbbr_2676)***	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **NRRL B-67689**

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 **September 26, 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.	(b)(6)

---

[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.

Appendix 002: Supplementary Methods and Results for *Clostridium beijerinckii* ASCUSBR67 In Vitro Biochemical Assays

Objectives:

The objective of this work was to assess the carbohydrate fermentation capabilities and metabolite production of *Clostridium beijerinckii* ASCUSBR67 through in vitro assays.

Methods:

Carbohydrate fermentation of *C. beijerinckii* ASCUSBR67 was qualitatively measured using the API 50CH carbon panel (b) (4) Results can be found in Table 1. *C. beijerinckii* ASCUSBR67 cells were grown to late exponential phase and recovered by centrifugation at 3,000 x g for 10 minutes. Cells were resuspended and (b) (4) (wt/vol) phenol red added as a pH indicator for acidification of carbohydrates (Avgustin et al. 1997).

Metabolite production of *C. beijerinckii* ASCUSBR67 fermentation run (b) (4) was measured at 8.2, 16.2, 24, 32.1, 40, 48, 54.1 and 60.3 hours using an (b) (4) series with RI detector operated at (b) (4) C. The column used was a (b) (4) with (b) (4) operated at (b) (4). The mobile phase was (b) (4) N Sulfuric Acid (b) (4) mL concentrated sulfuric to (b) (4)) at a flow rate of (b) (4) mL/min. Pure standards were used at varying concentrations to generate a standard curve.

Results:

C. beijerinckii ASCUSBR67 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.

Appendix 002: Supplementary Methods and Results for *Clostridium beijerinckii* ASCUSBR67 In Vitro Biochemical Assays

Table 1. Carbon Source Fermentation by *C. beijerinckii* ASCUSBR67

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	No Growth
D-Arabinose	No Growth	Methyl-aD-Mannopyranoside	No Growth
L-Arabinose	Growth	Methyl-aD-Glucopyranoside	Growth
D-Ribose	No Growth	N-AcetylGlucosamine	Growth
D-Xylose	Growth	Amygdalin	Growth
L-Xylose	No Growth	Arbutin	Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	Growth
Methyl-BD-xylopyranoside	Growth	Salicin	Growth
D-Galactose	Growth	D-Cellobiose	Growth
D-Glucose	Growth	D-Maltose	Growth
D-Fructose	Growth	D-Lactose	Growth
D-Mannose	Growth	D-Melibiose	No Growth
L-Sorbose	No Growth	D-Saccharose	Growth
L-Rhamnose	No Growth	D-Trehalose	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	Growth	D-Arabitol	No Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	Growth	Potassium Gluconate	No Growth
D-Turanose	Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth	Potassium 5-KetoGluconate	No Growth

Table 2. Metabolite Production by *C. beijerinckii* ASCUSBR67 on Complex Media with Glucose

	Glucose	**Pyruvic Acid**	**Succinic Acid**	**Lactic Acid**	**Glycerol**	**Acetic Acid**	**Propionic Acid**	**Ethanol**	**Butyric Acid**	**1-Butanol**
Fermentation Time (hrs)	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**
8.2	(b) (4)									
16.2										
24.0										
32.1										
40.0										
48.0										
54.1										
60.3										

Conclusions:

In vitro assays demonstrate that *C. beijerinckii* ASCUSBR67 grows on a variety monosaccharides, disaccharides, sugar alcohols, and various other glycosides including L-arabanose, D-xylose, Methyl-BD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, starch, glycogen, gentiobiose,D-turanose, Methyl-aD-Glucopyranoside, N-AcetylGlucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose. When grown on complex media with glucose *C. beijerinckii* ASCUSBR67 produces acetic acid, butyric acid, and butanol as major fermentation products.

Signed: (b)(6)________________________________ Date:________________

References

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Appendix 003A: *Clostridium beijerinckii* 16S and WGS Genomic Identification

Objectives

The objective of this work was to determine the identity of *Clostridium beijerinckii* ASCUSBR67 using genomic methods.

Methods

For 16S sequence analysis, the 16S gene was amplified from *C. beijerinckii* ASCUSBR67 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 *C. beijerinckii* ASCUSBR67 by a modified Sambrook phenol-chloroform extraction/purification protocol (Jain et al. 2018). Short read sequencing libraries were prepared using the (b) (4), (b)(6)) by manufacturer’s recommended protocol and the resulting libraries were sequenced (1x300bp) on an (b) (4). In parallel, long read libraries were prepared from the same extracted DNA using the (b) (4) (b) (4) (b)(6) using a modified version of the protocol outlined by (Jain et al. 2018) and 1D sequenced on the MinION (R9.4 flow cell) by (b) (4). Full details of the genome assembly can be found in appendix 003C. MUMmer 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 *C. beijerinckii* ASCUSBR67

Genus species (GenBank accession #)	Percent Match	Percent Coverage
Clostridium diolis DSM 15410 (CP043998)	99.7%	100%
Clostridium beijerinckii NRRL B-598 (CP011966)	99.7%	100%
Clostridium beijerinckii BAS/B3/I/124 (CP016090)	99.7%	100%
Clostridium beijerinckii ATCC 35702 (CP006777)	99.7%	100%
Clostridium beijerinckii JCM 8026 (AB647333)	99.7%	100%

Whole genome average nucleotide identity (ANI) was used to confirm the 16S identification. Genomes for ANI comparison were selected based on 16S similarity to *C. beijerinckii* ASCUSBR67. As shown in Table 2, the *C. beijerinckii* ASCUSBR67 genome most closely matched *Clostridium beijerinckii* ATCC

35702. Both *C. beijerinckii* and *C. diolis* matched *C. beijerinckii* ASCUSBR67 at 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 MUMmer

Average Nucleotide Identity (ANI) of Related *Clostridium* Species to *C. beijerinckii* ASCUSBR67		
Genus species (assembly)	**ANI (%)**	**Coverage (%)**
Clostridium beijerinckii ATCC 35702 (GCA_000767745)	98.2	88.6
Clostridium diolis WST (GCA_003015255)	97.6	83.5
Clostridium diolis NJP7 (GCA_002176895)	97.5	85.4
Clostridium beijerinckii NRRL B-598 (GCA_000506785)	97.5	81.4
Clostridium butyricum TOA (GCA_001646605)	84.9	8.9
Clostridium butyricum KNUL09 (GCA_001456065)	84.8	8.8
Clostridium acetobutylicum JB200 (GCA_002216285)	84.6	1.6
Clostridium acetobutylicum ATCC 824 (GCA_000008765)	84.3	1.4

Conclusions

Whole genome ANI and 16s comparisons suggest that *C. beijerinckii* ASCUSBR67 represents a strain of *C. beijerinckii*. *C. diolis* also provided a match within the species cutoffs for 16S and whole genome ANI. 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 Native Microbials 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
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RID: 1K25PC0G01R


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Length=300


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Appendix 003C: Supplementary Whole Genome Analysis Methods and Read Quality Metrics *Clostridium beijerinckii* ASCUSBR67

The *C. beijerinckii* ASCUSBR67 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) and (b) (4) quality metrics for the (b) (4) and (b) (4) reads respectively, metrics generated by (b) (4) for the completed assembly, and a visualization of the assembly graph generated by Bandage.

Assembly Pipeline in Detail

(b) (4)

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

(b) (4)

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

(b) (4)

Read distribution as related to quality score

(b) (4)

Metrics for *C. beijerinckii* ASCUSBR67 (b) (4) **reads as generated by** (b) (4).

General Summary

(b) (4)

Number, Percentage, and Megabases of Reads Above Quality Cutoffs

(b) (4)

Longest Reads in Base Pairs (base pairs)

(b) (4)

Assembly Statistics as reported by (b) (4)

(b) (4)

Flye Assembly Graph as Visualized by Bandage.

(b) (4)

References

De Coster, W., D'Hert, S., Schultz, D.T., Cruts, M. and Van Broeckhoven, C., 2018. NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics*, *34*(15), pp.2666-2669.

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Gurevich, A., Saveliev, V., Vyahhi, N. and Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics*, *29*(8), pp.1072-1075.

Kolmogorov, Mikhail, Jeffrey Yuan, Yu Lin, and Pavel A. Pevzner. 2019. "Assembly of Long, Error-Prone Reads Using Repeat Graphs." *Nature Biotechnology* 37 (5): 540–46.

Koren, S., Walenz, B.P., Berlin, K., Miller, J.R., Bergman, N.H. and Phillippy, A.M., 2017. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome research*, *27*(5), pp.722-736.

Vaser, R., Sović, I., Nagarajan, N. and Šikić, M., 2017. Fast and accurate de novo genome assembly from long uncorrected reads. *Genome research*, *27*(5), pp.737-746.

Walker, B.J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., Cuomo, C.A., Zeng, Q., Wortman, J., Young, S.K. and Earl, A.M., 2014. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PloS one*, *9*(11), p.e112963.

Wick, R.R., Schultz, M.B., Zobel, J. and Holt, K.E., 2015. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics*, *31*(20), pp.3350-3352.

(b) (4)

FINAL REPORT

TITLE: **Susceptibility Profile of Gram Positive Organism from Native Microbials: *Clostridium beijerinckii* (BR67)**

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

SPONSOR:
Native Microbials[1]
10255 Science Center Drive
Suites C2
San Diego, CA 92121

INVESTIGATOR: (b) (4), (b)(6)

VERSION: FINAL

SIGNATURE: (b)(6)
Principal Investigator

2/5/21
Date

---

[1] The sponsor name changed from Ascus Biosciences as indicated in the protocol to Native Microbials.

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TABLE OF TABLES

1. OBJECTIVES

To determine the Antimicrobial Susceptibility Profile of the Gram positive Ascus organism *Clostridium beijerinckii* ASCUSBR67 (Broiler-67) production strain to multiple 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 document M11 to the extent to which they are applicable as detailed in the protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) were referenced for determining non-wildtype 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*[2] as applicable and as detailed in the protocol.

3. STUDY SITE

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

4. MATERIALS AND METHODS

4.1. Isolates

The sponsor provided the Broiler-67 (BR67) production strain (i.e., *Clostridium beijerinckii* ASCUSBR67) on May 22, 2020. The culture was streaked to trypticase soy agar with 5% sheep blood agar (BA) and incubated in an anaerobic atmosphere to verify that the organism was viable, pure and morphologically typical of the purported species. The organism was additionally streaked to BA and incubated in 5±2% CO_2 to assure it was susceptible to oxygen.

5. SUSCEPTIBILITY PROFILE

5.1. Procedure

The procedures listed in the protocol were written to comply with CLSI document M11-A9 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Ninth Edition for anaerobes using the agar dilution procedure.

The organism was susceptibility tested according to (b) (4) (Appendix A).

---

[2] EFSA Journal 2018; 16(3): 5206

5.2. Preparation of agar dilution plates

Agar dilution plates were prepared by (b) (4). with doubling dilutions of the following antimicrobials.

Table 1. Antimicrobials and Doubling Dilution Ranges

Antimicrobial	Abbreviation	Doubling Dilution Concentration Range (µg/mL)
Ampicillin	AMP	0.5-128
Vancomycin	VAN	0.12-16
Gentamicin	GEN	0.12-32
Kanamycin	KAN	0.5-64
Streptomycin	STR	0.5-64
Erythromycin	ERY	0.12-16
Clindamycin	CLI	0.12-32
Tetracycline	TET	0.06-64
Chloramphenicol	CHL	0.5-64

Plates were prepared according to procedures in (b) (4)(Appendix A) using Supplemented Brucella agar as recommended by CLSI document M11-A9. Agar dilution plates were used on the date of preparation.

5.3. Incubation and Interpretation of Susceptibility Tests

Agar dilution plates were incubated and interpreted according to (b) (4) (Appendix A).

5.4. Quality Control

Bacteroides fragilis (ATCC 25285, (b) (4), and *Clostridioides difficile* (ATCC 700057, (b) (4) were tested on each testing date.

With each testing date, one dilution series was inoculated with sterile broth to assess procedural and media sterility. Inoculum counts were conducted according to (b) (4) (b) (4) (Appendix A).

6. DISPOSITIONS

6.1. Agar dilution plates were discarded after their expiration.

6.2. The isolate was discarded after autoclaving. No retention culture was maintained.

7. RESULTS

Susceptibility results of the *Clostridium beijerinckii* ASCUSBR67 (Broiler-67) isolate are presented in Table 2. *Clostridium* does not have specific EFSA Cut-off values; therefore, the Other Gram-positive Cut-off values are referenced. The isolate would be considered resistant to streptomycin and chloramphenicol according to the EFSA criteria for Other Gram-positive. Chloramphenicol would be considered wild-type or susceptible according to the EUCAST and CLSI Breakpoints. There are no EUCAST or CLSI breakpoints for streptomycin.

Table 2. Listing of Broiler-67 MIC results and Cut-off Values or Breakpoints

Antimicrobial	*Clostridium beijerinckii* (Broiler-67) MIC (µg/mL)	EFSA Microbiological Cut-off Values (µg/mL) for Other Gram-positive[3]	EUCAST Resistant Breakpoints (µg/mL) for Gram-positive anaerobes[4]	CLSI Resistant Breakpoints (µg/mL) for anaerobes[5]
Ampicillin	≤0.5	8	>8	≥2
Vancomycin	2	4	>2	---
Gentamicin	4	4	---	---
Kanamycin	4	16	---	---
Streptomycin	16	8	---	---
Erythromycin	1	1	---	---
Clindamycin	2	4	>4	≥8
Tetracycline	2	2	---	≥16
Chloramphenicol	8	4	>8	≥32

The MIC results of the quality control organisms were within the expected values as indicated in Table 3. The inoculum counts were within specifications.

---

[3] Guidance on microorganisms used as feed additives or as production organisms; EFSA Journal 2018;16(3):5206.
[4] EUCAST breakpoints for Gram-positive anaerobes, Clinical Breakpoint Tables V.11.0
[5] CLSI M100S-30th Ed. Table 2J

Table 3. Quality Control Organism Results and Acceptable MIC Ranges

	MIC and CLSI Acceptable Ranges (μg/mL)[6]			
	Bacteroides fragilis (b) (4)		*Clostridioides difficile* (b) (4)	
	ATCC 25285		ATCC 700057	
Antimicrobial	MIC	Acceptable Range	MIC	Acceptable Range
Ampicillin	(b) (4)	16-64	(b) (4)	1-4
Vancomycin	[redacted]	---	[redacted]	0.5-4
Gentamicin	[redacted]	---	[redacted]	---
Kanamycin	[redacted]	---	[redacted]	---
Streptomycin	[redacted]	---	[redacted]	---
Erythromycin	[redacted]	---	[redacted]	---
Clindamycin	[redacted]	0.5-2	[redacted]	2-8
Tetracycline	[redacted]	0.125-0.5	[redacted]	---
Chloramphenicol	[redacted]	2-8	[redacted]	---

---

[6] Obtained from CLSI document M-100

APPENDIX A. PROTOCOL AND STANDARD OPERATING PROCEDURES

(b) (4)

STUDY PROTOCOL

TITLE:	**Susceptibility Profile of Gram Positive Organisms from Ascus Biosciences ASCUSDY10, ASCUSDY19, ASCUSBF65 and ASCUSBR67**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 6450 Lusk Blvd Suites E109/209 San Diego, CA 92121
INVESTIGATOR:	(b) (4), (b)(6)
VERSION:	FINAL

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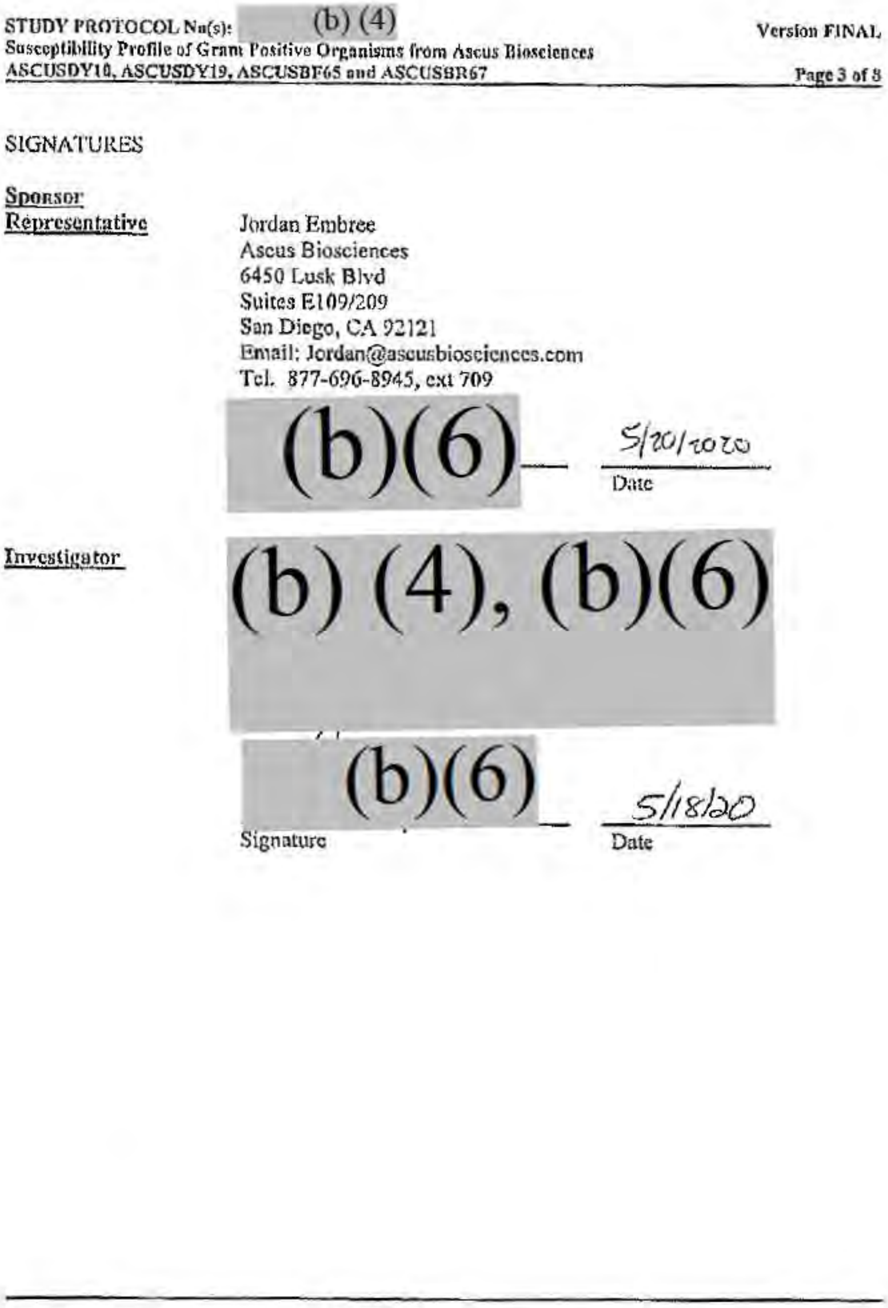

SIGNATURES

Sponsor
Representative

Jordan Embree
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6450 Lusk Blvd
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Tel. 877-696-8945, ext 709

(b)(6) 5/20/2020
Date

Investigator

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

(b)(6) 5/18/20
Signature Date

1. OBJECTIVES

To determine the Susceptibility Profile of Gram positive Ascus organism(s): *Ruminococcus bovis* ASCUSDY10 (Dairy-10), *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19), *Chordacoccus ruminofurens* ASCUSBF65 (Beef-65), and *Clostridium beijerinckii* ASCUSBR67 (Broiler-67) production strains to multiple antimicrobials.

2. STUDY TIMELINE

Anticipated study dates are:	
Susceptibility Testing:	May 2020

3. STANDARDS OF COMPLIANCE

This study will be conducted in a GSP-like (Good Scientific Practice) manner in accordance with testing facility SOPs and to CLSI document M11 to the extent to which they are applicable as detailed in this protocol. European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints or epidemiological cutoff values (ECOFFs) may be referenced for determining non-wildtype 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*[1] as applicable and as detailed in this protocol.

4. STUDY SITE

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

5. MATERIALS AND METHODS

5.1. Isolates

The sponsor will provide the production strain(s) to test. Lot number of each of the strains will be identified in the final report. The cultures will be streaked to an appropriate media (e.g., trypticase soy agar with 5% sheep blood agar [BA], Columbia Blood agar [CBA], Fastidious Blood Agar [FAA], or other agar that adequately supports the growth of the organism) to verify that the organisms are viable, pure and morphologically typical of the purported species. All organisms are anaerobic and will additionally be streaked to the appropriate agar and will be incubated aerobically and/or in 5±2% CO_2 to assure they are susceptible to oxygen.

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[1] EFSA Journal 2018; 16(3): 5206

6. SUSCEPTIBILITY PROFILE

6.1. Procedure

Each production strain will be tested. Additional strains may be tested upon direction of the sponsor. The MIC values of the production strain(s) will be compared to the "other Gram-positive" cut-off values published by EFSA or CLSI/EUCAST breakpoints to determine if a non-wild type strain (defined as potentially harboring resistance mechanisms) or if non-susceptible [refer to Table 3].

The procedures listed in this protocol were written to comply with CLSI document M11-A9 entitled Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Ninth Edition for anaerobes using the agar dilution procedure.

The isolates will be MIC tested according to (b) (4)

6.2. Preparation of agar dilution plates

Agar dilution plates will be prepared by (b) (4), with doubling dilutions of the following antimicrobials.

Table 1. Antimicrobials and Doubling Dilution Ranges

Antimicrobial	Abbreviation	Doubling Dilution Concentration Range (µg/mL)
Ampicillin	AMP	0.5-128
Vancomycin	VAN	0.12-16
Gentamicin	GEN	0.12-32
Kanamycin	KAN	0.5-64
Streptomycin	STR	0.5-64
Erythromycin	ERY	0.12-16
Clindamycin	CLI	0.12-32
Tetracycline	TET	0.06-64
Chloramphenicol	CHL	0.5-64

Plates will be prepared according to procedures in (b) (4) using Supplemented Brucella agar as recommended by CLSI document M11-A9. Agar dilution plates will be given an expiration date of one month from the date of preparation and will be considered acceptable as long as the quality control organism MIC is within the acceptable range.

6.3. Incubation and Interpretation of Susceptibility Tests

Agar dilution plates will be incubated and interpreted according to (b) (4)

6.4. Quality Control

Bacteroides fragilis (ATCC 25285, (b) (4) and *Clostridioides difficile* (ATCC 700057, (b) (4)) will be tested on each testing date when testing anaerobes.

Below are listings of the available CLSI acceptable ranges for each of the quality control organism/antimicrobial combinations.

With each testing date, one dilution series should be inoculated with sterile broth to assess procedural and media sterility. Results of the sterility testing should be recorded on the appropriate form. Conduct inoculum counts for bacteria according to (b) (4)

Table 2. Quality Control Organisms and Acceptable MIC ranges for Anaerobes[2]

	SBA	
Antimicrobial	***Bacteroides fragilis*** **(Br-1) ATCC 25285**	***Clostridioides difficile*** **(CL-16) ATCC 700057**
Ampicillin	16-64	1-4
Vancomycin	---	0.5-4
Gentamicin	---	---
Kanamycin	---	---
Streptomycin	---	---
Erythromycin	---	---
Clindamycin	0.5-2	2-8
Tetracycline	0.125-0.5	---
Chloramphenicol	2-8	---

[2] Obtained from CLSI document M100

Table 3. Listing of EFSA Microbiological Cut-off Values and EUCAST and CLSI Breakpoints for Bacteria

	EFSA Microbiological Cut-off Values (µg/mL) [3]	**EUCAST Resistant Breakpoints (µg/mL)**[4]	**CLSI Resistant Breakpoints (µg/mL)**[5]
Antimicrobial	Other Gram-positive	Gram-positive anaerobes	Anaerobes
Ampicillin	8	>8	≥2
Vancomycin	4	---	---
Gentamicin	4	---	---
Kanamycin	16	---	---
Streptomycin	8	---	---
Erythromycin	1	---	---
Clindamycin	4	---	---
Tetracycline	2	---	≥16
Chloramphenicol	4	---	≥32

[3] Guidance on microorganisms used as feed additives or as production organisms ; EFSA Journal 2018;16(3):5206.
[4] EUCAST breakpoints for Gram-positive anaerobes, Clinical Breakpoint Tables V.10.0
[5] CLSI M100S-30th Ed. Table 2J

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 study report will be issued for each of the production strain(s) for each of the tests performed. Hence, a total of four reports will be issued.

If additional production strains are tested, reports will be issued in a similar manner, depending upon the tests required.

8. DISPOSITIONS

8.1. Agar dilution plates will be discarded after their expiration.

8.2. Isolates will be discarded after autoclaving. No retention cultures 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.

CONFIDENTIAL

(b) (4)
Revision #3
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AGAR DILUTION SUSCEPTIBILITY TESTING

Section 1. Media and Preparation

(b) (4)

A. Media

(b) (4)

AGAR DILUTION SUSCEPTIBILITY TESTING

B. Antimicrobial dilution procedure

(b) (4)

C. Preparing agar plates

(b) (4)

AGAR DILUTION SUSCEPTIBILITY TESTING

(b) (4)

Section 2. Inoculum Preparation

(b) (4)

Section 3. Inoculation and Incubation of Test Plates

A. Filling the Steers replicator

(b) (4)

B. Inoculation of plates

(b) (4)

AGAR DILUTION SUSCEPTIBILITY TESTING

(b) (4)

C. Incubation of plates

(b) (4)

D. Cleaning of Steers Replicator

(b) (4)

Section 4. Reading and Interpretation of Plates

(b) (4)

AGAR DILUTION SUSCEPTIBILITY TESTING

Section 5. Quality Control

(b) (4)

(b) (4)
Revision #3
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AGAR DILUTION SUSCEPTIBILITY TESTING

(b) (4)

Prepared by: (b) (9), (b)(6), (b) (8)	Reviewed by: (b)(6)
Date: 5/26/16	Date: 5/26/16
Reference Verification by: (b)(6)	Approved by: (b)(6)
Date: 5/26/16	Date: 5/27/16 Effective Date: 6/10/16

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(b) (4)
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SUSCEPTIBILITY TESTING OF ANAEROBES

Section 1. General Considerations

(b) (4)

(b) (4)

(b) (4)

Section 2. Inoculum preparation

(b) (4)

(b) (4)
Revision #4
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SUSCEPTIBILITY TESTING OF ANAEROBES

Section 3. Agar Dilution

1. Media

(b) (4)

2. Inoculation of plates

(b) (4)

3. Reading plates

(b) (4)

SUSCEPTIBILITY TESTING OF ANAEROBES

(b) (4)

Section 4. Broth microdilution - (b) (4) prepared plates

1. Preparation of plates

(b) (4)

2. Inoculation of plates

(b) (4)

3. Interpretation of MIC

(b) (4)

SUSCEPTIBILITY TESTING OF ANAEROBES

Section 5. Broth microdilution – Sensititre

1. Inoculation of plates

(b) (4)

2. Interpretation of MIC

(b) (4)

Section 6. Quality Control Tests

1. Organisms

(b) (4)

2. Acceptable ranges

(b) (4)

(b) (4)
Revision #4
Page 5 of 5

SUSCEPTIBILITY TESTING OF ANAEROBES

3. Sterility checks

(b) (4)

4. Inoculum counts

Inoculum counts will be performed as described in (b) (4)

Reference:

CLSI Publication No. M11-A8, "Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Eighth Edition," Vol. 32, No. 5.

Prepared by: (b)(6)

Date: 11/24/14

Reference Verification by: (b)(6)

Date: 12/8/14

Reviewed by (b)(6)

Date: 12/9/14

Approved by: (b)(6)

Date: 12/9/14

Effective Date: 12/23/14

CONFIDENTIAL

(b) (4)
Revision #10
Page 1 of 5

ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

Section 1. General

(b) (4)

Section 2. Agar Dilution

A. General:

(b) (4)

ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

(b) (4)

B. Anaerobic Bacteria:

(b) (4)

(b) (4)
Revision #10
Page 3 of 5

ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

For *Clostridioides difficile*:

(b) (4)

Section 3. Disk Diffusion and/or E Test

(b) (4)

Section 4. Microdilution (MRI prepared MIC plates and/or Sensititre)

A. Bacteria

(b) (4)

ACCURACY VERIFICATION OF STANDARDIZED CULTURES FOR SUSCEPTIBILITY TESTING

B. Mycoplasma

(b) (4)

C. Anaerobic Bacteria

(b) (4)

ACCURACY VERIFICATION OF STANDARDIZED CULTURES
FOR SUSCEPTIBILITY TESTING

Section 5. Interpretation of Results

(b) (4)

Prepared by: (b)(6)
Date: 5/27/20

Reviewed by: (b)(6)
Date: 5/27/20
Approved by: (b)(6)
Date: 5/28/20
Effective Date: 6/4/20

(b) (4)

FINAL REPORT

TITLE: **Characterization of Native Microbials *Clostridium beijerinckii* (Broiler-67) Production Strain: Absence of Antimicrobial Activity**

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

CONDUCT DATES:

Receipt of supernatant: October 30, 2020
Testing of supernatant: October 29, 2020 through November 6, 2020

SPONSOR:

Native Microbials[1]
10255 Science Center Drive
Suites C2
San Diego, CA 92121

INVESTIGATOR: (b) (4), (b)(6)

VERSION: FINAL

SIGNATURE: (b)(6)

Donald J. Bade
Principal Investigator

12/17/20
Date

---

[1] The sponsor name changed from Ascus Biosciences as indicated in the protocol to Native Microbials.

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LIST OF TABLES AND APPENDICES

OBJECTIVES

To determine the antimicrobial properties of the *Clostridium beijerinckii* (Broiler-67) production strain supernatant.

STANDARDS OF COMPLIANCE

This study was conducted 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 growth medium supernatant from the *Clostridium beijerinckii* Broiler-67 (Lot number BR67_BTR_2244BB_EO1 CG29OCT2020) was prepared by the sponsor using centrifugation followed by sterile filtration with a 0.45µm membrane. The sample was received on October 30, 2020. The material was kept refrigerated (2-8°C) and used 5 days after receipt.

ANTIMICROBIAL PROPERTIES

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)

1.2. Disk Preparation

(b) (4)

1.3. Incubation

(b) (4)

1.4. Interpretation

(b) (4)

1.5. Quality Control

(b) (4)

DISPOSITIONS

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 Broiler-67 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 Broler-67 Supernatant and Controls

Organism	ATCC number	(b) (4) code	Zone Diameter for the indicated solution (mm)		
			Broiler-67 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 Broiler-67 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 *Clostridium beijerinckii* (Broiler-67) Production Strain: Absence of Antimicrobial Activity**
INVESTIGATOR'S STUDY NUMBER:	(b) (4)
SPONSOR:	Ascus Biosciences 10255 Science Center Drive, Suite C2 San Diego, CA 92121-1117
INVESTIGATOR:	(b) (4), (b)(6)
VERSION:	FINAL

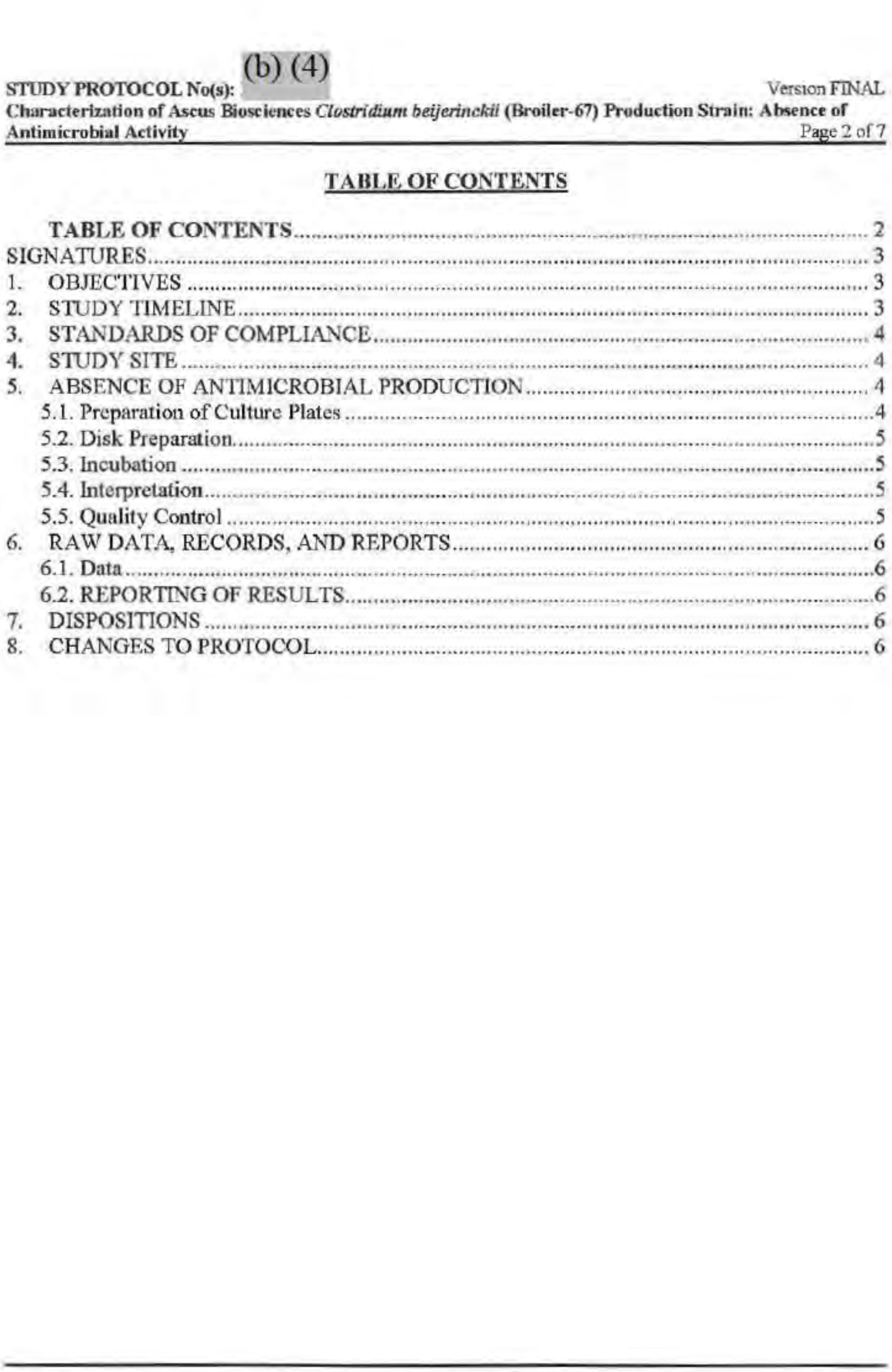
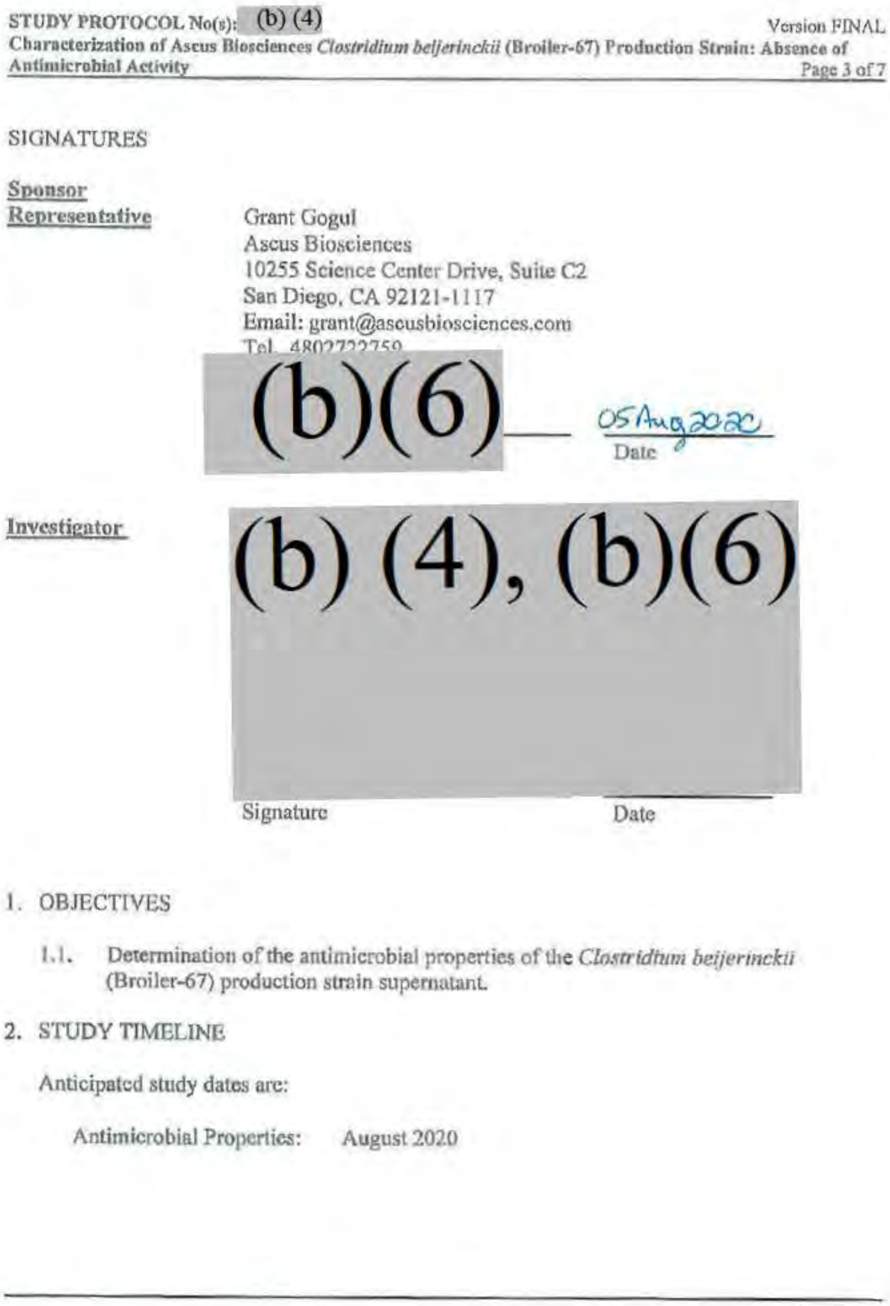


TABLE OF CONTENTS



SIGNATURES

Sponsor Representative

Grant Gogul
Ascus Biosciences
10255 Science Center Drive, Suite C2
San Diego, CA 92121-1117
Email: grant@ascusbiosciences.com
Tel.

(b)(6) 05Aug2020
Date

Investigator

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

Signature Date

1. OBJECTIVES

   1.1. Determination of the antimicrobial properties of the *Clostridium beijerinckii* (Broiler-67) production strain supernatant.

2. STUDY TIMELINE

Anticipated study dates are:

Antimicrobial Properties: August 2020

3. 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.

4. STUDY SITE

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

5. ABSENCE OF ANTIMICROBIAL PRODUCTION[1]

The presence of antimicrobial activity in the growth medium from the *Clostridium beijerinckii* (Broiler-67) supernatant will be tested. (b) (4) (b) (4)

5.1. Preparation of Culture Plates

The following six organisms will be tested against each 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)

---

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

(b) (4)

5.2. Disk Preparation

(b) (4)

5.3. Incubation

(b) (4)

5.4. Interpretation

(b) (4)

5.5. Quality Control

(b) (4)

6. RAW DATA, RECORDS, AND REPORTS

6.1. 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)

6.2. 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.

7. DISPOSITIONS

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

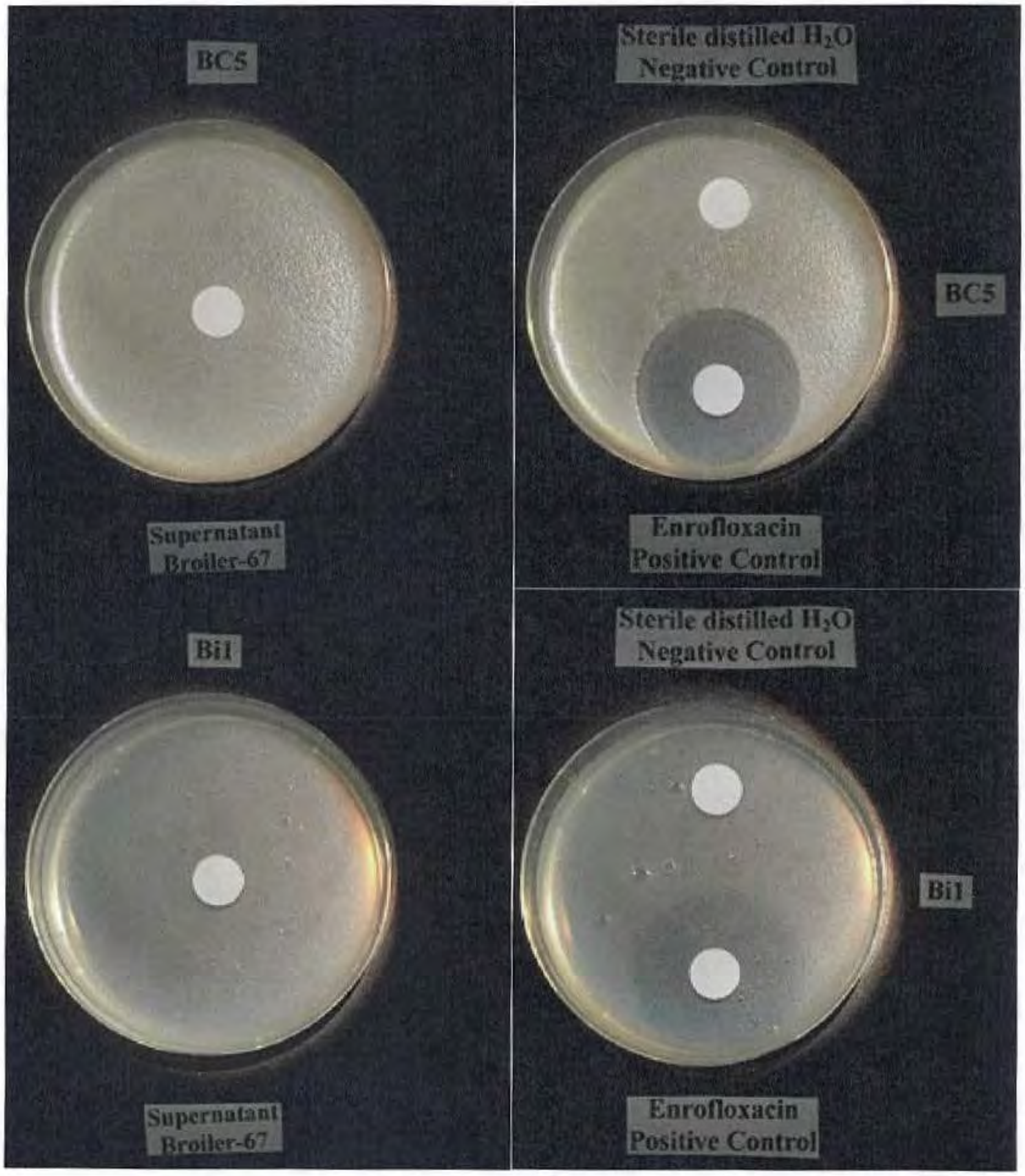
7.2. MIC plates will be discarded after their expiration.

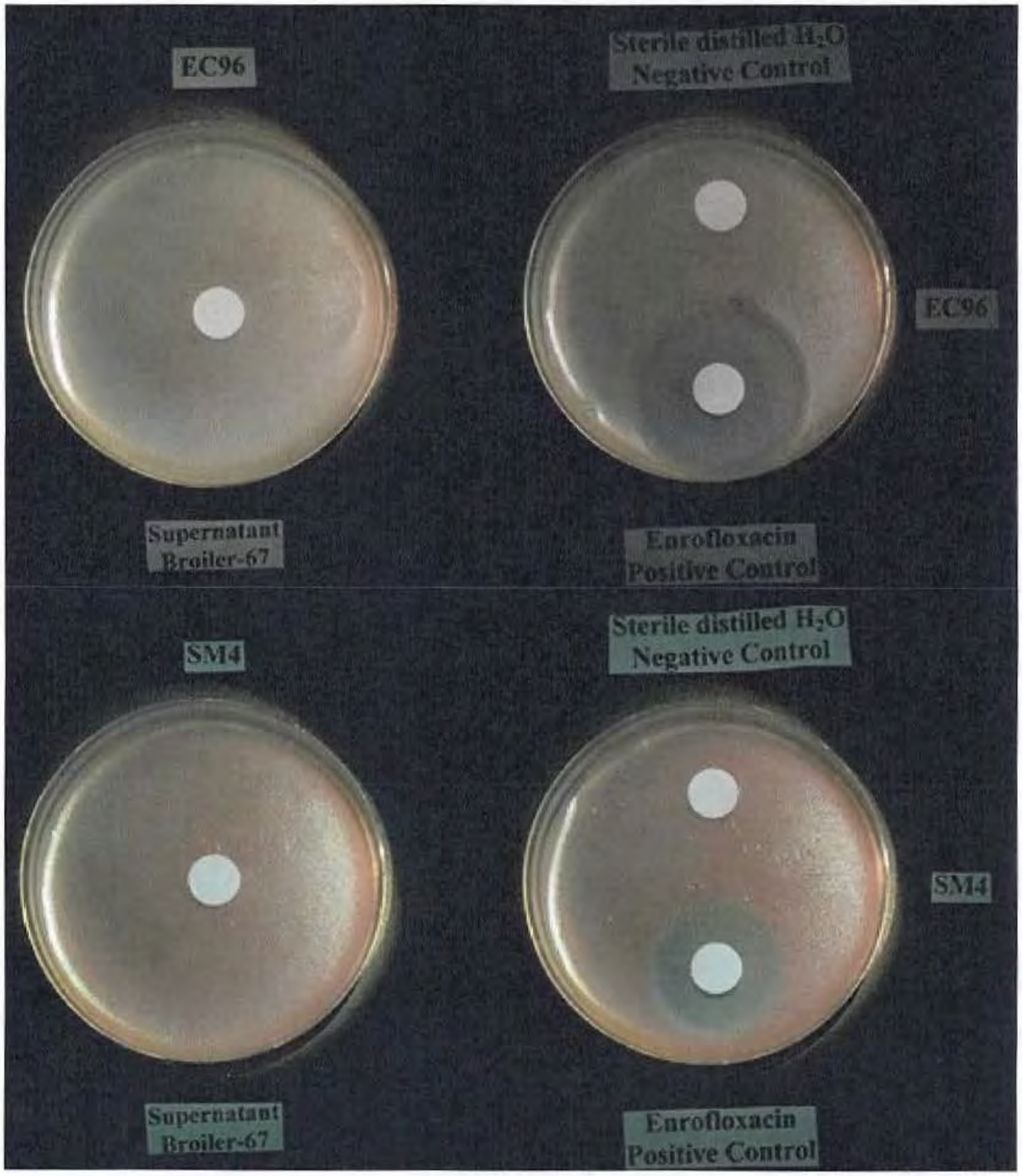
7.3. Isolates will be discarded after autoclaving. No retention cultures will be maintained.

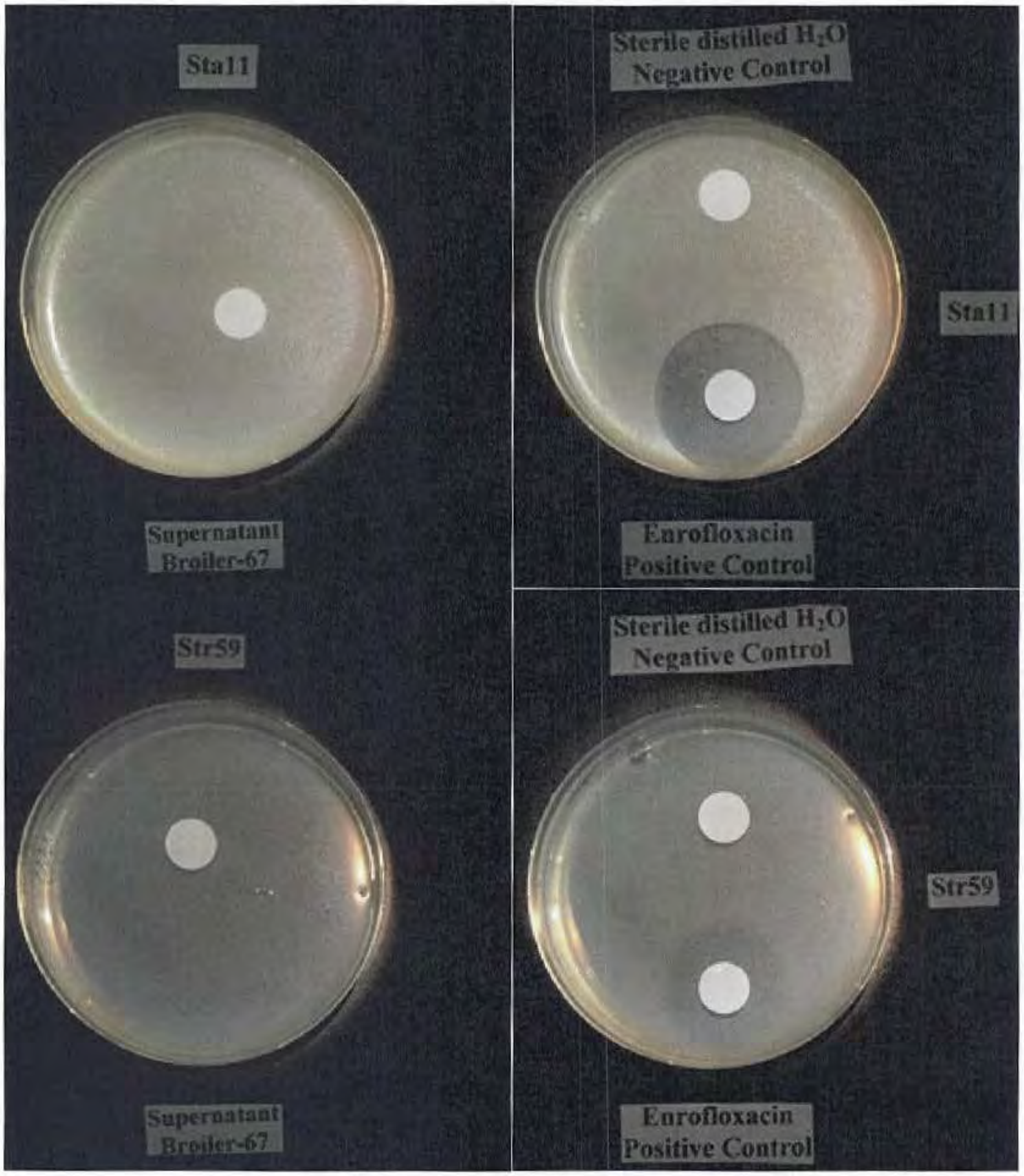
8. CHANGES TO PROTOCOL

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




EC96
Sterile distilled H2O Negative Control
EC96
Supernatant Broiler-67
Enrofloxacin Positive Control
SM4
Sterile distilled H2O Negative Control
SM4
Supernatant Broiler-67
Enrofloxacin Positive Control


Sta11
Supernatant Broiler-67
Sterile distilled H₂O Negative Control
Sta11
Enrofloxacin Positive Control
Str59
Supernatant Broiler-67
Sterile distilled H₂O Negative Control
Str59
Enrofloxacin Positive Control

(b) (4)
Method Folder

Method Identifier (b) (4)
Issue Date 2/28/19
Revision No.2

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) 1000/2000 ICP-MS

6.0 Method Folder

6.1 Instrumentation

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

6.2 Safety

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

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

6.3 Definitions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

7.0 Method Work Level Instructions

7.1 Equipment and materials

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

7.2 Reagents and Standards

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

(b) (4)

7.2.1 Working solutions

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

(b) (4)

(b) (4)

7.3 Test Sample Treatment

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

(b) (4)

[redacted]

(b) (4)

7.4 Instrumentation Set up

(b) (4)

(b) (4)

Appendix A - Calibration Concentrations

Calibrations

(b) (4)

Hg	1.6	0.2

(b) (4)

(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: 12/21/2020 11:46:20
Reported: 1/6/2021 16:39:41
P.O. #: N/A
Page: **1 of 1**

Report of Results

(b) (4) **Analysis Date:** 2020/12/21 **Receiving Temperature:** 7.6C **Sample Condition:** Okay

Description: BR21 Lot: 1801.2049

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

(b) (4) **Analysis Date:** 2020/12/21 **Receiving Temperature:** 7.6C **Sample Condition:** Okay

Description: BR67 Lot: 1801.2048

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

(b) (4) **Analysis Date:** 2020/12/21 **Receiving Temperature:** 7.6C **Sample Condition:** Okay

Description: BR67 Lot: 1801.2047

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

(b) (4) **Analysis Date:** 2020/12/21 **Receiving Temperature:** 7.6C **Sample Condition:** Okay

Description: BR67 Lot: 1801.2046

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

Login By: (b)(6) **Entered By:** (b)(6) **Approved** (b)(6)

(b) (4)

(b) (4)

CERTIFICATE OF ANALYSIS

Customer: (b) (4)

Product: (b) (4)

Sales Order #: (b) (4)

Purchase Order #: (b) (4)

Shipment Date: 6/24/2019

Lot #: 05-02-19-01

	Analysis
(b) (4) wt.%:	(b) (4)
Specific Gravity @ 60°F, g/mL:	
Appearance:	Clear, Colorless

NSF

Certified to NSF/ANSI 60

Maximum Usage Level:
10 mg/L

This document was produced electronically and no signature is required.

(b) (4) Antifoam

(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 acknowedging 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. (b) (4) 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 beow.

Updated January 16, 2017

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	1JD0338
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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)	SPECTRUM MATCHES REFERENCE		SPECTRUM 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)

ISO 9001:2015 REGISTERED

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	PO204	Lot Number	1JH0518
Item	(b) (4)	Manufacturer Lot	26660003
CAS Number		Manufacturer Code	283
Molecular Formula		Molecular Weight	(b) (4)

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		NO YELLOW COLOR
SODIUM (EP)		0.1 %	(b) (4)
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)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	PO204	Lot Number	1JH0518
Item	(b) (4)	Manufacturer Lot	26660003
CAS Number		Manufacturer Code	283
Molecular Formula		Molecular Weight	(b) (4)

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		NO YELLOW COLOR
SODIUM (EP)		0.1 %	(b) (4)
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)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	P1382	Lot Number	1JI0296
Item	(b) (4)		
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)		Lot Number	1JH0059
Item			Manufacturer Lot	4350064-A
CAS Number			Manufacturer Code	14941
Molecular Formula			Molecular Weight	(b) (4)

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)

ISO 9001:2015 REGISTERED

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	S1303	Lot Number	1GA0557
Item	(b) (4)		
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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	
LEAD (Pb)		2 mg/kg	
MERCURY		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)

ISO 9001:2015 REGISTERED

Certificate of Analysis Results Certified by:

(b) (4)

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

(b) (4)

(b) (4)

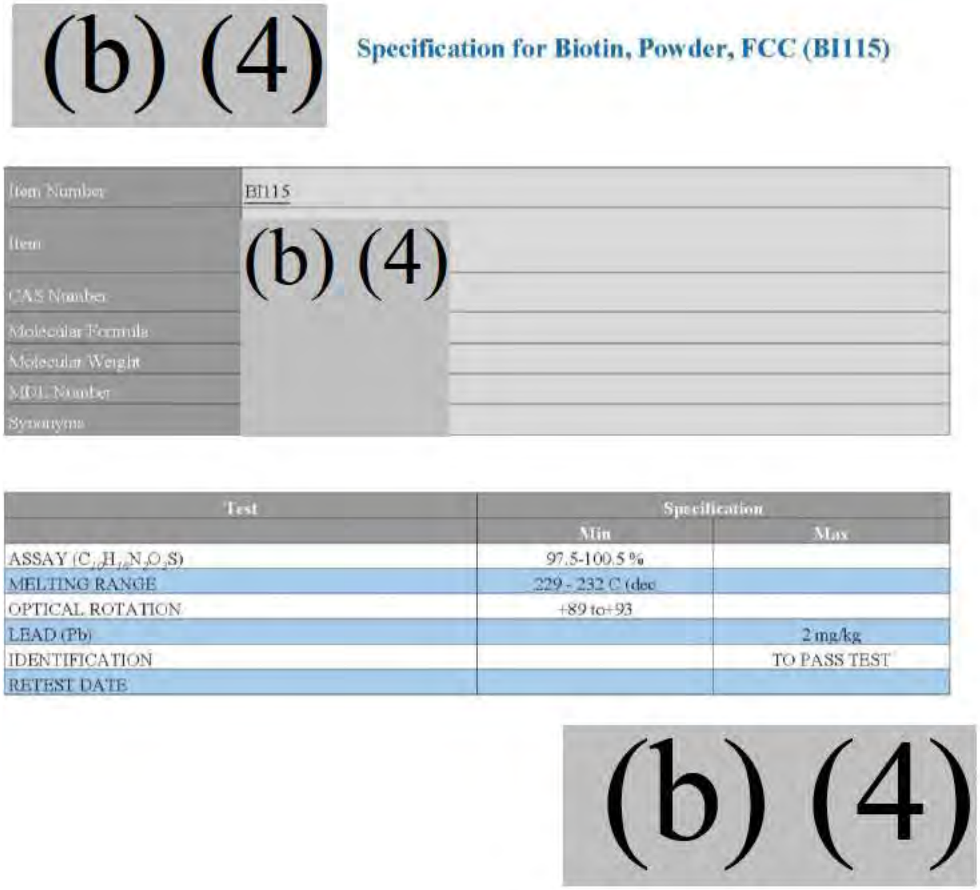
Specification for Ammonium Chloride, Granular, FCC (A1167)

Item Number	A1167
Item	(b) (4)
CAS Number	
Molecular Formula	
Molecular Weight	
MDL Number	
Synonyms	

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

(b) (4)

Biotin



(b) (4) **Specification for Biotin, Powder, FCC (BI115)**

Item Number	BI115
Item	(b) (4)
CAS Number	
Molecular Formula	
Molecular Weight	
MDL Number	
Synonyms	

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)

SPECIFICATIONS

Ref: (b) (4)

(b) (4)

PAGE 1/1

DEFINITION :

Spray-dried (b) (4)
CAS no.: 66071-94-1
EINECS : 266-113-4

SPECIFICATIONS :

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.)

(b) (4)

COMMENTS :

(b) (4) is a spray-dried version of the (b) (4). (b) (4) is a high quality (b) (4) 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.

MCL,MMC: (b) (4)

QUALITY ASSURANCE / INDUSTRY February 10, 2016

(b) (4) **Non-GMO**

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	2IA0400
Item	(b) (4)		
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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)

ISO 9001:2015 REGISTERED

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	1IJ0734
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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		PASSES TEST
ARSENIC		2 ppm	< 2 ppm
CERTIFIED KOSHER			CERTIFIED KOSHER
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			06-JUN-2022
DATE OF MANUFACTURE			06-JUN-2019
APPEARANCE			WHITE CRYSTALS
RESIDUAL SOLVENTS	TO PASS TEST		NO RESIUDUAL SOLVENTS USED

(b) (4)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	2FF0011
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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		PASSES TEST
ARSENIC (As)		3 mg/kg	(b) (4)
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)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	1IH1295
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

Test	Specification		Result
	min	max	
ASSAY	85.0 %		(b) (4)
ARSENIC (As)		3 mg/kg	
CADMIUM (Cd)		3 mg/kg	
FLUORIDE		10 mg/kg	
LEAD (Pb)		3 mg/kg	
IDENTIFICATION	POSITIVE FOR PHOSPHATE		POSITIVE FOR PHOSPHATE
CERTIFIED HALAL			CERTIFIED HALAL
EXPIRATION DATE			12-JUN-2021
DATE OF MANUFACTURE			13-JUN-2019
APPEARANCE			CLEAR COLORLESS LIQUID

(b) (4)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	1JI0681
Item		Manufacturer Lot	RI20191040
CAS Number		Manufacturer Code	12349
Molecular Formula		Molecular Weight	(b) (4)

Test	Specification		Result
	min	max	
ASSAY (DRIED BASIS)	99.0	100.5 %	(b) (4)
APPEARANCE OF SOLUTION	CLEAR COLORLESS		CLEAR COLORLESS
ACIDITY OR ALKALINITY		0.5 ml	(b) (4)
LOSS ON DRYING		0.5%	
ALUMINUM		0.2 ppm	
BROMIDES		100 ppm	
PHOSPHATES		25 ppm	
POTASSIUM		500 ppm	
IODIDES	NO BLUE COLOR		NO BLUE COLOR
MAGNESIUM AND ALKALINE-EARTH METALS (as Ca)		100 ppm	(b) (4)
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)

Certificate of Analysis Results Certified by:

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

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

(b) (4)

(b) (4)

Certificate Of Analysis

Item Number	(b) (4)	Lot Number	2HG0513
Item			
CAS Number			
Molecular Formula		Molecular Weight	(b) (4)

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)

Certificate of Analysis Results Certified by:

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

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

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

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

Dear Mr. Yingling:

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

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

(b) (4)

(b) (4)

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
(b) [redacted]	(4) [redacted]	Average MW: 2000
[redacted]	[redacted]	
[redacted]	[redacted]	Average MW: 2000
[redacted]	[redacted]	
[redacted]	[redacted]	
[redacted]	[redacted]	
[redacted]	[redacted]	
[redacted]	[redacted]	
[redacted]	[redacted]	
[redacted]	[redacted]	Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
[redacted]	[redacted]	
[redacted]	[redacted]	

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
(b)(4)		Cationic polyamine
		Cationic polyamine
		Cationic polyamine
		Cationic polyacrylamide
		Cationic polyacrylamide
		Anionic polyacrylamide

TECHNICAL DATA SHEET
P_N581PW
Rev 0; 2017.01.20 - Page1/1

(b) (4)

Product range:
(b) (4)

(b) (4) - Powder

(b) (4)

APPLICATION

Suitable for a broad range of mesophilic and thermophilic lactic bacteria, (b) (4)ssociated with (b) (4)extract promotes microorganisms growth and can be used during the strain conservation as viability protective agent.

DESCRIPTION

(b) (4) is a primary (b) (4) obtained by the autolysis of a selected strain of (b) (4) especially grown on a molasses-based media.

STATEMENTS

(b) (4)s guaranteed to be Non-GM, free of animal origin ingredient, halal and kosher.

COMPOSITION

(b) (4)s a high quality source of readily available soluble, amino acids, medium-sized peptides, vitamins and essential elements.

PHYSICO-CHEMICAL SPECIFICATIONS

Expressed on product as is

Dry matter	Min.	94,0	g / 100 g
Total nitrogen	Min.	10,00	g / 100 g
Amino nitrogen	Min.	1,60	g / 100 g
Proteins	Min.	62,5	g / 100 g
pH		6,2-7,2	

MICROBIOLOGICAL SPECIFICATIONS

Total mesophilic bacteria	Max.	5000	CFU/g
Total coliforms	Max.	5	CFU/g
Spores of clostridium perfringens	Max.	10	CFU/g
Yeasts	Max.	50	CFU/g
Moulds	Max.	50	CFU/g
Bacillus cereus	Max.	100	CFU/g
Enterobacteria	Max.	10	CFU/g
Salmonella		Neg.	CFU/25g
E.coli		Neg.	CFU/g
Pathogenic Staphylococcus		Neg.	CFU/g
Listeria		Neg.	CFU/25g

PACKAGING & PALLET

20 kg (44 lbs) sealed paper bags with polyethylene liner/ pallets of 600 kg (1,322 lbs).

SHELF LIFE & STORAGE

3 years in their original packaging, stored in a dry place at a cool to ambient temperature and protected from direct sunlight.

(b) (4)

(b) (4)

Order N°	:
Delivery note	:
Vendor Code	:
Letter of crédit	:
Invoice	:

CERTIFICATE OF ANALYSIS / CONFORMITY

Customer

Consignee

F.A.O

Page : 1 / 1

Item	(b) (4)
Your reference	: /
Batch N°	: 071603160
Use by	: 08/13/2019

Production date : 08/13/2016

(b) (4) is a ISO 9001, FSSC22000 certified company

Characteristic	Value	Unit
DRY MATTER	95.9	g%g
TOTAL NITROGEN	10.80	g%g
AMINO NITROGEN	4.50	g%g
PH	7.0	
PROTEINS (N X 6.25)	67.5	g%g
TOTAL MESO BACTERIA	5	/g
TOTAL COLIFORMS	0	/g
SPORES CLOS PERF.	< 5	/g
YEASTS	< 10	/g
MOULDS	< 10	/g
BACILLUS CEREUS	< 20	/g
SALMONELLA	0	/25g
E. COLI	0	/g
PATHOG. STAPHYLOCOCCUS	0	/g

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

(b) (4)

Quality Manager / (b)(6)

(b) (4)

(b) (4)

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

(b) (4)

(b) (4) Certificate of Analysis

(b) (4)

Sc o: 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 (b) (4) 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	[redacted]
Total Coliform (3 Tube MPN) /g	AOAC 966.24	0	10	[redacted]
E. Coli (3 Tube MPN) /g	AOAC 966.24	ND	ND	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	(b) (4)
pH (5% solution)	pH Meter	5.3	6.3	[redacted]
Protein (N x 6.25) %	AOAC 990.03	55.0	100.0	[redacted]
Salmonella /750g	AOAC RI 100201	NEGATIVE	NEGATIVE	NEGATIVE
Salt as Chlorides %	AOAC 971.27	0	1.50	(b) (4)
Standard Plate Count cfu/g	AOAC 990.12	0	10000	[redacted]
Yeast and Mold cfu/g	AOAC 121301	0	100	[redacted]

(b) (4)

(b)(6)
Quality Assurance Manager

*ND = NOT DETECTED

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 (b) (4)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	ND
Listeria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	(b) (4)
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	NEGATIVE

(b) (4)

(b)(6)
Quality Assurance Manager

*ND = NOT DETECTED

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

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

(b) (4): Certificate of Analysis

d To: (b) (4)

Customer PO No.:
Customer Order No.: (b) (4)
Item No.: SN2000027196 (b) (4)
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	PASS
APPEAR		PASS	PASS	PASS
ODOR		PASS	PASS	PASS
AEROBIC PLATE COUNT (CFU/G)		<10000 /G	<10000 /G	(b) (4)
COLIFORM (CFU /G)		<10 /G	<10 /G	
YEAST & MOLD (CFU/G)		<100 /G	<100 /G	
SALMONELLA ELFA METHOD 375G		ND	ND	ND
E. COLI MPN/g		ND	ND	ND

*ND = NOT DETECTED

(b)(6)
Quality Assurance Manager

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

Page 0 of 0

(b) (4)

(b) (4)

(b) (4) Certificate of Analysis

d 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	(b) (4) 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	ND
steria monocytogenes /25g	AOAC2003.12	NEGATIVE	NEGATIVE	NEGATIVE
Moisture Loss on Drying %	AOAC 930.15	0	6.0	(b) (4)
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	NEGATIVE
Protein (N x 6.25) %	AOAC 990.03	50.0	100.0	(b) (4)

(b) (4)

(b)(6)
Quality Assurance Manager

*ND = NOT DETECTED

(b) (4)

Confidential Detailed Manufacturing Summary of Spray Dried *Clostridium beijerinckii* ASCUSBR67

(b) (4)

Continued on next page

Table 1. Raw Materials and Processing Aids Used in the Manufacture of *C. beijerinckii* ASCUSBR67

(b) (4)

2 Master Cell Bank / Working Cell Bank

(b) (4)

3 Fermentation

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Appendix A. Process Diagram of the Production of Spray Dried *C. beijerinckii* ASCUSBR67

Clostridium beijerinckii ASCUSBR67 Manufacturing Process

CONFIDENTIAL

11 May2021

(b) (4)

Native Microbial Mix Uniformity for *Clostridium beijerinckii* ASCUSBR67

Protocol (b) (4)

Approvers:

DocuSigned by:
Martin Mayhew
CA3DAF452B8A47C...

3/22/2021

Martin Mayhew

Date

Vice President – Product Development

& Manufacturing

(b)(6)

3/18/2021

(b)(6)

Date

Quality

DocuSigned by:
kevin korth
1C2149273B2345F...

3/17/2021

Kevin Korth

Date

Regulatory

(b)(6)

3/17/2021

Date

Scientist

The following protocol was approved via email on 03Dec2020 prior to circulating for internal signatures.

Prepared By

Native Microbials, Inc

San Diego, CA

March 2021

Native Microbial Mix Uniformity for *Clostridium beijerinckii* ASCUSBR67

Protocol (b) (4)

Objective:

1. To ensure the homogeneity of a Native Microbials test ingredient *Clostridium beijerinckii* ASCUSBR67 within a typical broiler diet.

Diet Manufacturing:

(b) (4)

Figure 1: Sampling locations

(b) (4)

Shipping Addresses:

1) Send one composite sample

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

Appendix A: Experimental diets

INGREDIENTS	AMOUNT (%)
Corn, ground 600µm	(b) (4)
SBM, 46.5 %	
Soy oil	
Monocal phosphate, 21%	
Limestone	
Sodium chloride	
Choline chloride, 60%	
NB 3000 Poultry vit/min. premix	
L-Lys HCl	
DL- Met	
L- Thr	
Quantum Blue	
Total	100.00

Appendix B: Batch Records

Components	**Ingredient, lbs**	**Actual Amount in Batch**	**Initial & Date**
Corn, ground 600µm	(b) (4)		
SBM, 46.5 %	[redacted]		
Soy oil	[redacted]		
Monocal phosphate, 21%	[redacted]		
Limestone	[redacted]		
Sodium chloride	[redacted]		
Choline chloride, 60%	[redacted]		
NB 3000 Poultry vit/min. premix	[redacted]		
L-Lys HCl	[redacted]		
DL- Met	[redacted]		
L- Thr	[redacted]		
Quantum Blue	[redacted]		
***Test ingredient will be added in as a replacement of corn**			
Total:	**200.00**		

- **Initials will be writing at bottom of batch records followed by a full signature.**

APPENDIX C- Mixing Verification

	Batch Number	**Initial & Date**
Avius Protect Lot #		
Mixing Start Time		
Mixing End time		

APPENDIX D- Scale Verification

Scale Verification Report

Date	Scale model	Standard weight, unit	As found Reading, unit	Inspector

Documentation of lot number of *Clostridium beijerinckii* ASCUSBR67 used, mixing times, scales used, personnel involved, and actual ingredient weights will be records and compiled in study records. (b) (4)

Native Microbial Mix Uniformity for *Clostridium beijerinckii* ASCUSBR67

Protocol (b) (4)

Approvers:

DocuSigned by:
Martin Mayhew
CA3DAF452B8A47C...

3/22/2021

Martin Mayhew

Date

Vice President – Product Development

& Manufacturing

(b)(6)

3/18/2021

Date

Quality

DocuSigned by:
kevin korth
1C2149273B2345F...

3/17/2021

Kevin Korth

Date

Regulatory

(b)(6)

3/17/2021

Date

Scientist

The following protocol was approved via email on 03Dec2020 prior to circulating for internal signatures.

Prepared By

Native Microbials, Inc

San Diego, CA

March 2021

Native Microbial Mix Uniformity for *Clostridium beijerinckii* ASCUSBR67

Protocol (b) (4)

Objective:

1. To ensure the homogeneity of a Native Microbials test ingredient *Clostridium beijerinckii* ASCUSBR67 within a typical broiler diet.

Diet Manufacturing:

(b) (4)

Figure 1: Sampling locations

(b) (4)

Shipping Addresses:

1) Send one composite sample

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

Appendix A: Experimental diets

INGREDIENTS	AMOUNT (%)
Corn, ground 600µm	(b) (4)
SBM, 46.5 %	
Soy oil	
Monocal phosphate, 21%	
Limestone	
Sodium chloride	
Choline chloride, 60%	
NB 3000 Poultry vit/min. premix	
L-Lys HCl	
DL- Met	
L- Thr	
Quantum Blue	
Total	100.00

Appendix B: Batch Records

Components	**Ingredient, lbs**	**Actual Amount in Batch**	**Initial & Date**
Corn, ground 600µm	(b) (4)		
SBM, 46.5 %			
Soy oil			
Monocal phosphate, 21%			
Limestone			
Sodium chloride			
Choline chloride, 60%			
NB 3000 Poultry vit/min. premix			
L-Lys HCl			
DL- Met			
L- Thr			
Quantum Blue			
***Test ingredient will be added in as a replacement of corn**			
Total:	**200.00**		

- **Initials will be writing at bottom of batch records followed by a full signature.**

APPENDIX C- Mixing Verification

	Batch Number	Initial & Date
Avius Protect Lot #		
Mixing Start Time		
Mixing End time		

APPENDIX D- Scale Verification

Scale Verification Report

Date	Scale model	Standard weight, unit	As found Reading, unit	Inspector

Documentation of lot number of *Clostridium beijerinckii* ASCUSBR67 used, mixing times, scales used, personnel involved, and actual ingredient weights will be records and compiled in study records. Copies will be stored electronically in XXXX

Native Microbial Mix Uniformity for *Clostridium beijerinckii* ASCUSBR67

Protocol (b) (4)

Approvers:

DocuSigned by:
Martin Mayhew
ACBDDAD433BF491...

3/5/2021

Martin Mayhew

Date

Vice President – Product Development

& Manufacturing

(b)(6)

3/5/2021

(b)(6)

Date

Quality

DocuSigned by:
kevin korth
869AE3638AA543B...

3/5/2021

Kevin Korth

Date

Regulatory

(b)(6)

3/5/2021

(b)(6)

Date

Scientist

DocuSigned by:
(b)(6)

3/8/2021

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

Date

Research Coordinator

Prepared By
Native Microbials, Inc
San Diego, CA
March 2021

Native Microbial Mix Uniformity for *Clostridium beijerinckii* ASCUSBR67

Protocol #(b) (4)

Objective:

1. To ensure the homogeneity of a Native Microbials test ingredient *Clostridium beijerinckii* ASCUSBR67 within a typical broiler diet.

Diet Manufacturing:

(b) (4)

Figure 1: Sampling locations

(b) (4)

Shipping Addresses:

1) Send 10 samples

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

Appendix A: Experimental diets

INGREDIENTS	AMOUNT (%)
Corn, ground 600µm	(b) (4)
SBM, 46.5 %	
Soy oil	
Monocal phosphate, 21%	
Limestone	
Sodium chloride	
Choline chloride, 60%	
NB 3000 Poultry vit/min. premix	
L-Lys HCl	
DL- Met	
L- Thr	
Quantum Blue	
Total	100.00

Appendix B: Batch Records

Components	**Ingredient, lbs**	**Actual Amount in Batch**	**Initial & Date**
Corn, ground 600µm	(b) (4)		
SBM, 46.5 %	[redacted]		
Soy oil	[redacted]		
Monocal phosphate, 21%	[redacted]		
Limestone	[redacted]		
Sodium chloride	[redacted]		
Choline chloride, 60%	[redacted]		
NB 3000 Poultry vit/min. premix	[redacted]		
L-Lys HCl	[redacted]		
DL- Met	[redacted]		
L- Thr	[redacted]		
Quantum Blue	[redacted]		
BR67 (Grams)	[redacted]		
***Test ingredient will be added in as a replacement of corn**			
Total:	**200.00**		

- **Initials will be writing at bottom of batch records followed by a full signature.**

APPENDIX C- Mixing Verification

	Batch Number	**Initial & Date**
Avius Protect Lot #		
Mixing Start Time		
Mixing End time		

APPENDIX D- Scale Verification

Scale Verification Report

Date	Scale model	Standard weight, unit	As found Reading, unit	Inspector

Homogeneity Study of Broiler-67 (ASCUSBR67) in Mash

Lab Performing the Study: Native Microbials, Inc.

Pre-Execution Approval:

Name & Title	Signature & Date	
Martin Mayhew – VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/5/2021
Kevin Korth Regulatory	DocuSigned by: kevin korth 869AE3838AA543B...	3/4/2021
(b)(6) Quality	(b)(6)	3/5/2021

Post-Execution Approval:

Name & Title	Signature & Date	
Martin Mayhew – VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	5/5/2021
Kevin Korth Regulatory	DocuSigned by: kevin korth 869AE3838AA543B...	5/5/2021
(b)(6) Quality	(b)(6)	5/5/2021

Objective:

This Homogeneity Study will demonstrate that broiler mash can be prepared with uniformly distributed *Clostridium beijerinckii* ASCUSBR67 (aka BR67 or Broiler-67) throughout the mash.

Method Overview:

The method (b) (4) will be used to determine the amount of viable BR67 in mash samples. For this method, the mash sample is weighed, diluted in buffer, plated on TSB+FAC plates, incubated anaerobically, and colonies are counted after approximately 24 hours.

The approved and validated method is retained on the Native Microbials (b) (4) (b) (4)

Equipment calibration and monitoring records are maintained in the Native Microbials lab documentation.

Sample Preparation:

For the study, mash was prepared at (b) (4) to contain 0.5 lbs of Broiler-67 SDP per ton of mash. After blending, approximately 10 samples were taken from across the

lot. (b) (4)
(b) (4)

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

Acceptance Criteria:
- The Coefficient of Variation (CV) is <75% for results of all samples from the same mash.
- Greater than 1E+04 CFU/g of mash

Summary and Conclusion:
A summary report will be prepared and approved. The report will include the following information:
- Objective of the study
- Table of results
- Comparison of data to the acceptance criteria
- Graph of results
- Discussion of any deviations
- Discussion of any changes to the protocol or method
- Location of raw data supporting the study

PROTOCOL EXECUTION

Personnel Executing the Protocol:

Your signature indicates that you have read and understood this protocol.

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

Sample Preparation:

		Initial & Date
Broiler 67 lot # and date prepared	Lot# BR67 20-0202-049-B6 14Dec2020	(b)(6) 06Apr2021
Mash ingredients	See 1113 Data.pdf	(b)(6) 06Apr2021
Mash manufacturing date	Se 1113 Data.pdf	(b)(6) 06Apr2021
Date samples received	10Mar2021	(b)(6) 06Apr2021
Date samples assayed	11 Mar 2021 12 Mar 2021	(b)(6) 06Apr2021

DocuSign Envelope ID: E54F4FB3-C764-476A-B3DA-2B614AEBA188

	(b)(4)	Analysis				
Sample number	[redacted]	Avg. CFU/g	Std. Dev.	CV		
1	[redacted]	1.67E+05	4.44E+04	27%	Plated: Counted	GG 11Mar21 GG 3/12/2021
2	[redacted]	2.11E+05	4.44E+04	21%	Plated: Counted	GG 11Mar21 GG 3/12/2021
3	[redacted]	2.37E+05	6.44E+04	27%	Plated: Counted	GG 11Mar21 GG 3/12/2021
4	[redacted]	2.00E+05	5.46E+04	27%	Plated: Counted	GG 11Mar21 GG 3/12/2021
5	[redacted]	2.45E+05	1.30E+04	5%	Plated: Counted	GG 11Mar21 GG 3/12/2021
6	[redacted]	1.80E+05	5.00E+03	3%	Plated: Counted	GG 11Mar21 GG 3/12/2021
7	[redacted]	2.03E+05	1.05E+04	5%	Plated: Counted	12-Mar-21 13-Mar-21
8	[redacted]	2.58E+05	4.36E+04	17%	Plated: Counted	12-Mar-21 13-Mar-21
9	[redacted]	3.03E+05	5.55E+04	18%	Plated: Counted	12-Mar-21 13-Mar-21
10	[redacted]	2.56E+05	9.43E+03	4%	Plated: Counted	12-Mar-21 13-Mar-21

Homogeneity Study of Broiler-67 (*Clostridium beijerinckii* ASCUSBR67) in Mash

Objective:

This homogeneity study was designed to demonstrate that broiler chicken feed mash can be prepared with uniformly distributed *Clostridium beijerinckii* ASCUSBR67 (aka BR67 or Broiler-67) throughout the mash.

Method Overview:

The method (b) (4) was used to determine the amount of viable *Clostridium beijerinckii* ASCUSBR67 in mash samples. For this method, the mash sample is weighted, diluted in a buffer and plated on TSB+FAC plates, incubated anaerobically, and colonies are counted after approximately 24 hours.

The approved and validated method is retained on the Native Microbials (b) (4)

Equipment calibration and monitoring records are maintained in the Native Microbials lab documentation.

Sample Preparation:

For the study, mash was prepared at (b) (4) to contain 0.5 lbs of BR67 Spray Dried Powder (SDP) per ton of mash. After blending, approximately 10 samples were taken from across the lot. (b) (4)

Data Analysis:

The calculations for converting raw colony numbers to CFU/g is listed in the method. The Coefficient of Variation and Standard Deviation calculations are also included in the method.

Acceptance Criteria:

- The Coefficient of Variation (CV) is <75% for results of all samples from the same mash.
- Greater than 1.00E+04 CFU/g of mash

Results:

All 10 samples had an average CFU/g of mash greater than the minimum CFU/g of 1.00E+04 CFU/g of mash with all samples having a CV less than 75% (Table 1 and Figure 1). Analysis of all 10 samples demonstrated an average CFU/g of mash of 1.76E+05 CFU/g of with an overall CV of 37%, meeting the acceptance Criteria (see Table 1 and Figure 1).

Table 1

	Individual Samples			All Samples		
Sample	AVG CFU/g	STD Dev.	CV	AVG CFU/g	STD Dev.	CV
1	(b) (4)			2.26E+05	5.21E+04	23%
2						
3						
4						
5						
6						
7						
8						
9						
10						

Figure 1

(b) (4)

Deviations and Method Changes:
There were no deviations or changes from the method or study protocol(s).

Conclusions:
The results indicate that broiler chicken feed mash can be prepared with uniformly distributed *Clostridium beijerinckii* ASCUSBR67, using standard industry methods.

Raw Data:
Raw Data can be found on the Native Microbials (b) (4)
(b) (4)

Approvals:

Name & Title	**Signature & Date**	
Martin Mayhew VP – Process Development and Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	5/3/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F...	5/3/2021
(b)(6) Quality	DocuSigned by: (b)(6)	5/3/2021

Method

Title	BR67 Liquid Intermediate Microbe Enumeration
Version	01
Effective Date	16Nov2020
Author	(b)(6)
Approver (Signature & Date)	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 11/16/2020 Martin Mayhew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable spores of *Clostridium beijerinckii* in Broiler-67 End of Fermentation (EoF) and Broiler-67 Cell Concentrate (CC) samples by counting colony forming units (CFU) on solid media. The methods are the same for each fermentation intermediate.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath and hot liquids. Wear appropriate personal protective equipment (safety glasses and gloves) at all times.

Materials

(b) (4) EZ large incubation container (b) (4)
EZ anaerobe container system sachets (b) (4)
(b) (4) 15mL Polypropylene Centrifuge Tubes (b) (4)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL pipette
200 µL pipette
100 µL positive displacement pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
100 µL positive displacement tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Autoclave
Water bath set to (b) (4)
Laboratory Vortexer
Class I/II Biosafety Cabinet

Media & Reagents

NOTE: *Comparable quality ingredients (Laboratory, NF, USP, Reagent, or ACS grade) from different suppliers may be used.*

TSB+FAC plates
(b) (4) 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (b) (4)
1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (b) (4)

Method

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

BR67 Liquid Intermediate Microbe Enumeration

(b) (4)

2. Prepare the Primary Dilution Mix and Heat Shock

(b) (4)

3. BR67 End of Fermentation Dilutions and Plating

(b) (4)

(b) (4)

4. BR67 Cell Concentrate Dilutions and Plating

(b) (4)

5. Negative Control Plating

(b) (4)

(b) (4)

BR67 Liquid Intermediate Microbe Enumeration

(b) (4)

Method Validation Protocol, Version 2

Method Title and Versions

Title	BR67 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	DocuSigned by: Martin Mayhew 53219323BAF64C2	10/29/2020
[redacted]	(b)(6)	10/29/2020

Post Execution Approval:

Printed Name & Title	**Signature**	
Martin Mayhew – VP-Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF49	11/16/2020
(b) (4) – Quality	(b)(6)	11/11/2020

Personnel Executing the Validation:

Your signature indicates that you have read and understand this protocol.

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

Purpose:

This validation will demonstrate that the BR67 Liquid Intermediate Microbe Enumeration method can quantify the amount of BR67 (*Clostridium beijerinckii*) in liquid intermediates, such as Cell Concentrate and End of Fermentation samples. The following parameters will be tested in this validation:

- Repeatability – closeness of results obtained on the same sample when assayed multiple times by the same person with the same reagents and equipment.

- Robustness – reliability of the method to withstand small variations such as different technicians and reagent preparations.
- Linearity – the assay produces reliable results over a range of concentrations.

Background:

BR67 (*Clostridium beijerinckii)* liquid intermediates are produced during the fermentation process of the organism. Samples may be tested at the end of the fermentation and after concentration of cells. The cell concentrate is further processed into a powder that will be used in the final product. The microbe enumeration assay was developed by Native Microbials.

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

Method Overview:

Agar plates are prepared a day before test commencement. (b) (4)

(b) (4)

Sample Preparation:

(b) (4)

(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)

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

Samples 2 – 5 will be assayed one time by analyst 1.

(b) (4)

Acceptance Criteria:

(b) (4)

Summary and Conclusions:

(b) (4)

Data Collection – Analyst 1 **Name** (b)(6)

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
1-1	(b)(4)		(b)(6) 06Nov2020		1-1	(b)(4)		(b)(6) 06Nov2020
1-1			(b)(6) 06Nov2020					

(b)(4)

[redacted] (b) (4)

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
1-2	[redacted] (b)(4)	[redacted]	[redacted] (b)(6) 06Nov2020		1-3	[redacted] (b)(4)	[redacted]	[redacted] (b)(6) 06Nov2020
1-2	[redacted]	[redacted]	[redacted] (b)(6) 06Nov2020		1-3	[redacted]	[redacted]	[redacted] (b)(6) 06Nov2020
1-2	[redacted]	[redacted]	[redacted] (b)(6) 06Nov2020		1-3	[redacted]	[redacted]	[redacted] (b)(6) 06Nov2020

[redacted] (b)(6)

[redacted] (b) (4)

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
2	(b)(4)	(b)(4)	(b)(6) 06Nov2020		3	(b)(4)	(b)(4)	(b)(6) 06Nov2020
2	(b)(4)	(b)(4)	(b)(6) 06Nov2020		3	(b)(4)	(b)(4)	(b)(6) 06Nov2020
2	(b)(4)	(b)(4)	(b)(6) 06Nov2020		3	(b)(4)	(b)(4)	(b)(6) 06Nov2020

(b)(4)

[redacted] (b) (4)

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
4	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020		5	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020
4	(b)(4) [redacted]	[redacted]	(b)(6) [redacted] 06Nov2020		5	(b)(4) [redacted]	[redacted]	(b)(6) [redacted] 06Nov2020
4	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020		5	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020

Calculations and Results

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
1-1	[redacted]	[redacted]	[redacted] 06Nov2020		1-2	[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
1-3	[redacted]	[redacted]	[redacted] 06Nov2020		2	[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
3	[redacted]	[redacted]	[redacted] 06Nov2020		4	[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
5	[redacted]	[redacted]	[redacted] 06Nov2020					
	[redacted]	[redacted]	[redacted] 06Nov2020					
	[redacted]	[redacted]	[redacted] 06Nov2020					

(b)(4)

Sample			Initial/Date		Sample			Initial/Date
1-1 [redacted] (b) (4)	Final Result (CFU/g)	4.39E9	[redacted] (b)(6) 06Nov2020		1-2 [redacted] (b) (4)	Final Result (CFU/g)	[redacted] (b)(4)	[redacted] (b)(6) 06Nov2020
	Standard Deviation	3.17E8	[redacted] (b)(6) 06Nov2020			Standard Deviation		[redacted] (b)(6) 06Nov2020
	CV	7%	[redacted] (b)(6) 06Nov2020			CV		[redacted] (b)(6) 06Nov2020
	Valid Results? (circle)	[✓ Yes] No	06Nov2020			Valid Results? (circle)		06Nov2020
Sample			Initial/Date		Sample			Initial/Date

1-3 [redacted] (b) (4)	Final Result (CFU/g)	4.16E9	[redacted]	ov2020		2 [redacted] (b) (4)	Final Result (CFU/g)	1.11E9	[redacted]	ov2020
	Standard Deviation	7.52E8	[redacted]	ov2020			Standard Deviation	1.84E7	[redacted]	ov2020
	CV	18%	[redacted]	06Nov2020			CV	2%	[redacted]	06Nov2020
	Valid Results? (circle)	[✓ Yes] No	[redacted]	ov2020			Valid Results? (circle)	[✓ Yes] No	[redacted]	ov2020
3 [redacted] (b) (4)	Final Result (CFU/g)	7.36E8	[redacted]	ov2020		4 [redacted] (b) (4)	Final Result (CFU/g)	2.35E7	[redacted]	ov2020
	Standard Deviation	1.56E8	[redacted]	ov2020			Standard Deviation	1.94E6	[redacted] (b)(6)	ov2020
	CV	20%	[redacted]	6Nov2020			CV	8%	[redacted]	06Nov2020
	Valid Results? (circle)	[✓ Yes] No	[redacted]	ov2020			Valid Results? (circle)	[✓ Yes] No		06Nov2020
5 [redacted] (b) (4)	Final Result (CFU/g)	2.01E8	[redacted]	ov2020						
	Standard Deviation	7.57E6	[redacted]	ov2020						
	CV	4%	[redacted] (b)(6)	06Nov2020						
	Valid Results? (circle)	[✓ Yes] No	[redacted]	ov2020						

(b)(4) [redacted]

Data Collection – Analyst 2 Name_(b)(6) [redacted]

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
1	(b)(4) [redacted]	[redacted]	(b)(6) [redacted] 06Nov2023		1	(b)(4) [redacted]	[redacted]	(b)(6) [redacted] 06Nov2023
1	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2023					

(b)(4) [redacted]

(b) (4) [redacted]

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
2	(b) (4) [redacted]	[redacted]	(b)(6) [redacted] 06Nov2020		3	(b) (4) [redacted]	[redacted]	(b)(6) [redacted] 06Nov2020
2	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020		3	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020
2	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020		3	[redacted]	[redacted]	(b)(6) [redacted] 06Nov2020

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
4	(b)(4)		(b)(6) 06Nov2020		5	(b)(4)		(b)(6) 06Nov2020
4			(b)(6) 06Nov2020		5			(b)(6) 06Nov2020
4			(b)(6) 06Nov2020		5			(b)(6) 06Nov2020

(b) (4)

(b)(4) [redacted]

Calculations and Results

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
1	(b)(4), (b)(6) [redacted]	[redacted]	[redacted] 06Nov2020		2	(b)(4), (b)(6) [redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
3	[redacted]	[redacted]	[redacted] 06Nov2020		4	[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
	[redacted]	[redacted]	[redacted] 06Nov2020			[redacted]	[redacted]	[redacted] 06Nov2020
5	[redacted]	[redacted]	[redacted] 06Nov2020					
	[redacted]	[redacted]	[redacted] 06Nov2020					
	[redacted]	[redacted]	[redacted] 06Nov2020					

(b)(4) [redacted]

Sample			Initial/Date		Sample			Initial/Date	
1 PDM 1-3	Final Result (CFU/g)	1.92E9	(b)(6)	06Nov2020	2 PDM 1-3	Final Result (CFU/g)	6.89E8	(b)(6)	06Nov2020
	Standard Deviation	4.65E8		06Nov2020		Standard Deviation	1.15E8		06Nov2020
	CV	34%		06Nov2020		CV	17%		06Nov2020
	Valid Results? (circle)	[✓ Yes] No		06Nov2020		Valid Results? (circle)	[✓ Yes] No		06Nov2020
3 PDM 1-3	Final Result (CFU/g)	4.14E8		06Nov2020	4 PDM 1-3	Final Result (CFU/g)	4.39E7		06Nov2020
	Standard Deviation	1.37E8		06Nov2020		Standard Deviation	1.29E7		06Nov2020
	CV	33%		06Nov2020		CV	29%		06Nov2020
	Valid Results? (circle)	[✓ Yes] No		06Nov2020		Valid Results? (circle)	[✓ Yes] No		06Nov2020
5 PDM 1-3	Final Result (CFU/g)	1.00E8		06Nov2020					
	Standard Deviation	1.08E7		06Nov2020					
	CV	11%		06Nov2020					
	Valid Results? (circle)	[✓ Yes] No		06Nov2020					

(b)(4)

Reasons for Revision

(b)(4)

(b) (4)

(b) (4)

Draft Method

Title	BR67 Liquid Intermediate Microbe Enumeration
Version	01 Draft
Effective Date	
Author	(b)(6)
Approver (Signature & Date)	Martin Mayhew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable spores of *Clostridium beijerinckii* in Broiler-67 End of Fermentation (EoF) and Broiler-67 Cell Concentrate (CC) samples by counting colony forming units (CFU) on solid media. The methods are the same for each fermentation intermediate.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath and hot liquids. Wear appropriate personal protective equipment (safety glasses and gloves) at all times.

Materials

(b) (4) EZ large incubation containe (b) (4)
EZ anaerobe container system sachets (b) (4)
(b) (4) 15mL Polypropylene Centrifuge Tubes (b) (4)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL pipette
200 µL pipette
100 µL positive displacement pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
100 µL positive displacement tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Autoclave
Water bath set to 50°C
Laboratory Vortexer
Class I/II Biosafety Cabinet

Media & Reagents

NOTE: *Comparable quality ingredients (Laboratory, NF, USP, Reagent, or ACS grade) from different suppliers may be used.*

TSB+FAC plates

(b) (4) 10X Phosphate Buffered Saline pH 7.4 (PBS), steril (b) (4)

X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (b) (4)

Method

1. (b) (4)

(b) (4)

2. Prepare the Primary Dilution Mix and Heat Shock

(b) (4)

3. BR67 End of Fermentation Dilutions and Plating

(b) (4)

(b) (4)

(b) (4)

(b) (4)

4. BR67 Cell Concentrate Dilutions and Plating

(b) (4)

Initials

(b) (4)

5. Negative Control Plating

(b) (4)

6. Plate Counting

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Validation Summary Report

BR67 Liquid Intermediate Validation Protocol, V2

Method

BR67 Liquid Intermediate Microbe Enumeration

Objective

The objective of this validation was to demonstrate that the BR67 Liquid Intermediate Microbe Enumeration method can quantify the amount of BR67 (*Clostridium beijerinckii*) in liquid intermediates such as End of Fermentation (EOF) and Cell Concentrate (CC). The method was evaluated for repeatability, robustness, and linearity.

Repeatability was assessed through the closeness of results obtained on the same sample (BR67 1006 V4 CC) when assayed multiple times by the same person with the same reagents and equipment.

Robustness was assessed through the closeness of results obtained on the same set of samples (BR67 1006 V4 CC, BR67 1006 V4 CC /5, BR67 1006 V4 CC /10, BR67 1006 V4 EOF, BR67 1006 V2 EOF) across multiple analysts and reagent preparations.

Linearity was assessed by enumerating the same sample at a concentration of 20% and 10% of the original sample (BR67 1006 V4 CC, BR67 1006 V4 CC /5, BR67 1006 V4 CC /10).

Results

Repeatability

The average of samples 1-1, 1-2, and 1-3 is 4.36E+09 CFU/mL with a standard deviation of 6.10E+08 CFU/mL. The coefficient of variation from these samples is 14%. The low CV resulting from repeated measurements of the same sample demonstrates the repeatability of the assay.

Table 1: Summary table of BR67 liquid enumeration 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
Samples 1-5 were enumerated by an independent analyst. All measurements yielded a CV less than 44% for both analysts, and the data measured for samples across analysts yielded CVs less than 41%, suggesting that the measurement is robust to different analysts and reagent preparations.

Table 2: Summary of Repeatability, Linearity, and Robustness

Repeatability			
Average Sample 1	STDEV	CV	
4.36E+09	6.10E+08	14%	
Linearity			
R2 = 0.9999			
Robustness across analysts			
	Average	STDEV	CV
Sample 1	(b) (4)		
Sample 2			
Sample 3			
Sample 4			
Sample 5			

Linearity

(b) (4)

(b) (4)

(b) (4)

Location of Raw Data

All original and replate data and analysis can be found on the company (b) (4) [redacted]

Conclusion

Repeatability, robustness, and linearity of the assay were demonstrated. The BR67 Liquid Intermediate Microbe Enumeration method is validated and may be used on commercial product.

(b) (4)

Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD4338F491...	11/16/2020
(b)(6) Quality	(b)(6)	11/11/2020

Method Validation Protocol, Version 1

Method Titles and Versions

Title	BR67 Pellet and Mash Microbe Enumeration
Version	01 Draft

Lab Performing the Validation: Native Microbials Inc.

Pre-Execution Approval:

Printed Name & Title	Signature
Martin Mayhew – VP Process Development and Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
(b)(6) Quality	(b)(6) 12/1/2020

Post Execution Approval:

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

Personnel Executing the Validation:

Your signature indicates that you have read and understand this protocol.

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

(b) (4)

Method Overview:

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Sample Preparation:

Sample #	Lot Number	Location of Lot Preparation Details
1	BR67 M01122020-1	①
2	BR67 M01122020-2	①
3	1103C AScus BR671175F	①
4	ASC20-1-FD #4(5)	①

(b) (4)

[redacted] (b) (4)

[redacted] (b) (4)

[redacted] (b) (4)

(b)(4) [redacted]

Data [redacted] (b) (4)

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4) [redacted]

(b) (4) [redacted]

Negative Control Beads________________________

Negative Control Buffer________________________

Calculations and Results

(b) (4) [redacted]

(b) (4) [redacted]

(b) (4)
[redacted]

(b) (4)
[redacted]

(b) (4)
[redacted]

(b) (4)
[redacted]

(b) (4)
[redacted]

(b) (4)

[redacted]

Data Collection – Analyst 2 **Analyst Name:** ____________________

(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4)

[redacted]

(b) (4)

(b) (4)

(b) (4)

[redacted]

[redacted]

(b) (4)

(b) (4)

[redacted]

DRAFT METHOD

Title	BR67 Pellet and Mash 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 spores of *Clostridium beijerinckii* in broiler pellet and mash feed samples.

Safety

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

Materials, Equipment, and Media

P200 pipette
P200 pipette tip
Sterile 1x PBS + 0.05% Tween20
Sterile glass beads
P1000 pipette
P1000 pipette tips
50 mL conical
Sterile 1.5 microcentrifuge tubes
Sterile microcentrifuge tubes containing 0.9 mL of PBS 0.05% Tween 20
37°C incubator
(b) (4) Coffee Mill (b) (4) or equivalent
Biological safety cabinet
Anaerobic Boxes
Anaerobic Pouches
TSB+FAC plates

Method

1. Preparation of Primary Dilution Mixtures

(b) (4)

(b) (4)

2. Dilutions

(b) (4)

3. Plating

(b) (4)

4. Colony Forming Unit Determination

(b) (4)

(b) (4)

(b) (4)

Validation Summary Report

Broiler - 67 Pellet Mash Method Validation V1

Objective

(b) (4)

Table 1: Summary table of BR67 Pellet and Mash method validation results

Analyst	Lot #		Sample Type	Average CFU/g	STDEV	CV
1	BR67 M01122020-1	Sample 1-1	Mash	(b) (4)		
		Sample 1-2	Mash			
		Sample 1-3	Mash			
	BR67 M01122020-2	Sample 2	Mash			
	1103C ASCUSBR67 175F	Sample 3-1	Pellet			
		Sample 3-2	Pellet			
		Sample 3-3	Pellet			
	ASC-20-1FD trt4 (5)	Sample 4	Pellet			

2	BR67 M01122020-1	Sample 1	Mash	(b) (4)
	BR67 M01122020-2	Sample 2	Mash	
	1103C ASCUS BR67 175F	Sample 3	Pellet	
	ASC-20-1FD trt4 (5)	Sample 4	Pellet	

Robustness

(b) (4)

Table 2: Summary of Repeatability and Robustness

Repeatability			
Sample	**Avg. CFU/g**	**Std. Dev.**	**CV**
1	1.37E+06	4.29E+05	31%
3	2.65E+05	7.77E+04	29%
Robust Across Analyst			
Sample	**Avg. CFU/g**	**STD DEV**	**CV %**
1	(b) (4)		
2			
3			
4			

Conclusion

The BR67 Pellet and Mash Microbe Enumeration method V1 is valid. The protocol was executed as written except that raw data was recorded electronically. Repeatability and robustness of the assay were demonstrated and the assay may be used for commercial purposes.

(b) (4)

Approvals

Name & Title	**Signature & Date**
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew 1/13/2021
(b)(6) Quality	(b)(6) 1/13/2021

Title	BR67 Pellet and Mash Microbe Enumeration
Version	01
Effective Date	15Jan2021
Author	(b)(6)
Approver (Signature & Date)	DocuSigned by: Martin Mayhew ACBDDAD433BF491 1/13/2021 Martin Mayhew – VP Product Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable spores of *Clostridium beijerinckii* in broiler pellet and mash feed samples.

Safety

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

Materials, Equipment, and Media

P200 pipette
P200 pipette tip
Sterile 1x PBS + 0.05% Tween20
Sterile glass beads
P1000 pipette
P1000 pipette tips
50 mL conical
Sterile 1.5 microcentrifuge tubes
Sterile microcentrifuge tubes containing 0.9 mL of PBS 0.05% Tween 20
37°C incubator
(b) (4) Coffee Mill (b) (4) or equivalent
Biological safety cabinet
Anaerobic Boxes
Anaerobic Pouches
TSB+FAC plates

Method

1. Preparation of Primary Dilution Mixtures

(b) (4)

(b) (4)

2. Dilutions

(b) (4)

3. Plating

(b) (4)

4. Colony Forming Unit Determination

(b) (4)

(b) (4)

(b) (4)

Method

Title	BR67 SDP Microbe Enumeration
Version	01
Effective Date	09Nov2020
Author	(b)(6)
Approver (Signature & Date)	DocuSigned by: Martin Mayhew 53249323BAF84C2... 10/29/2020 Martin Mayhew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable spores of *Clostridium beijerinckii* in Broiler-67 Spray Dried Powder (BR67-SDP) by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath and hot liquids. Wear appropriate personal protective equipment (safety glasses and gloves) at all times.

Materials

(b) (4) EZ large incubation container (b) (4)
EZ anaerobe container system sachets (b) (4)
(b) (4) 15mL Polypropylene Centrifuge Tubes (b) (4)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Water bath set to 50°C
Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Magnet Stir Plate

Media & Reagents

NOTE: *Comparable quality ingredients (Laboratory, NF, USP, Reagent, or ACS grade) from different suppliers may be used.*

(b) (4) 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (b) (4)
1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile (b) (4)
TSB+FAC plates
Reagent grade 95% Ethanol

Method

1. Preparation of sterile 1X Phosphate Buffered Saline (PBS), pH 7.4 with 0.05% Polysorbate 20

(b) (4)

BR67-SDP Microbe Enumeration

(b) (4)

2. Prepare the Primary Dilution Mix

(b) (4)

3. BR67-SDP Heat Shock Anaerobic Plating

(b) (4)

(b) (4)

4. Negative Control Plating

(b) (4)

5

BR67-SDP Microbe Enumeration

(b) (4)

Method Validation Protocol, Version 1

Method Titles and Versions

Title	BR67 SDP Microbe Enumeration
Version	01 Draft

Lab Performing the Validation: Native Microbials Inc.

Pre-Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP Process Development and Manufacturing	DocuSigned by: ACBDDAB433BF491...	9/25/2020
(b)(6) Quality	(b)(6)	9/25/2020

Post Execution Approval:

Printed Name & Title	Signature	
Martin Mayhew – VP Process Development and Manufacturing	DocuSigned by: Martin Mayhew 53249323BAF84C2...	10/29/2020
(b)(6) Quality	(b)(6)	10/29/2020

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:

This validation will demonstrate that the BR67 SDP Microbe Enumeration method can quantify the amount of Broiler-67 (*Clostridium beijerinckii)* in the intermediate Broiler-67 Spray Dried Powder (BR67 SDP). The following parameters will be tested in this validation:

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

Background:

(b) (4)

Method Overview:

(b) (4)

The full method is attached to this protocol and will be approved upon successful completion of this validation protocol.

Validation Approach:

(b) (4)

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

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

Acceptance Criteria:

(b) (4)

Summary and Conclusions:

(b) (4)

Sample Preparation:

(b) (4)

Sample #	Approximate Concentration	Approximate Grams of BR-67 SDP to Use
1	Lot A Normal	(b) (4)
2	Lot A 200% of Normal	
3	Lot A 10% of Normal	
4	Lot B Normal	
5	Lot B 200% of Normal	
6	Lot B 10% of Normal	

(b) (4)

(b) (4) [redacted]

(b) (4) [redacted]

Analyst Name: (b)(6) [redacted]

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
1-1	(b) (4), (b)(6) [redacted]	[redacted]	T2020		1-2	(b) (4) [redacted]	[redacted]	(b)(6) [redacted]
1-1	[redacted]	[redacted]	T2020		1-2	[redacted]	[redacted]	[redacted]
1-1	[redacted]	[redacted]	T2020		1-2	[redacted]	[redacted]	02OCT2020

(b) (4) [redacted]

(b) (4) [redacted]

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
1-3	(b) (4) [redacted]	[redacted]	(b)(6) [redacted] 02OCT2020		2	(b) (4) [redacted]	[redacted]	(b)(6) [redacted] 02OCT2020
1-3	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020		2	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020
1-3	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020		2	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020

(b)(4) [redacted]

(b) (4) [redacted]

(b) (4) [redacted]

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
5	[redacted] (b)(4)	[redacted]	(b)(6) [redacted] 02OCT2020		6	[redacted] (b)(4)	[redacted]	(b)(6) [redacted] 02OCT2020
5	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020		6	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020
5	[redacted]	[redacted]	(b)(6) [redacted] 02OCT2020		6	[redacted]	[redacted]	(b)(6) [redacted] 02 OCT2020

(b) (4)

(b) (4)

Sample	Plate	Colonies	Initial/Date	Sample	Plate	Colonies	Initial/Date
3	(b) (4)	(b) (4)	(b)(6) 02 OCT2020	4	(b) (4)	(b) (4)	(b)(6) 02 OCT2020
3	(b) (4)	(b) (4)	(b) (4) 02OCT2020	4	(b) (4)	(b) (4)	(b)(6) 02OCT2020
3	(b) (4)	(b) (4)	(b) (4) 02OCT2020	4	(b) (4)	(b) (4)	(b)(6) 02OCT2020

(b) (4)

[redacted] (b) (4)

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
3	[redacted] (b) (4), (b)(6)	[redacted]	[redacted] 2Oct2020		4	[redacted] (b) (4), (b)(6)	[redacted]	[redacted] 02Oct2020
	[redacted]	[redacted]	[redacted] Oct2020			[redacted]	[redacted]	[redacted] 02Oct2020
	[redacted]	[redacted]	[redacted] 2Oct 2020			[redacted]	[redacted]	[redacted] 02Oct2020
5	[redacted]	[redacted]	[redacted] 2Oct2020		6	[redacted]	[redacted]	[redacted] 02Oct2020
	[redacted]	[redacted]	[redacted] 2Oct2020			[redacted]	[redacted]	[redacted] 02Oct2020
	[redacted]	[redacted]	[redacted] 2Oct 2020			[redacted]	[redacted]	[redacted] 02Oct2020

Sample			Initial/Date		Sample			Initial/Date
3 PDM 1-3	Final Result (CFU/g)	3.28E9	[redacted] (b)(6) 2020		4 PDM 1-3	Final Result (CFU/g)	1.48E9	[redacted] (b)(6) ct 2020
	Standard Deviation	~~1.6E①~~ 8.49E8	[redacted] 2020			Standard Deviation	1.66E8	[redacted] t 2020
	CV	26%	[redacted] Oct 2020			CV	11%	[redacted] 2Oct2020
	Valid Results? (circle)	[✓ Yes] No	[redacted] 2020			Valid Results? (circle)	[✓ Yes] No	[redacted] ct 2020
5 PDM 1-3	Final Result (CFU/g)	1.54E9	[redacted] 2020		6 PDM 1-3	Final Result (CFU/g)	2.13E9	[redacted] ct 2020
	Standard Deviation	2.34E8	[redacted] 2020			Standard Deviation	4.46E8	[redacted] ct 2020
	CV	15%	[redacted] Oct 2020			CV	21%	[redacted] 02Oct2020
	Valid Results? (circle)	[✓ Yes] No	[redacted] 02Oct 2020			Valid Results? (circle)	[✓ Yes] No	[redacted] 02Oct 2020

[redacted] (b) (4)

(b) (4)

Calculations and Results

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
1-1	(b) (4), (b)(6)		2OCT2020		1-2	(b) (4)		0 2OCT2020
			2OCT2020					O2OCT2020
			2OCT2020					02OCT2020
1-3			2OCT2020		2			O2OCT2020
			2OCT2020					O2OCT2020
			2OCT2020					O2OCT2020

Sample			Initial/Date		Sample			Initial/Date
1-1 PDM 1-3	**Final Result (CFU/g)**	7.17E9	(b)(6) 2OCT2020		1-2 PDM 1-3	**Final Result (CFU/g)**	8.83E9	(b)(6) 02OCT2020
	Standard Deviation	3.73E9	2OCT2020			**Standard Deviation**	5.35E9	(b)(6) 02OCT2020
	CV	52%	2OCT2020			**CV**	60.6%	(b)(6) 02OCT2020
	Valid Results? (circle)	[✓ Yes] No	2OCT2020			**Valid Results? (circle)**	Yes [✓ No]	02OCT2020
1-3 PDM 1-3	**Final Result (CFU/g)**	5.60E9	T2020		2 PDM 1-3	**Final Result (CFU/g)**	6.68E9	(b)(6) 02OCT2020
	Standard Deviation	9.73E8	2020			**Standard Deviation**	4.22E8	(b)(6) 02OCT2020
	CV	17%	2OCT2020			**CV**	6%	(b)(6) 02OCT2020
	Valid Results? (circle)	[✓ Yes] No	02OCT2020			**Valid Results? (circle)**	[✓ Yes] No	OCT2020

[redacted] (b)(4)

Data Collection – Analyst 2 **Analyst Name:** [redacted] (b)(4)

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
1	[redacted] (b)(4)	[redacted]	[redacted] (b)(6) 09OCT2020		2	[redacted] (b)(4)	[redacted]	[redacted] (b)(6) 09 OCT 2020
1	[redacted]	[redacted]	[redacted] (b)(6) 09 OCT 2020		2	[redacted]	[redacted]	[redacted] (b)(6) 09 OCT 2020
1	[redacted]	[redacted]	[redacted] (b)(6) 09OCT2020		2	[redacted]	[redacted]	[redacted] (b)(6) 09 OCT 2020

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
3	(b)(4)	(b)(4)	(b)(6) 09 OCT2020		4	(b)(4)	(b)(4)	(b)(6) 09 OCT2020
3	(b)(4)	(b)(4)	(b)(6) 09 OCT2020		4	(b)(4)	(b)(4)	(b)(6) 09 OCT2020
3	(b)(4)	(b)(4)	(b)(6) 09 OCT2020		4	(b)(4)	(b)(4)	(b)(6) 09 OCT2020

(b) (4)

[redacted] (b) (4)

Sample	Plate	Colonies	Initial/Date		Sample	Plate	Colonies	Initial/Date
5	[redacted] (b)(4)	[redacted]	[redacted] (b)(6) 09OCT2020		6	[redacted] (b)(4)	[redacted]	[redacted] (b)(6) 09OCT2020
5	[redacted]	[redacted]	[redacted] (b)(6) 09OCT2020		6	[redacted]	[redacted]	[redacted] (b)(6) 09OCT2020
5	[redacted]	[redacted]	[redacted] (b)(6) 09OCT2020		6	[redacted]	[redacted]	[redacted] (b)(6) 09OCT2020

Calculations and Results

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
1	[redacted]	[redacted]	(b) (4), (b)(6) [redacted] 09 OCT2020		2	[redacted]	[redacted]	(b) (4), (b)(6) [redacted] 09 OCT2020
			09 OCT2020					09 OCT2020
			09 OCT2020					09 OCT2020
3			09 OCT2020		4			09 OCT 2020
			09 OCT2020					09 OCT2020
			09 OCT2020					09 OCT2020

Sample			Initial/Date		Sample			Initial/Date
1 PDM 1-3	Final Result (CFU/g)	1.79E09	(b)(6) [redacted] OCT2020		2 PDM 1-3	Final Result (CFU/g)	2.20E09	(b)(6) [redacted] 09 OCT2020
	Standard Deviation	4.09E08	09 OCT2020			Standard Deviation	2.52E08	09 OCT2020
	CV	23%	09 OCT2020			CV	11%	09 OCT2020
	Valid Results? (circle)	[✓ Yes] No	09 OCT2020			Valid Results? (circle)	[✓ Yes] No	09 OCT2020
3 PDM 1-3	Final Result (CFU/g)	4.54E09	09 OCT2020		4 PDM 1-3	Final Result (CFU/g)	1.80E09	09 OCT2020
	Standard Deviation	8.57E08	09 OCT2020			Standard Deviation	1.40E08	09 OCT2020
	CV	19%	09 OCT2020			CV	8%	09 OCT2020
	Valid Results? (circle)	[✓ Yes] No	09 OCT2020			Valid Results? (circle)	[✓ Yes] No	09 OCT2020

[redacted] (b) (4)

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
5	(b) (4), (b)(6)		09 OCT 2020		6	(b) (4), (b)(6)		09 OCT 2020
			09 OCT 2020					09 OCT 2020
			09 OCT 2020					09 OCT 2020

Sample			Initial/Date		Sample			Initial/Date
5 PDM 1-3	Final Result (CFU/g)	(b) (4)	(b)(6) 09 OCT 2020		6 PDM 1-3	Final Result (CFU/g)	1.74E09	(b)(6) 09 OCT 2020
	Standard Deviation		09 OCT 2020			Standard Deviation	6.74E08	09 OCT 2020
	CV		09 OCT 2020			CV	39%	09 OCT 2020
	Valid Results? (circle)	[✓ Yes] No	09 OCT 2020			Valid Results? (circle)	[✓ Yes] No	09 OCT 2020

(b) (4)

Method

Title	BR67 SDP Microbe Enumeration
Version	01Draft
Effective Date	
Author	(b)(6)
Approver (Signature & Date)	Martin Mayhew VP – Process Development & Manufacturing

Scope

The purpose of this assay is to determine the number of viable spores of *Clostridium beijerinckii* in Broiler-67 Spray Dried Powder (BR67-SDP) by counting colony forming units (CFU) on solid media.

Safety

Consult the Safety Data Sheet for all reagents prior to handling. Use caution in working with a hot water bath and hot liquids. Wear appropriate personal protective equipment (safety glasses and gloves) at all times.

Materials

(b) (4) EZ large incubation container (b) (4)
EZ anaerobe container system sachets (b) (4)
15mL Polypropylene Centrifuge Tubes (b) (4)
Test tubes, 13x100 mm, sterile
Test tube cap, 16 mm, polypropylene
1.5 mL polypropylene microcentrifuge tube with snap cap
1000 µL Pipette
200 µL Pipette
1000 µL pipette tips, sterile
200 µL pipette tips, sterile
Glass beads, 3 mm, sterile, new

Equipment

Water bath set to 50°C
Laboratory Vortexer
Class I/II Biosafety Cabinet
pH meter
Magnet Stir Plate

Media & Reagents

NOTE: *Comparable quality ingredients (Laboratory, NF, USP, Reagent, or ACS grade) from different suppliers may be used.*

(b) (4) 10X Phosphate Buffered Saline pH 7.4 (PBS), sterile (b) (4)
1X Phosphate Buffered Saline with 0.05% TWEEN pH 7.4, sterile ((b) (4)
plates

Reagent grade 95% Ethanol

Method

1. (b) (4)

(b) (4)

2. Prepare the Primary Dilution Mix

(b) (4)

3. BR67-SDP Heat Shock Anaerobic Plating

(b) (4)

(b) (4)

4. Negative Control Plating

(b) (4)

5 Plate Counting

(b) (4)

(b) (4)

(b) (4)

(b) (4)

Sample	(b) (4)
1-3	[redacted]
1-3	[redacted]
1-3	[redacted]

Sample	(b) (4)
2	[redacted]
2	[redacted]
2	[redacted]

[redacted] (b) (4)

Data Collection – Analyst 1 **Analyst Name:** (b)(6)

Sample		Sample	
1-1	(b) (4)	1-2	(b) (4)
1-1		1-2	
1-1		1-2	

(b) (4)

[redacted] (b)(4)

Calculations and Results

Sample	PDM	Avg CFU/g	Initials/Date		Sample	PDM	Avg CFU/g	Initials/Date
1-1	[redacted]	[redacted] (b)(4), (b)(6)	06OCT2020		1-2	[redacted]	[redacted] (b)(4), (b)(6)	6OCT2020
	[redacted]	[redacted]	06OCT2020			[redacted]	[redacted]	6OCT2020
	[redacted]	[redacted]	06OCT2020			[redacted]	[redacted]	6OCT2020
1-3	[redacted]	[redacted]	06OCT2020		2	[redacted]	[redacted]	
	[redacted]	[redacted]	06OCT2020			[redacted]	[redacted]	
	[redacted]	[redacted]	06OCT2020			[redacted]	[redacted]	

Sample			Initial/Date		Sample			Initial/Date
1-1 PDM 1-3	Final Result (CFU/g)	[redacted] (b)(4)	[redacted] (b)(6) T2020		1-2 PDM 1-3	Final Result (CFU/g)	[redacted] (b)(4)	[redacted] (b)(6) 06OCT2020
	Standard Deviation	[redacted]	[redacted] (b)(6) 06OCT2020			Standard Deviation	[redacted]	[redacted] (b)(6) 06OCT2020
	CV	[redacted]	[redacted] (b)(6) 6OCT2020			CV	[redacted]	[redacted] (b)(6) 06OCT2020
	Valid Results? (circle)	[redacted]	06OCT2020			Valid Results? (circle)	[redacted]	06OCT2020
1-3 PDM 1-3	Final Result (CFU/g)	[redacted] (b)(4)	[redacted] (b)(6) 06OCT2020		2 PDM 1-3	Final Result (CFU/g)		
	Standard Deviation	[redacted]	[redacted] (b)(6) 06OCT2020			Standard Deviation		
	CV	[redacted]	[redacted] (b)(6) 06OCT2020			CV		
	Valid Results? (circle)	[redacted]	06OCT2020			Valid Results? (circle)	Yes No	

(b) (4)

Validation Summary Report

BR67-SDP Microbe Enumeration, V1

Objective

The objective of this validation study was to demonstrate that the BR67-SDP Microbe Enumeration method can quantify the amount of BR67 (*Clostridium beijerinckii* ASCUSBR67) in SDP samples. The method was evaluated for repeatability, robustness, and linearity.

Repeatability was assessed through the closeness of results obtained on the same sample (19-0202-007-P48) when assayed multiple times by the same person with the same reagents and equipment.

Robustness was assessed through the closeness of results obtained on the same set of samples (19-0202-007-P48 and 19-0202-007-P69) across multiple analysts and reagent preparations.

Linearity was assessed by enumerating the same sample at a concentration of 200% and 10% of the original sample (19-0202-007-P48 and 19-0202-007-P69).

Results

Repeatability

The average of samples 1-1, 1-2, and 1-3 is 3.04E+09 CFU/g with a standard deviation of 4.86E+08 CFU/g. The coefficient of variation from these samples is 16%. The low CV resulting from repeated measurements of the same sample demonstrates the repeatability of the assay.

Table 1: Summary table of BR67-SDP method validation results

Analyst	Lot #		Average CFU/g	STDEV	CV
1	19-0202-007-P48 (0.1g)	Sample 1-1	(b) (4)	[redacted]	[redacted]
		Sample 1-2	[redacted]	[redacted]	[redacted]
		Sample 1-3	[redacted]	[redacted]	[redacted]
	19-0202-007-P48 (0.2g)	Sample 2	[redacted]	[redacted]	[redacted]
	19-0202-007-P48 (0.01g)	Sample 3	[redacted]	[redacted]	[redacted]
	19-0202-007-P69 (0.1g)	Sample 4	[redacted]	[redacted]	[redacted]
	19-0202-007- P69 (0.2g)	Sample 5	[redacted]	[redacted]	[redacted]
	19-0202-007- P69 (0.01g)	Sample 6	[redacted]	[redacted]	[redacted]

Analyst	Lot #		Average CFU/g	STDEV	CV
2	19-0202-007-P48 (0.1g)	Sample 1	(b) (4)	[redacted]	[redacted]
	19-0202-007-P73 (0.2g)	Sample 2	[redacted]	[redacted]	[redacted]
	19-0202-007-P73 (0.01g)	Sample 3	[redacted]	[redacted]	[redacted]
	19-0202-007-P48 (0.2g)	Sample 4	[redacted]	[redacted]	[redacted]
	19-0202-007-P48 (0.01g)	Sample 5	[redacted]	[redacted]	[redacted]
	19-0202-007-P69 (0.1g)	Sample 6	[redacted]	[redacted]	[redacted]

Robustness

Samples 1-6 were enumerated by an independent analyst. All samples met the criteria of a CV less than 60%. The CV when all sample were averaged for analyst one and analyst two is 24% for lot 19-0202-007-P48 and 21% for lot 19-0202-007-P69 demonstrating that the assay is robust.

	19-0202-007-P48							19-0202-007-P69						
Analyst	Average (g)	AVG CFU/g	STD Dev	CV	AVG CFU/g	AVG STDEV	AVG CV	Average (g)	AVG CFU/g	STD Dev	CV	AVG CFU/g	AVG STDEV	AVG CV
1	(b) (4)	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
2	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
AVG 1&2	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

Linearity

(b) (4)

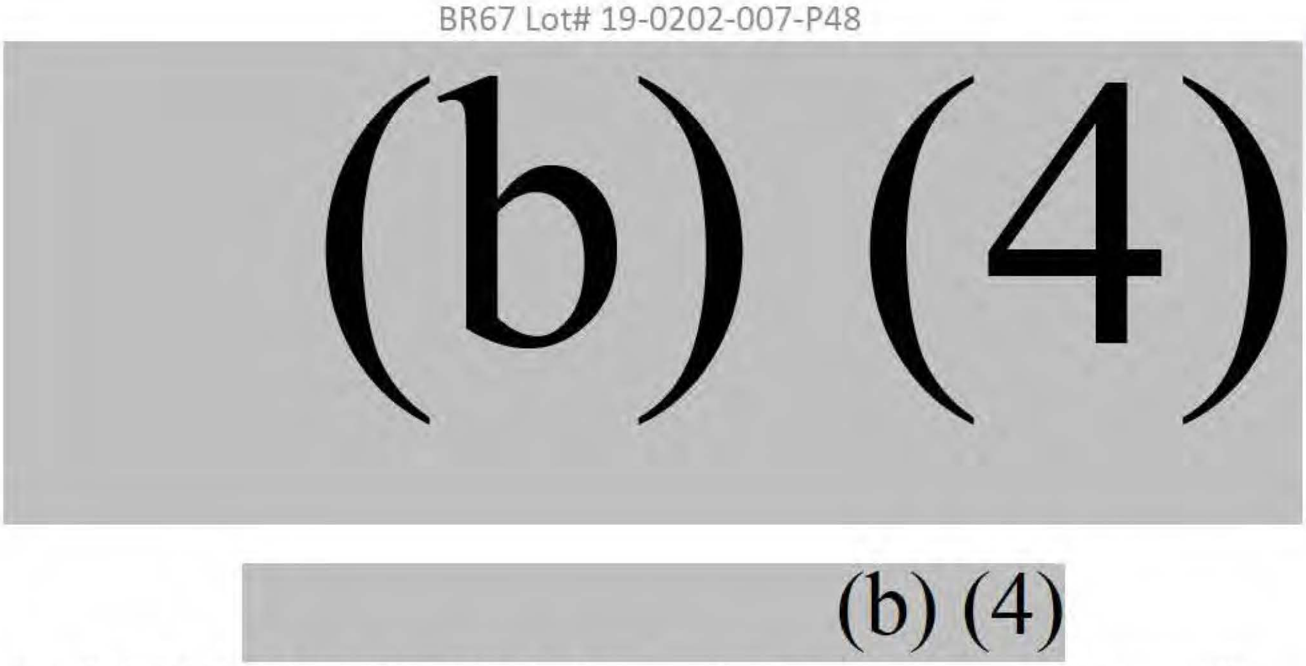


Figure 1. BR67 SDP Lot# 19-0202-007-P48 is plotted with average weight vs CFU/g. Average Analyst 1 and 2 is represented by triangles (R^2=0.1135)

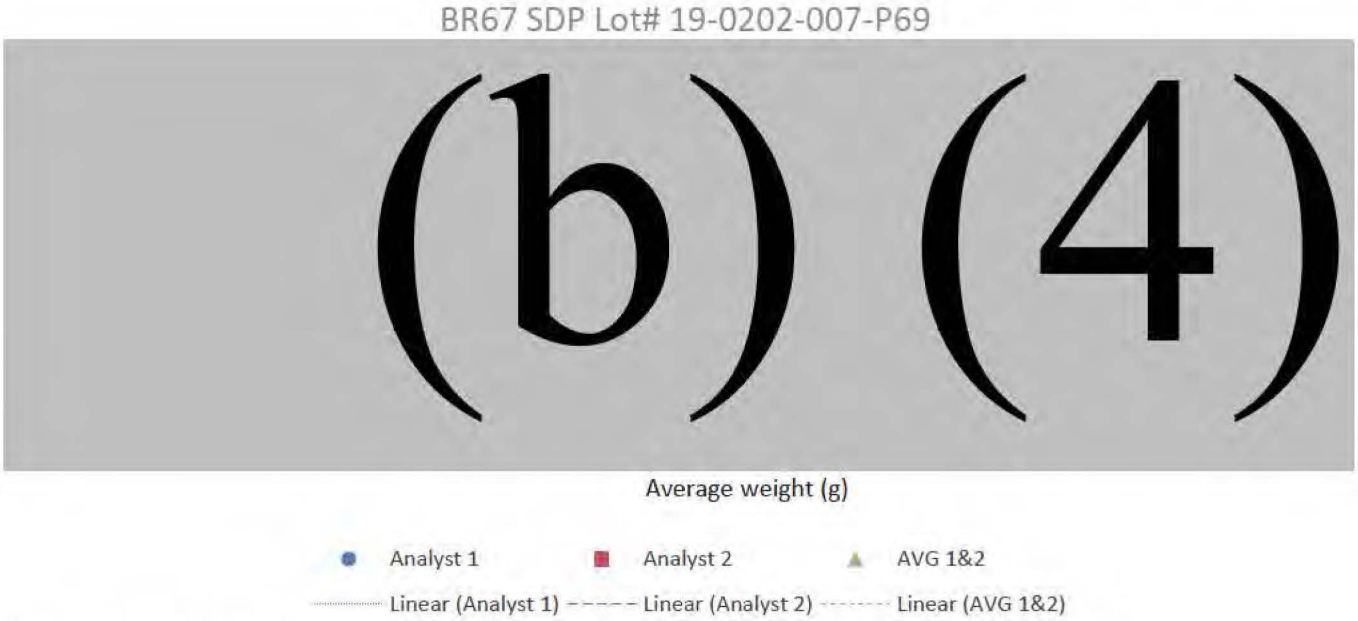


Figure 2. BR67 SDP Lot# 19-0202-007-P69 is plotted with average weight vs CFU/g. Average Analyst 1 and 2 is plotted represented by triangles (R^2=0.9975)

Excursions from the Protocol

(b) (4)

Conclusion

(b) (4)

[redacted].

Location of Data

(b) (4)

Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew 53249323BAF84C2...	10/29/2020
(b)(6) Quality	(b)(6)	10/29/2020

native

nativemicrobials.com

Product Certificate of Analysis

Product Name	*C. beijerinckii* ASCUSBR67 Spray Dried Powder
Batch Number	20-0202-049-P6
Date of Manufacture	30Nov2020
Expiration Date	N/A
Retest Date	30Nov2021
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) 2/18/2021

Quality

(b) (4)

native

nativemicrobials.com

Product Certificate of Analysis

Product Name	*C. beijerinckii* ASCUSBR67 Spray Dried Powder
Batch Number	20-0202-049-P7
Date of Manufacture	30Nov2020
Expiration Date	N/A
Retest Date	30Nov2021
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) 2/18/2021

Quality

native

nativemicrobials.com

Product Certificate of Analysis

Product Name	*C. beijerinckii* ASCUSBR67 Spray Dried Powder
Batch Number	20-0202-049-P12A
Date of Manufacture	07Dec2020
Expiration Date	N/A
Retest Date	07Dec2021
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) 2/18/2021

Quality

Analysis of *Clostridium beijerinckii* ASCUSBR67 (BR67) for Heavy Metals & Microbial Contamination

Approvers:

DocuSigned by:
Martin Mayhew
CA3DAF452B8A47C...

2/10/2021

Martin Mayhew
Vice President – Product Development
& Manufacturing

Date

(b)(6)

2/11/2021

Date

Quality

DocuSigned by:
kevin korth
1C2149273B2345F...

2/5/2021

Kevin Korth
Regulatory

Date

Prepared by
Native Microbials, Inc
San Diego, CA

February 2021

[redacted] (b) (4)

Analysis of *Clostridium beijerinckii* ASCUSBR67 for Heavy Metals & Microbial Contamination

Three lots of *Clostridium beijerinckii* ASCUSBR67 were sent for heavy metal and microbial contamination analysis at [redacted] (b) (4) (Note: *C. beijerinckii* ASCUSBR67 is listed on certificate of analysis as BR67 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 *Clostridium beijerinckii* ASCUSBR67

Lot Number	Arsenic, ppm	Cadmium, ppm	Lead, ppm	Mercury, ppm
Detection Limit	0.004	0.0008	0.001	0.001
20-0202-049-P6	0.286	0.215	0.017	ND
20-0202-049-P7	0.266	0.054	0.050	ND
20-0202-049-P12A	0.133	0.043	0.019	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 *Clostridium beijerinckii* ASCUSBR67

Lot Number	Coliform, CFU/g	E. coli, CFU/g	Salmonella, per 25g	Listeria, per 25g
Requirement	<10	<10	Negative	Negative
20-0202-049-P6	<10	<10	Negative	Negative
20-0202-049-P7	<10	<10	Negative	Negative
20-0202-049-P12A	<10	<10	Negative	Negative

Clostridium beijerinckii ASCUSBR67 is intended to be fed at a rate of 0.5 lbs per ton of broiler feed. Given the low inclusion rate in the broiler feed, no heavy metal specification is needed. However, all lots will be tested for microbial contamination at the end of the production of *Clostridium beijerinckii* ASCUSBR67.

Attachment 1. Certificate of Analysis – Heavy Metal Analysis (b) (4)

(b) (4)

Certificate of Analysis

December 31, 2020

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

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

SAMPLE INFORMATION

Description	BR67
Lot Number	20-0202-049-P6
Received	December 18, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 18, 2020 to December 31, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	0.286
Cadmium	0.001/0.001	0.215
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	0.017

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

Reported by
(b) (4), (b)(6)

December 31, 2020

If there are any questions with this report, please contac (b) (4)

(b) (4)

page 1 of 1

Attachment 2. Certificate of Analysis – Heavy Metal Analysis (b) (4)

(b) (4)

Certificate of Analysis

December 31, 2020

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

Order N (b) (4)
Sample No

SAMPLE INFORMATION

Description	BR67
Lot Number	20-0202-049-P7
Received	December 18, 2020

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	December 18, 2020 to December 31, 2020

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.004	0.266
Cadmium	0.001/0.001	0.054
Mercury	0.001/0.001	None detected
Lead	0.001/0.001	0.050

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

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

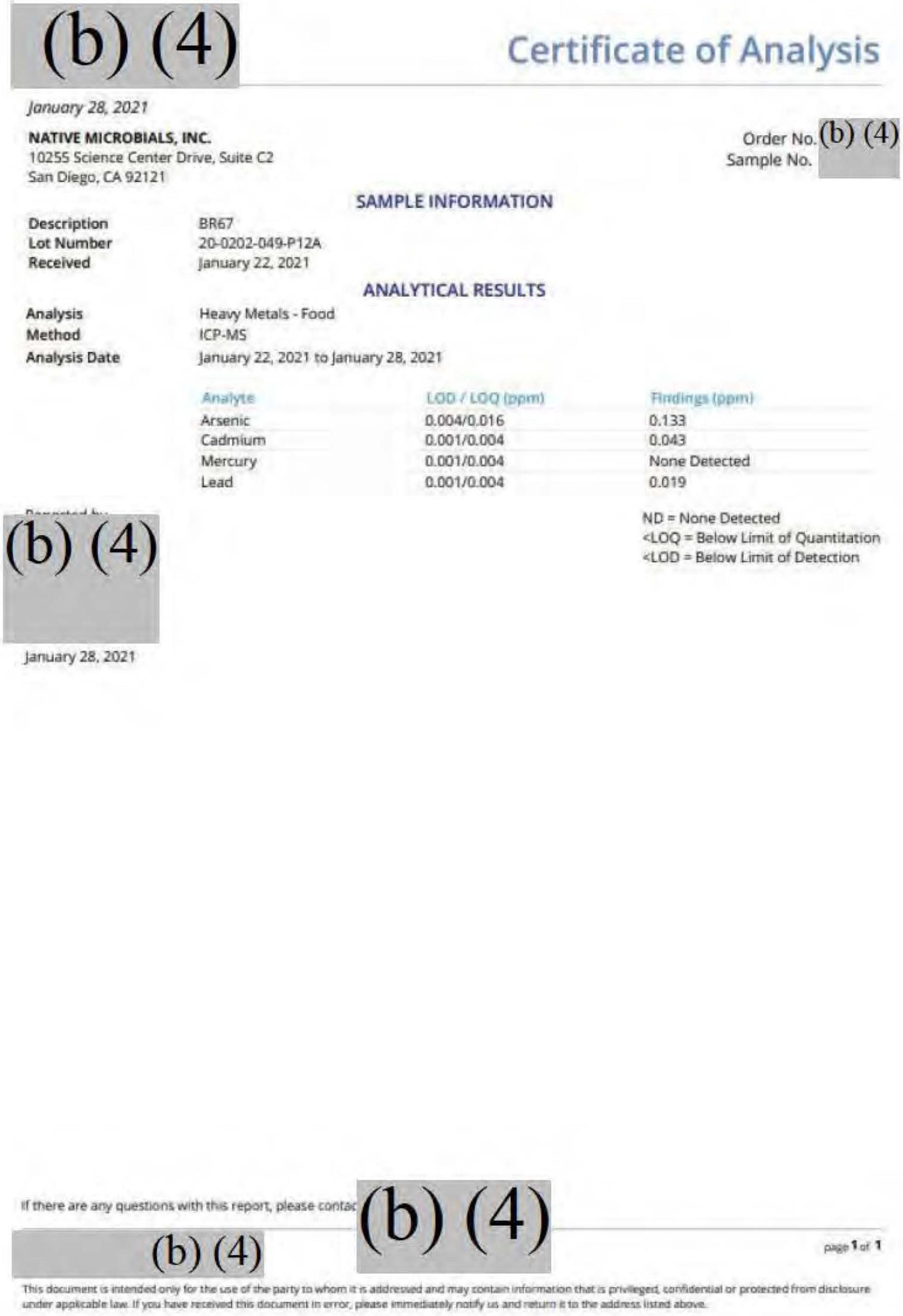
December 31, 2020

If there are any questions with this report, please contac (b) (4)

(b) (4)

page 1 of 1

Attachment 3. Certificate of Analysis –Heavy Metal Analysis (b) (4)



(b) (4)

Certificate of Analysis

January 28, 2021

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

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

SAMPLE INFORMATION

Description	BR67
Lot Number	20-0202-049-P12A
Received	January 22, 2021

ANALYTICAL RESULTS

Analysis	Heavy Metals - Food
Method	ICP-MS
Analysis Date	January 22, 2021 to January 28, 2021

Analyte	LOD / LOQ (ppm)	Findings (ppm)
Arsenic	0.004/0.016	0.133
Cadmium	0.001/0.004	0.043
Mercury	0.001/0.004	None Detected
Lead	0.001/0.004	0.019

(b) (4)

January 28, 2021

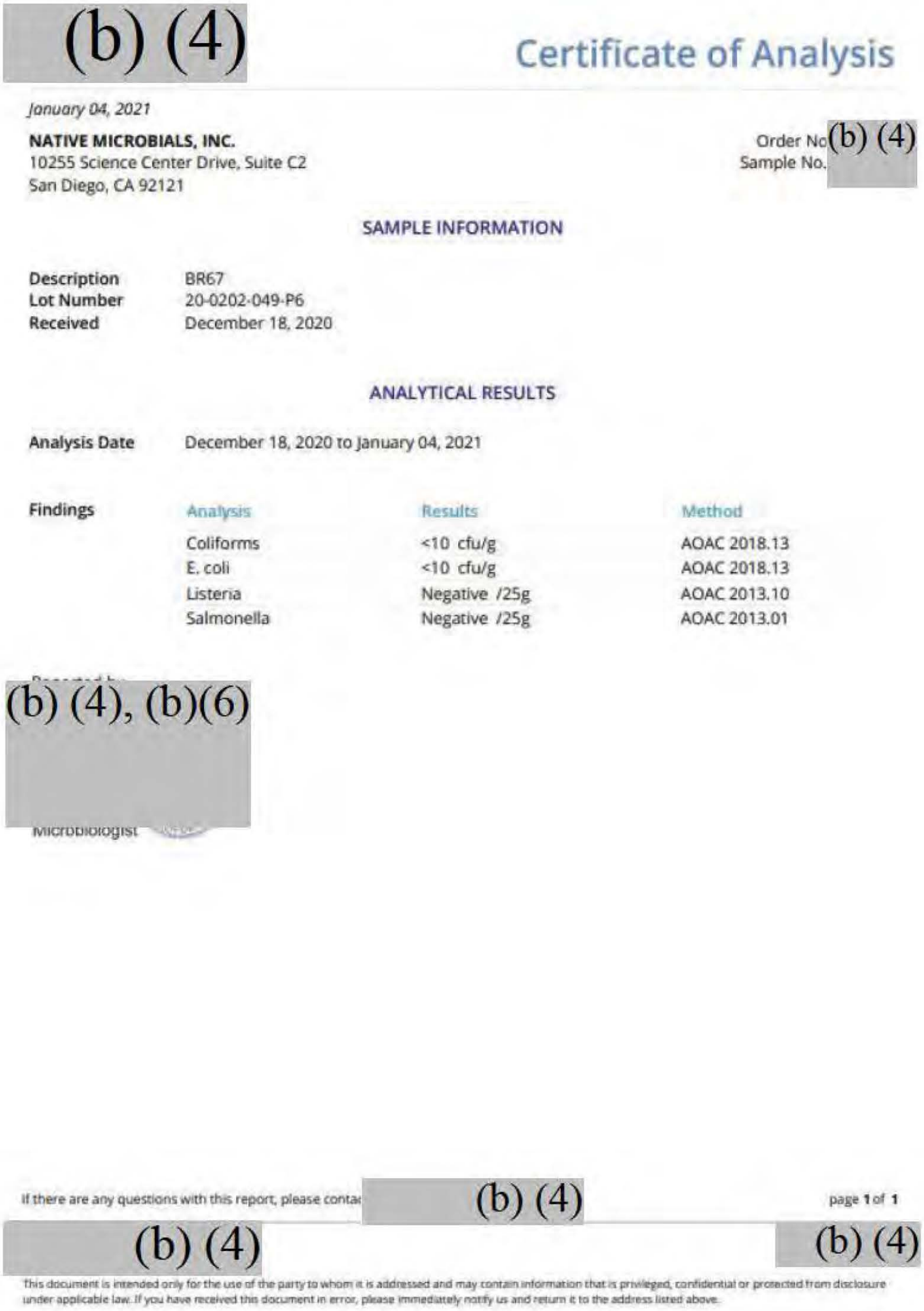
ND = None Detected
<LOQ = Below Limit of Quantitation
<LOD = Below Limit of Detection

If there are any questions with this report, please contac (b) (4)

(b) (4)

page 1 of 1

Attachment 4. Certificate of Analysis – Microbial Contamination Testing (b) (4)



(b) (4)

Certificate of Analysis

January 04, 2021

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

Order No (b) (4)
Sample No.

SAMPLE INFORMATION

Description	BR67
Lot Number	20-0202-049-P6
Received	December 18, 2020

ANALYTICAL RESULTS

Analysis Date December 18, 2020 to January 04, 2021

Findings

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

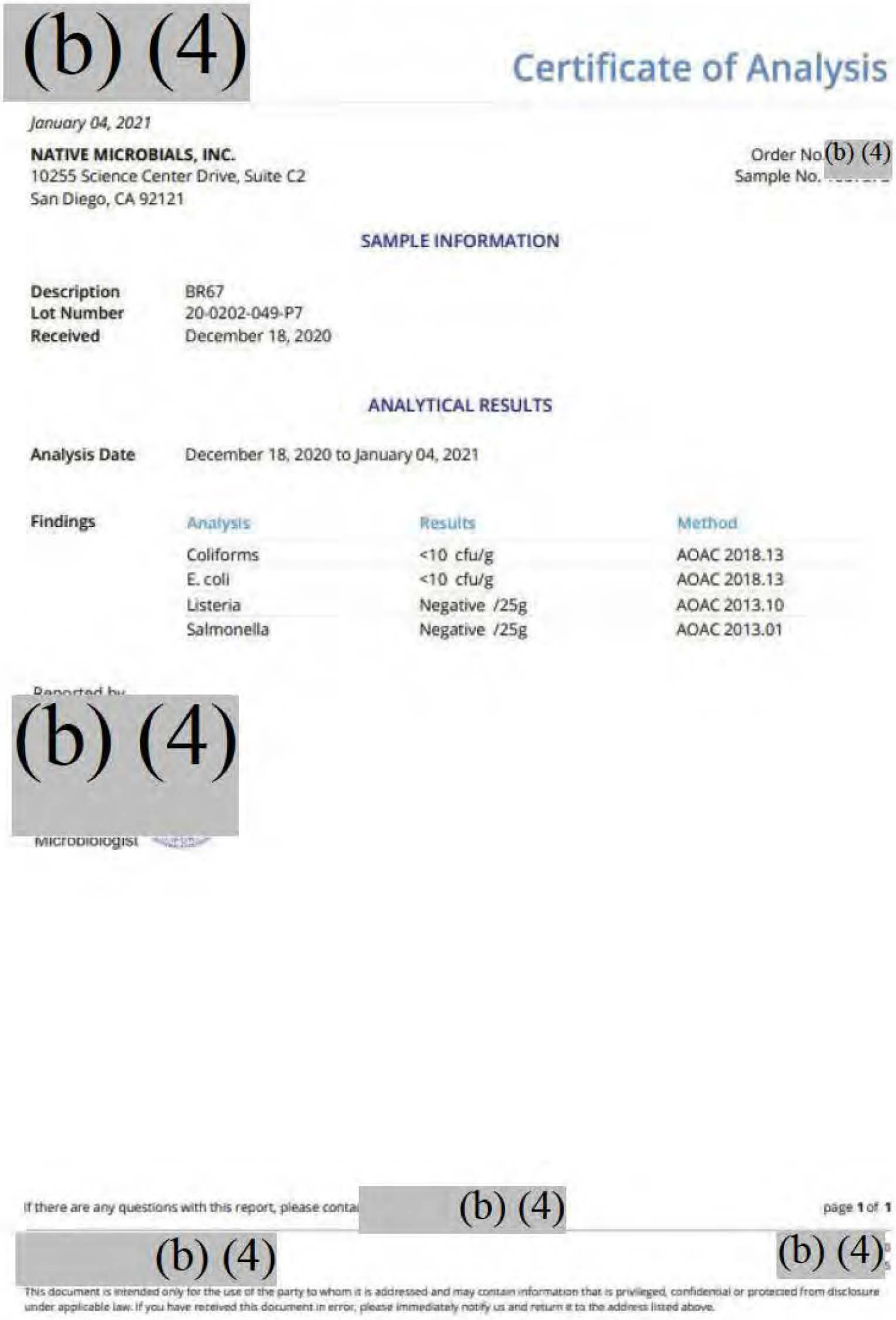
(b) (4), (b)(6)

If there are any questions with this report, please conta (b) (4)

page 1 of 1

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

Attachment 5. Certificate of Analysis – Microbial Contamination Testing (b) (4)



(b) (4)

Certificate of Analysis

January 04, 2021

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

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

SAMPLE INFORMATION

Description	BR67
Lot Number	20-0202-049-P7
Received	December 18, 2020

ANALYTICAL RESULTS

Analysis Date December 18, 2020 to January 04, 2021

Findings

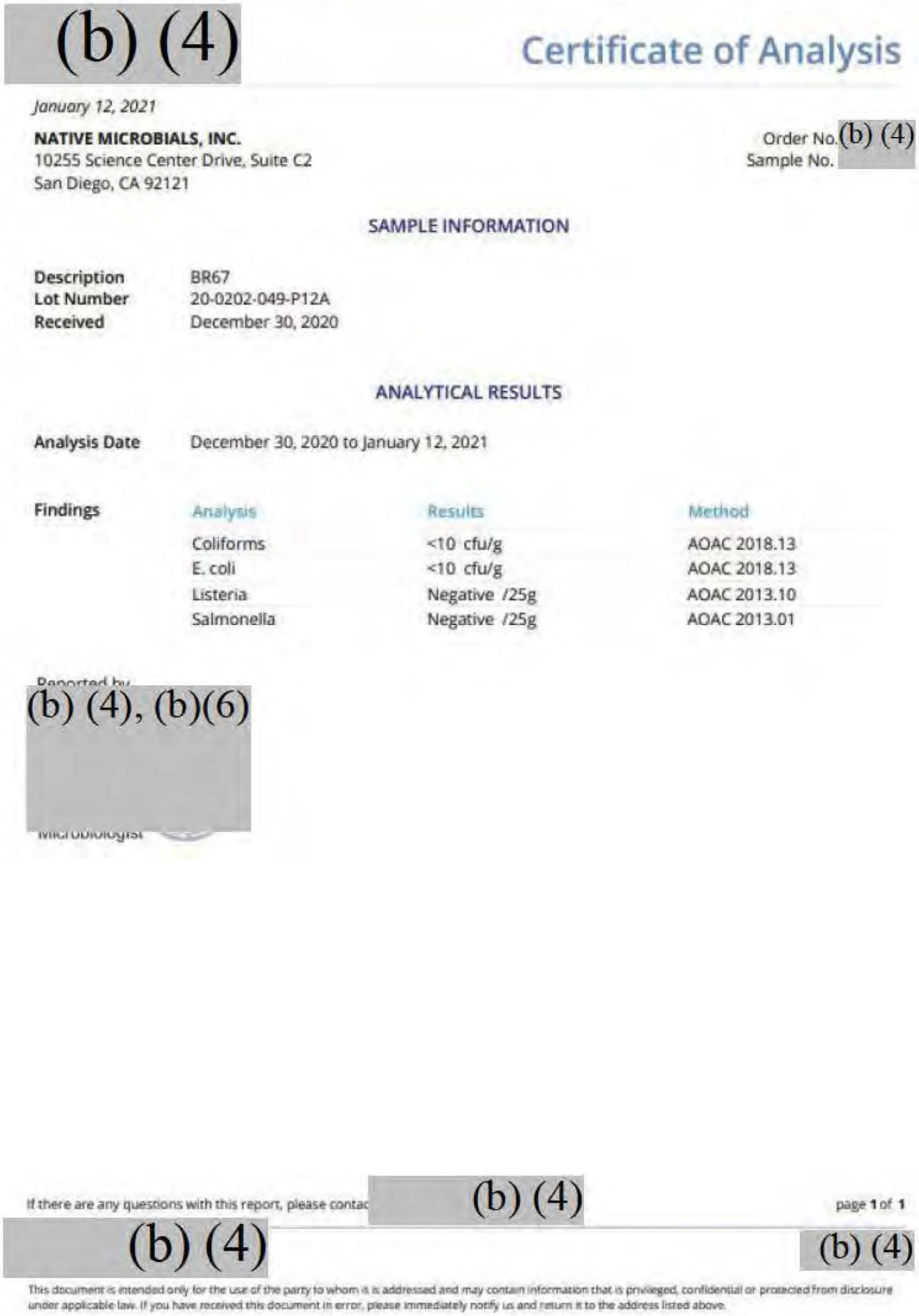
Analysis	Results	Method
Coliforms	<10 cfu/g	AOAC 2018.13
E. coli	<10 cfu/g	AOAC 2018.13
Listeria	Negative /25g	AOAC 2013.10
Salmonella	Negative /25g	AOAC 2013.01

(b) (4)

If there are any questions with this report, please conta (b) (4) page 1 of 1

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

Attachment 6. Certificate of Analysis – Microbial Contamination Testing (b) (4)



(b) (4)

Certificate of Analysis

January 12, 2021

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

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

SAMPLE INFORMATION

Description	BR67
Lot Number	20-0202-049-P12A
Received	December 30, 2020

ANALYTICAL RESULTS

Analysis Date December 30, 2020 to January 12, 2021

Findings

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

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

If there are any questions with this report, please contac (b) (4) page 1 of 1

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

Stability Protocol Titles:	*Clostridium beijerinckii* ASCUSBR67 Mash 25°C
Organism	*Clostridium beijerinckii* ASCUSBR67
Purpose:	To test the stability of the final product in mash under conditions that the mash may be stored at.
Number of Samples to Place on Stability:	6 samples per temperature condition.
Sample Storage Container:	Unsealed craft Poly Lined bag
Temperature Conditions:	25°C ± 2°C > 60% Relative humidity *
Acceptance Criteria:	See acceptance criteria section
Method:	BR67 Pellet and Mash Microbe Enumeration method

1. Introduction

A stability plan was developed for *Clostridium beijerinckii* ASCUSBR67 mash in accordance with Native Microbials stability procedure[1], CVM Guidelines[2], WHO Guidelines[3], and ICH Guidelines[4]. Stability studies will be conducted according to pre-approved protocols (b) (4)

(b) (4)

2. Sampling Plan

(b) (4)

(b) (4)

(b) (4)

3. Container Description

(b) (4)

4. Testing Conditions

(b) (4)

5. Testing Frequency

(b) (4)

6. Selection of Batches

(b) (4)

(b) (4)

7. Preparation of Samples

(b) (4)

8. Equipment & Monitoring

(b) (4)

9. Data & documentation

(b) (4)

10. Analysis

(b) (4)

11. Acceptance Criteria

(b) (4)

12. Summary and Conclusions

(b) (4)

13. Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/16/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F...	3/16/2021
(b)(6) Quality	(b)(6)	3/16/2021

Stability Protocol Titles:	*Clostridium beijerinckii* ASCUSBR67 Pellet 25°C
Organism	*Clostridium beijerinckii* ASCUSBR67
Purpose:	To test the stability of the final product in pelleted feed under conditions that the product may be stored at.
Number of Samples to Place on Stability:	6 samples per temperature condition.
Sample Storage Container:	Unsealed craft Poly Lined bag
Temperature Conditions:	25°C ± 2°C > 60% Relative humidity *
Acceptance Criteria:	See acceptance criteria section
Method:	BR67 Pellet and Mash Microbe Enumeration method

1. Introduction

A stability plan was developed for *Clostridium beijerinckii* ASCUSBR67 pellet in accordance with Native Microbials stability procedure[1], CVM Guidelines[2], WHO Guidelines[3], and ICH Guidelines[4]. Stability studies will be conducted according to pre-approved protocols (b) (4)

(b) (4)

2. Sampling Plan

(b) (4)

(b) (4)

(b) (4)

3. Container Description

(b) (4)

4. Testing Conditions

(b) (4)

(b) (4)

5. Testing Frequency

(b) (4)

6. Selection of Batches

(b) (4)

(b) (4)

7. Preparation of Samples

[redacted] (b) (4)

8. Equipment & Monitoring

[redacted] (b) (4)

9. Data & documentation

[redacted] (b) (4)

10. Analysis

[redacted] (b) (4)

11. Acceptance Criteria

[redacted] (b) (4)

12. Summary and Conclusions

(b) (4)

(b) (4)

13. Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/16/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645E	3/16/2021
(b)(6) Quality	(b)(6)	3/16/2021

Stability Protocol Titles:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 25°C
Purpose:	Support recommended storage conditions.
Number of Samples to Place on Stability:	9
Sample Storage Container:	Metalized polyester with linear low-density polyethylene liner, heat sealed.
Temperature Conditions:	25°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	**T$_0$**	**1 Month**	**2 Month**	**3 Month**	**6 Month**	**9 Month**	**12 Month**
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

* (b) (4)

Protocol Approvals

Name & Title	**Signature & Date**	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/16/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F...	3/16/2021
(b)(6) Quality	(b)(6)	3/16/2021

Stability Protocol Titles:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 50°C
Purpose:	Support excursions during shipping and storage. Support an accelerated prediction of stability at 25°C through an Arrhenius prediction.
Number of Samples to Place on Stability:	9
Sample Storage Container:	Metalized polyester with linear low-density polyethylene liner, heat sealed.
Temperature Conditions:	50°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	**T_0**	**Day 1**	**Day 2**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

* (b) (4)

Protocol Approvals

Name & Title	**Signature & Date**	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/16/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F...	3/16/2021
(b) (4), (b)(6) Quality	(b)(6)	3/16/2021

Stability Protocol Title:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 50°C
Purpose:	Support excursions during shipping and storage. Support an accelerated prediction of stability at 25°C through an Arrhenius prediction.
Number of Samples to Place on Stability:	9
Sample Storage Container:	Metalized polyester bag with linear low-density polyethylene liner, heat sealed.
Temperature & Humidity Conditions:	50°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	**T_0**	**Day 1**	**Day 2**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

Protocol Approvals:

Name & Title	**Signature & Date**	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F...	12/1/2020
(b)(6) Quality	(b)(6)	12/1/2020

Stability Protocol Titles:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 60°C
Purpose:	Support an accelerated prediction of stability at 25°C through an Arrhenius prediction.
Number of Samples to Place on Stability:	9
Sample Storage Container:	Metalized polyester with linear low-density polyethylene liner, heat sealed.
Temperature Conditions:	60°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	T_0	Day 1	Day 2	Day 7	Day 14	Day 21	Day 28
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

(b) (4)

Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/16/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F	3/16/2021
(b)(6) Quality	(b)(6)	3/16/2021

Stability Protocol Title:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 60°C
Purpose:	Support an accelerated prediction of stability at 25°C through an Arrhenius prediction.
Number of Samples to Place on Stability:	9
Sample Storage Container:	Metalized polyester bag with linear low-density polyethylene liner, heat sealed.
Temperature & Humidity Conditions:	60°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	**T_0**	**Day 1**	**Day 2**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

Protocol Approvals:

Name & Title	**Signature & Date**
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491... 12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F 12/1/2020
(b)(6) Quality	(b)(6) 12/1/2020

Stability Protocol Titles:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 70°C
Purpose:	Support an accelerated prediction of stability at 25°C through an Arrhenius prediction.
Number of Samples to Place on Stability:	9
Sample Storage Container:	Metalized polyester with linear low-density polyethylene liner, heat sealed.
Temperature Conditions:	70°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	**T$_0$**	**Day 1**	**Day 2**	**Day 4**	**Day 5**	**Day 7**	**Day 10**
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

(b) (4) .

Protocol Approvals

Name & Title	**Signature & Date**	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	3/16/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F	3/16/2021
(b)(6) Quality	(b)(6)	3/16/2021

Stability Protocol Title:	BR67 SDP *Clostridium beijerinckii* ASCUSBR67 70°C
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature & Humidity Conditions:	70°C ± 2°C
Acceptance Criteria:	(b) (4)

Tests and Timepoints:

Assay	**T_0**	**Day 1**	**Day 2**	**Day 4**	**Day 5**	**Day 7**	**Day 10**
BR67 SDP Microbe Enumeration method	X	X	X	X	X	X	X

Protocol Approvals:

Name & Title	**Signature & Date**	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	12/1/2020
Howard Green Regulatory	DocuSigned by: Howard B Green	12/1/2020
(b)(6) Quality	(b)(6)	12/1/2020

Accelerated Stability Study Report for *Clostridium beijerinckii* ASCUSBR67 Spray Dried Powder

Approvers:

DocuSigned by:
Martin Mayhew
ACBDDAD433BF491...

Martin Mayhew
Vice President – Product Development
& Manufacturing

3/15/2021
Date

(b)(6)
Quality

3/17/2021
Date

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kevin korth
869AE3638AA543B...

Kevin Korth
Regulatory

3/16/2021
Date

(b)(6)
Scientist

3/15/2021
Date

Prepared By
Native Microbials, Inc
San Diego, CA

March 2021

Accelerated Stability Study Report for *Clostridium beijerinckii* ASCUSBR67 Spray Dried Powder

Objective

The objective of this study was to utilize accelerated stability data obtained from *Clostridium beijerinckii* ASCUSBR67 held at three different temperatures to establish a shelf life under normal storage conditions (25°C ± 2°C) using Arrhenius analysis (Wirunpan, Savedboworn, and Wanchaitanawong 2016; King, Lin, and Liu 1998).

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

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

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

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

Protocols:

The following protocols were used in this report:
BR67 SDP *Clostridium beijerinckii* ASCUSBR67 50°C
BR67 SDP *Clostridium beijerinckii* ASCUSBR67 60°C
BR67 SDP *Clostridium beijerinckii* ASCUSBR67 70°C

Results

Samples from each lot were placed at 50°C, 60°C, and 70°C and analyzed over time for viable cell count. The results are shown in Tables 1-3 and plotted in Figure 1.

Table 1: Stability at 50°C

	Lot 20-0202-049-P6		Lot 20-0202-049-P7		Lot 20-0202-049-P12A	
Time (days)	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV
0	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
1	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
2	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
7	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
14	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
21	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
28	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

(b) (4)

Table 2: Stability at 60°C

	Lot 20-0202-049-P6		Lot 20-0202-049-P7		Lot 20-0202-049-P12A	
Time (days)	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV
0	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

(b) (4)

1	(b) (4)
2	
7	
14	
21	
28	

Table 3: Stability at 70°C

	Lot 20-0202-049-P6		Lot 20-0202-049-P7		Lot 20-0202-049-P12A	
Time (days)	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV	Average (CFU/g)	STDEV
0			(b) (4)			
1						
2						
4						
5						
7						
10						

(b) (4)

Figure 1: Rate of Decay at 50°C, 60°C, and 70°C. The decay over time is plotted for each lot at each temperature. A rate of decay was calculated from the slope of the regression, displayed as a dark line. The light shaded area represents the 95% confidence interval for the regression.

Rates of decay for each lot at each temperature were calculated from the slope of decay over time. As shown in Figure 2, the probability distributions of predicted rates of decay for the 3 lots at 60°C were not overlapping. Therefore, independent shelf life analysis of each lot was required and the rate data from all 3 lots could not be pooled for a combined analysis.

(b) (4)

Figure 2: Probability Distributions for the Rate of Decay at 60°C. The probability distribution of decay rates for the three lots are represented. The distributions are non-overlapping, demonstrating that the rate of decay for the three lots are significantly different at 60°C. Therefore, independent analysis of each lot is required.

The rates of decay for each lot were fit to the linear form of the Arrhenius equation in order to provide a prediction for the rate of decay at 25°C, given storage temperatures of 23-27°C. The linear Arrhenius regression for the 3 lots are shown in Figure 3.

(b) (4)

Figure 3: Linear Arrhenius Regression. Decay rate and temperature were transformed to fit the linear form of the Arrhenius equation. The linear regression of the decay rate probability distributions for each lot are displayed. From the linear regression, a rate of decay at 25°C was predicted for each lot, which was then used to determine the shelf life. 25°C corresponds to a value of 0.00335 on the x-axis.

From the linear regression, the upper-tailed 95% confidence interval for decay rate at 25°C was calculated and used to define the shelf life of each batch. The results are displayed in Table 4. (b) (4) (b) (4), which resulted in the shortest predicted shelf life at 25°C of days.

Table 4: Predicted Decay Rate and Shelf Life at 25°C

Lot	Initial CFU/g	Log CFU/g to minimum	ln(Decay Rate)	Predicted Decay Rate (Log CFU/day)	Predicted Shelf Life (days)
20-0202-049-P6	(b) (4)				
20-0202-049-P7					
20-0202-049-P12A					

Conclusion

Accelerated stability analysis using the Arrhenius equation leads to a minimum predicted time of 4516 days at 25°C until the minimum label claim of 1.00E+08 is reached. The analysis was conducted on three representative lots of manufactured product and justifies a tentative shelf life of 12 months under 23-27°C storage conditions.

The stability study was carried out according to protocols with no deviations noted.

Data Availability

Stabilit data Arrhenius analysis, and the original rotocol can be found on the com an drive under (b) (4)/

References

1. King, V. An-Erl, Haur-Jie Lin, and Chia-Fung Liu. 1998. "Accelerated Storage Testing of Freeze-Dried and Controlled Low-Temperature Vacuum Dehydrated Lactobacillus Acidophilus." *The Journal of General and Applied Microbiology* 44 (2): 160–65.
2. Wirunpan, Maneerat, Wanticha Savedboworn, and Penkhae Wanchaitanawong. 2016. "Survival and Shelf Life of *Lactobacillus lactis* 1464 in Shrimp Feed Pellet after Fluidized Bed Drying." *Agriculture and Natural Resources* 50 (1): 1–7.

Stability Protocol Titles:	*Clostridium beijerinckii* ASCUSBR67 Mash 25°C
Organism	*Clostridium beijerinckii* ASCUSBR67
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature Conditions:	
Acceptance Criteria:	See acceptance criteria section
Method:	BR67 Pellet and Mash Microbe Enumeration method

1. Introduction

A stability plan was developed for *Clostridium beijerinckii* ASCUSBR67 mash in accordance with Native Microbials stability procedure[1], CVM Guidelines[2], WHO Guidelines[3] and ICH Guidelines[4] Stability studies will be conducted according to pre-approved protocols (b) (4)

(b) (4)

2. Sampling Plan

(b) (4)

(b) (4)

(b) (4)

3. Container Description

(b) (4)

4. Testing Conditions

(b) (4)

5. Testing Frequency

(b) (4)

6. Selection of Batches

(b) (4)

(b) (4)

7. Preparation of Samples

(b) (4)

8. Equipment & Monitoring

(b) (4)

9. Data & documentation

(b) (4)

10. Analysis

(b) (4)

11. Acceptance Criteria

(b) (4)

(b) (4)

12. Summary and Conclusions

(b) (4)

13. Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	1/14/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F...	1/14/2021
(b)(6) Quality	(b)(6)	1/14/2021

Stability Protocol Titles:	*Clostridium beijerinckii* ASCUSBR67 Pellet 25°C
Organism	*Clostridium beijerinckii* ASCUSBR67
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature Conditions:	
Acceptance Criteria:	
Method:	BR67 Pellet and Mash Microbe Enumeration method

1. **Introduction**

   A stability plan was developed for *Clostridium beijerinckii* ASCUSBR67 pellet in accordance with Native Microbials stability procedure[1], CVM Guidelines[2], WHO Guidelines[3], and ICH Guidelines[4]. Stability studies will be conducted according to pre-approved protocols using (b) (4) (b) (4)

2. **Sampling Plan**

   (b) (4)

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

3. Container Description

(b) (4)

4. Testing Conditions

(b) (4)

5. Testing Frequency

(b) (4)

6. Selection of Batches

(b) (4)

(b) (4)

7. Preparation of Samples

[redacted] (b) (4)

8. Equipment & Monitoring

[redacted] (b) (4)

9. Data & documentation

[redacted] (b) (4)

10. Analysis

[redacted] (b) (4)

11. Acceptance Criteria

[redacted] (b) (4)

[redacted] (b) (4)

12. Summary and Conclusions

(b) (4)

13. Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491...	1/14/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645E	1/14/2021
(b)(6) Quality	(b)(6)	1/14/2021

Clostridium beijerinckii ASCUSBR67 Mash Stability – Summary Report

Approvers:

DocuSigned by:
Martin Mayhew
ACBDDAD433BF491...
Martin Mayhew
Vice President – Product Development
& Manufacturing

3/9/2021
Date

(b)(6)
(b)(6)
Quality

3/11/2021
Date

DocuSigned by:
kevin korth
869AE3638AA543B...
Kevin Korth
Regulatory

3/9/2021
Date

(b)(6)
Scientist

3/9/2021
Date

Prepared By
Native Microbials, Inc
San Diego, CA

March 2021

Clostridium beijerinckii ASCUSBR67 Mash Stability – Summary Report

Organism: *Clostridium beijerinckii* ASCUSBR67

Testing Condition: 25°C ± 2°C

>60% Relative Humidity (RH)

Purpose: To test the stability of the final product in mash under conditions that the mash may be stored at.

Study Numbers: (b) (4)

Acceptance Criteria: Not Less Than 1 X 10^2 CFU/g

Results

Table 1. Results for each lot at each time point. Results are reported in average colony forming units (CFU)/gram.

Time (Days)	Stability Study Number								
		(b) (4)							
	Avg. CFU/g	Standard Deviation	CV	Avg. CFU/g	Standard Deviation	CV	Avg. CFU/g	Standard Deviation	CV
0	(b) (4)								
7									
14									

(b) (4)

Figure 1. Graph of results for each lot at each time point. Results are reported in average colony forming units (CFU)/gram.

(b) (4)

Discussion

(b) (4)

(b) (4)

Deviations

No deviations to protocol or method were noted.

Changes

No significant changes to protocol or assay method occurred during the stability study.

(b) (4)

Location of raw data

(b) (4)

(b) (4)

Appendix 1. Protocol

(b) (4)

native

nativemicrobials.com

Stability Protocol Titles:	*Clostridium beijerinckii* ASCUSBR67 Mash 25°C
Organism	*Clostridium beijerinckii* ASCUSBR67
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature Conditions:	
Acceptance Criteria:	See acceptance criteria section
Method:	BR67 Pellet and Mash Microbe Enumeration method

1. **Introduction**

(b) (4)

2. **Sampling Plan**

(b) (4)

(b) (4)

7. Preparation of Samples

(b) (4)

8. Equipment & Monitoring

(b) (4)

(b) (4)

9. Data & documentation

(b) (4)

10. Analysis

(b) (4)

11. Acceptance Criteria

(b) (4)

(b) (4)

(b) (4)

3. Container Description

(b) (4)

4. Testing Conditions

(b) (4)

5. Testing Frequency

(b) (4)

6. Selection of Batches

(b) (4)

(b) (4)

(b) (4)

12. Summary and Conclusions

(b) (4)

13. Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BE491	1/14/2021
Howard Green Regulatory	DocuSigned by: Howard B Green 432FC873A25645F	1/14/2021
(b)(6) Quality	(b)(6)	1/14/2021

(b) (4)

Clostridium beijerinckii ASCUSBR67 Pellet Stability – Summary Report

Approvers:

DocuSigned by:
Martin Mayhew
ACBDDAD433BF491...
Martin Mayhew
Vice President – Product Development
& Manufacturing

3/9/2021
Date

(b)(6)
Quality

3/11/2021
Date

DocuSigned by:
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Kevin Korth
Regulatory

3/9/2021
Date

(b)(6)
Scientist

3/9/2021
Date

Prepared By
Native Microbials, Inc
San Diego, CA

March 2021

(b) (4)

Clostridium beijerinckii ASCUSBR67 Pellet Stability – Summary Report

Organism:	*Clostridium beijerinckii* ASCUSBR67
Testing Condition:	25°C ± 2°C >60% Relative Humidity (RH)
Purpose:	To test the stability of the final product in Pellet under condition that the Pellet may be stored at.
Study Numbers:	(b) (4)
Acceptance Criteria:	Not Less Than 1 X 10^2 CFU/g

Pelleting Parameters:

(b) (4)

Table 1. Shows pelleting conditions for each lot of pellets.

Lot	Production Rate (lbs/min)	Conditioning Temp (F)	Hot Pellet Temp (F)	Feeder Rate (%)	Conditioner Rate (%)	Motor Load (%)	Mash Temp (F)	Indoor Temp (F)	Indoor RH (%)
(b) (4)									

(b) (4)

Results

Table 2. Results for each lot at each time point. Results are reported in average colony forming units (CFU)/gram.

Time (Days)	Stability Study Number								
		(b) (4)							
	Ave CFU/g	Standard Deviation	CV	Ave CFU/g	Standard Deviation	CV	Ave CFU/g	Standard Deviation	CV
0	(b) (4)								
7									
14									

(b) (4)

(b) (4)

Figure 1. Graph of results for each lot at each time point. Results are reported in average colony forming units (CFU)/gram.

Discussion

(b) (4)

Deviations

No deviations to protocol or method were noted.

Changes

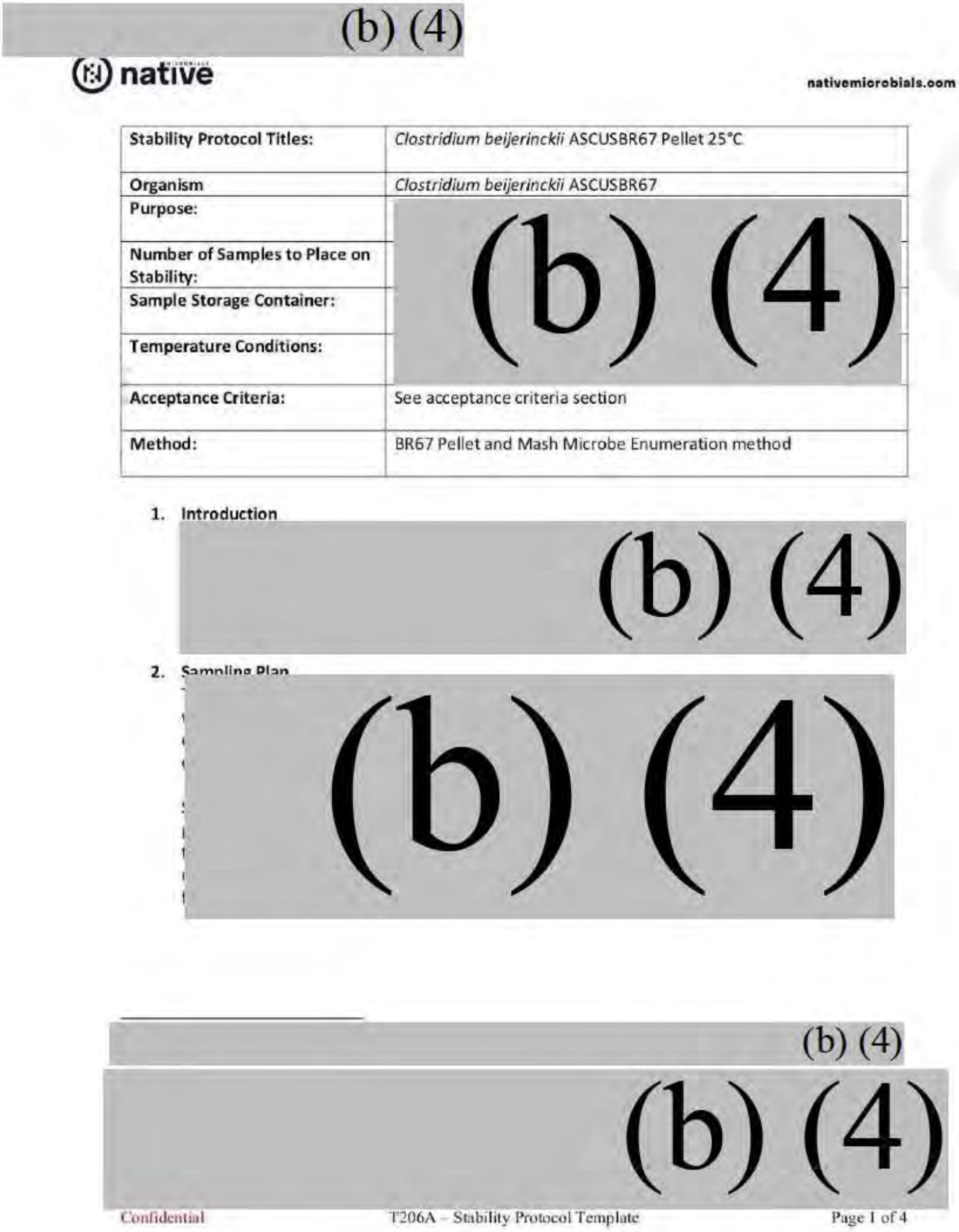
No significant changes to protocol or assay method occurred during the stability study.

Location of raw data

(b) (4)

(b) (4)

Appendix 1. Protocol



(b) (4)

native

nativemicrobials.com

Stability Protocol Titles:	*Clostridium beijerinckii* ASCUSBR67 Pellet 25°C
Organism	*Clostridium beijerinckii* ASCUSBR67
Purpose:	(b) (4)
Number of Samples to Place on Stability:	
Sample Storage Container:	
Temperature Conditions:	
Acceptance Criteria:	See acceptance criteria section
Method:	BR67 Pellet and Mash Microbe Enumeration method

1. **Introduction**

(b) (4)

2. **Sampling Plan**

(b) (4)

(b) (4)

(b) (4)

Confidential T206A – Stability Protocol Template Page 1 of 4

(b) (4)

3. **Container Description**

(b) (4)

4. **Testing Conditions**

(b) (4)

5. **Testing Frequency**

(b) (4)

6. **Selection of Batches**

(b) (4)

(b) (4)

(b) (4)

native

nativemicrobials.com

7. **Preparation of Samples**

(b) (4)

8. **Equipment & Monitoring**

(b) (4)

9. **Data & documentation**

(b) (4)

10. **Analysis**

(b) (4)

11. **Acceptance Criteria**

(b) (4)

12. Summary and Conclusions

(b) (4)

13. Protocol Approvals

Name & Title	Signature & Date	
Martin Mayhew VP – Process Development & Manufacturing	DocuSigned by: Martin Mayhew ACBDDAD433BF491	1/14/2021
Howard Green Regulatory	DocuSigned by: Howard B Green	1/14/2021
(b)(6) Quality	(b)(6)	1/14/2021

Clostridium beijerinckii ASCUSBR67 Appendix 16

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Appendix 017: Literature Search Strategy for *Clostridium beijerinckii* ASCUSBR67

A literature search was conducted by Native Microbials on May 7, 2021 in order to identify potential information related to the safety and utility of *Clostridium beijerinckii* as a direct fed microbial (DFM) strain for poultry. The overall search strategy is described in Table 1. The Google Scholar database was searched using the keyword/search terms listed in Table 2. The search was verified by reviewing the primary hits from a Google Scholar search.

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 published literature to identify all recognized taxonomic classification of the species Strains falling in this species have previously been classified as *Clostridium acetobutylicum* and *Clostridium butylicum*. Moreover, data on the closely related species, *Clostridium butyricum* was also considered relevant. The relevant database was searched using the keyword/search terms listed in Tables 2A, B, C, and D. There were many duplicates identified between the different searches on account of the different nomenclature being widely acknowledged in publications, and of there being a number of pertinent reviews on closely related *Clostridia* species. Considering the relatively large body of available information on closely related *Clostridia* species, the objective of the search was to identify a representative body of information on the species.

We also performed a literature search based on the Clostridial virulence genes identified using the virulence factor database (http://www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Clostridium). A total number of 42 Clostridial virulence genes and terms "hemolysin" and "neurotoxin" in combination with *C. beijerinckii, C. acetobutylicum*, *C. butylicum,* and *C. butyricum* were searched in Google Scholar. The number of results are reported in Table 3. The potential presence of *C. beijerinckii, C. acetobutylicum*, *C. butylicum,* and *C. butyricum* in bird eggs has also been evaluated and are reported in Table 4.

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 (exclude patents and citations)	
Step 2	Exclude duplicates by searching Google Scholar using conditional terms	
Step 3	Screen titles/abstracts and exclude obviously irrelevant records	
Step 4	Review full texts and assess for relevance and eligibility for inclusion	

Table 2A: Topic Specific Search Terms using Species [*Clostridium beijerinckii*]

	Google Scholar Search		Google Scholar Search (exclude duplicates)	
	Input terms in search box	Results	Input terms in search box	Results
Search strategy for safety of species [Safety Search]	"Clostridium beijerinckii"+"tox"	73	"Clostridium beijerinckii"+"tox" OR "toxin" OR "toxins" OR "toxicity" OR "toxicities" OR "pathogen" OR "pathogens" OR "safe" OR "safety" OR "infection" OR "infections" OR "disease" OR "diseases" OR "mortality" OR "mortalities"	5,430
	"Clostridium beijerinckii"+"toxin"	725		
	"Clostridium beijerinckii"+"toxins"	586		
	"Clostridium beijerinckii"+"toxicity"	2,760		
	"Clostridium beijerinckii"+"toxicities"	76		
	"Clostridium beijerinckii"+"pathogen"	1,010		
	"Clostridium beijerinckii"+"pathogens"	1,340		
	"Clostridium beijerinckii"+"safe"	935		
	"Clostridium beijerinckii"+"safety"	1,230		
	"Clostridium beijerinckii"+"infection"	1,320		
	"Clostridium beijerinckii"+"infections"	872		
	"Clostridium beijerinckii"+"disease"	1,470		
	"Clostridium beijerinckii"+"diseases"	1,260		
	"Clostridium beijerinckii"+"mortality"	300		
	"Clostridium beijerinckii"+"mortalities"	12		
Search strategy for safety Clostridium beijerinckii [Target Animal]	"Clostridium beijerinckii"+"broiler"	123	"Clostridium beijerinckii"+"broiler" OR "broilers" OR "chicken" OR "chickens" OR "avian" OR "avians" OR "poultry" OR "turkey" OR "turkeys" OR "bird" OR "birds"	1,260
	"Clostridium beijerinckii"+"broilers"	55		
	"Clostridium beijerinckii"+"chicken"	478		
	"Clostridium beijerinckii"+"chickens"	153		
	"Clostridium beijerinckii"+"avian"	120		
	"Clostridium beijerinckii"+"avians"	2		
	"Clostridium beijerinckii"+"poultry"	474		
	"Clostridium beijerinckii"+"turkey"	319		
	"Clostridium beijerinckii"+"turkeys"	39		
	"Clostridium beijerinckii"+"bird"	199		
	"Clostridium beijerinckii"+"birds"	146		
Search strategy for history of use of Clostridium beijerinckii	"Clostridium beijerinckii"+"food"	5,220	"Clostridium beijerinckii"+"food" OR "foods" OR "feed" OR "feeds"	5,850
	"Clostridium beijerinckii"+"foods"	1,010		
	"Clostridium beijerinckii"+"feed"	2,800		
	"Clostridium beijerinckii"+"feeds"	516		

Search: Must include quotes. Quotes ensure words being included in the search, although the words are not guaranteed to be in the found results. Exclude patents and citations.

Table 2B: Topic Specific Search Terms using Species [*Clostridium acetobutylicum*]

	Google Scholar Search		Google Scholar Search (exclude duplicates)	
	Input terms in search box	Results	Input terms in search box	Results
Search strategy for safety of species [Safety Search]	"Clostridium acetobutylicum"+"tox"	229	"Clostridium acetobutylicum"+"tox" OR "toxin" OR "toxins" OR "toxicity" OR "toxicities" OR "pathogen" OR "pathogens" OR "safe" OR "safety" OR "infection" OR "infections" OR "disease" OR "diseases" OR "mortality" OR "mortalities"	13,600
	"Clostridium acetobutylicum"+"toxin"	2,250		
	"Clostridium acetobutylicum"+"toxins"	1,690		
	"Clostridium acetobutylicum"+"toxicity"	6,210		
	"Clostridium acetobutylicum"+"toxicities"	212		
	"Clostridium acetobutylicum"+"pathogen"	3,100		
	"Clostridium acetobutylicum"+"pathogens"	3540		
	"Clostridium acetobutylicum"+"safe"	2,000		
	"Clostridium acetobutylicum"+"safety"	2,540		
	"Clostridium acetobutylicum"+"infection"	3,970		
	"Clostridium acetobutylicum"+"infections"	2,300		
	"Clostridium acetobutylicum"+"disease"	4,270		
	"Clostridium acetobutylicum"+"diseases"	3,340		
	"Clostridium acetobutylicum"+"mortality"	856		
	"Clostridium acetobutylicum"+"mortalities"	38		
Search strategy for safety Clostridium acetobutylicum [Target Animal]	"Clostridium acetobutylicum"+"broiler"	218	"Clostridium acetobutylicum"+"broiler " OR "broilers" OR "chicken" OR "chickens" OR "avian" OR "avians" OR "poultry" OR "turkey" OR "turkeys" OR "bird" OR "birds"	2,980
	"Clostridium acetobutylicum"+"broilers"	111		
	"Clostridium acetobutylicum"+"chicken"	1,080		
	"Clostridium acetobutylicum"+"chickens"	312		
	"Clostridium acetobutylicum"+"avian"	372		
	"Clostridium acetobutylicum"+"avians"	3		
	"Clostridium acetobutylicum"+"poultry"	927		
	"Clostridium acetobutylicum"+"turkey"	486		
	"Clostridium acetobutylicum"+"turkeys"	50		
	"Clostridium acetobutylicum"+"bird"	510		
	"Clostridium acetobutylicum"+"birds"	376		
Search strategy for history of use of Clostridium acetobutylicum	"Clostridium acetobutylicum"+"food"	11,000	"Clostridium acetobutylicum"+"food" OR "foods" OR "feed" OR "feeds"	13,100
	"Clostridium acetobutylicum"+"foods"	2,150		
	"Clostridium acetobutylicum"+"feed"	6,130		
	"Clostridium acetobutylicum"+"feeds"	1,360		

Search: Must include quotes. Quotes ensure words being included in the search, although the words are not guaranteed to be in the found results. Exclude patents and citations.

Table 2C: Topic Specific Search Terms using Species [*Clostridium butylicum*]

	Google Scholar Search		Google Scholar Search (exclude duplicates)	
	Input terms in search box	Results	Input terms in search box	Results
Search strategy for safety of species [Safety Search]	"Clostridium butylicum"+"tox"	9	"Clostridium butylicum"+"tox" OR "toxin" OR "toxins" OR "toxicity" OR "toxicities" OR "pathogen" OR "pathogens" OR "safe" OR "safety" OR "infection" OR "infections" OR "disease" OR "diseases" OR "mortality" OR "mortalities"	545
	"Clostridium butylicum"+"toxin"	98		
	"Clostridium butylicum"+"toxins"	62		
	"Clostridium butylicum"+"toxicity"	286		
	"Clostridium butylicum"+"toxicities"	4		
	"Clostridium butylicum"+"pathogen"	79		
	"Clostridium butylicum"+"pathogens"	90		
	"Clostridium butylicum"+"safe"	91		
	"Clostridium butylicum"+"safety"	104		
	"Clostridium butylicum"+"infection"	135		
	"Clostridium butylicum"+"infections"	88		
	"Clostridium butylicum"+"disease"	142		
	"Clostridium butylicum"+"diseases"	129		
	"Clostridium butylicum"+"mortality"	35		
	"Clostridium butylicum"+"mortalities"	0		
Search strategy for safety *Clostridium butylicum* [Target Animal]	"Clostridium butylicum"+"broiler"	21	"Clostridium butylicum"+"broiler" OR "broilers" OR "chicken" OR "chickens" OR "avian" OR "avians" OR "poultry" OR "turkey" OR "turkeys" OR "bird" OR "birds"	151
	"Clostridium butylicum"+"broilers"	10		
	"Clostridium butylicum"+"chicken"	72		
	"Clostridium butylicum"+"chickens"	28		
	"Clostridium butylicum"+"avian"	25		
	"Clostridium butylicum"+"avians"	0		
	"Clostridium butylicum"+"poultry"	59		
	"Clostridium butylicum"+"turkey"	47		
	"Clostridium butylicum"+"turkeys"	8		
	"Clostridium butylicum"+"bird"	40		
	"Clostridium butylicum"+"birds"	29		
Search strategy for history of use of *Clostridium butylicum*	"Clostridium butylicum"+"food"	483	"Clostridium butylicum"+"food" OR "foods" OR "feed" OR "feeds"	556
	"Clostridium butylicum"+"foods"	108		
	"Clostridium butylicum"+"feed"	293		
	"Clostridium butylicum"+"feeds"	63		

Search: Must include quotes. Quotes ensure words being included in the search, although the words are not guaranteed to be in the found results. Exclude patents and citations.

Table 2D: Topic Specific Search Terms using Species [*Clostridium butyricum*]

	Google Scholar Search		Google Scholar Search (exclude duplicates)	
	Input terms in search box	Results	Input terms in search box	Results
Search strategy for safety of species [Safety Search]	"Clostridium butyricum"+"tox"	368	"Clostridium butyricum"+"tox" OR "toxin" OR "toxins" OR "toxicity" OR "toxicities" OR "pathogen" OR "pathogens" OR "safe" OR "safety" OR "infection" OR "infections" OR "disease" OR "diseases" OR "mortality" OR "mortalities"	14,800
	"Clostridium butyricum"+"toxin"	3,750		
	"Clostridium butyricum"+"toxins"	3,250		
	"Clostridium butyricum"+"toxicity"	4,620		
	"Clostridium butyricum"+"toxicities"	311		
	"Clostridium butyricum"+"pathogen"	4,530		
	"Clostridium butyricum"+"pathogens"	5,760		
	"Clostridium butyricum"+"safe"	3,420		
	"Clostridium butyricum"+"safety"	5,040		
	"Clostridium butyricum"+"infection"	6,010		
	"Clostridium butyricum"+"infections"	4,290		
	"Clostridium butyricum"+"disease"	8,770		
	"Clostridium butyricum"+"diseases"	7,350		
	"Clostridium butyricum"+"mortality"	3,030		
	"Clostridium butyricum"+"mortalities"	236		
Search strategy for safety Clostridium butyricum [Target Animal]	"Clostridium butyricum"+"broiler"	1,260	"Clostridium butyricum"+"broiler" OR "broilers" OR "chicken" OR "chickens" OR "avian" OR "avians" OR "poultry" OR "turkey" OR "turkeys" OR "bird" OR "birds"	5,140
	"Clostridium butyricum"+"broilers"	1,000		
	"Clostridium butyricum"+"chicken"	2,160		
	"Clostridium butyricum"+"chickens"	1,550		
	"Clostridium butyricum"+"avian"	932		
	"Clostridium butyricum"+"avians"	25		
	"Clostridium butyricum"+"poultry"	2,330		
	"Clostridium butyricum"+"turkey"	1,110		
	"Clostridium butyricum"+"turkeys"	278		
	"Clostridium butyricum"+"bird"	877		
	"Clostridium butyricum"+"birds"	1,130		
Search strategy for history of use of Clostridium butyricum	"Clostridium butyricum"+"food"	13,700	"Clostridium butyricum"+"food" OR "foods" OR "feed" OR "feeds"	15,200
	"Clostridium butyricum"+"foods"	4,390		
	"Clostridium butyricum"+"feed"	6,620		
	"Clostridium butyricum"+"feeds"	1,680		

Search: Must include quotes. Quotes ensure words being included in the search, although the words are not guaranteed to be in the found results. Exclude patents and citations.

Table 3. Specific Search and Evaluation on Clostridial Toxin Genes.

Species	Clostridium virulence factor groups		Input terms in search box	Displayed Counts❖	Retrievable Counts◆	With Combination of the Keywords ₸	Combined (dereplicated by titles)	Results containing pathogen keywords[A]	Results containing pathogen keywords and animal keywords[B]	Final relevant results
Clostridium beijerinckii	Adherence factors		"Clostridium beijerinckii"+"cbpA" OR "CD0873" OR "CD2831" OR "CD3246" OR "cna" OR "cwp66" OR "cwp84" OR "cwpV" OR "fbpA" OR "fbp68" OR "groEL" OR "slpA"	402	399	16	118	57	4	2
	Exoenzyme factors		"Clostridium beijerinckii"+"zmp1"	2	2	0				
	Regulation factors		"Clostridium beijerinckii"+"virS" OR "virR"	40	30	0				
	Toxin factors (2 searches due to terms were too long)	search 1	"Clostridium beijerinckii"+"cloSI" OR "plc" OR "cpb2" OR "atx" OR "tcnA" OR "cpe" OR "cdtA" OR "cdtB" OR "entA" OR "entC" OR "entD" OR "entB" OR "Hemolysin"	321	310	63				
		search 2	"Clostridium beijerinckii"+"colA" OR "nagH" OR "nagI" OR "nagJ" OR "nagK" OR "nagL" OR "pfoA" OR "nanH" OR "nanI" OR "nanJ" OR "tetX" OR "toxA" OR "toxB"	156	120	3				
	neurotoxin		"Clostridium beijerinckii"+"neurotoxin" OR "neurotoxins"	175	163	49				
Clostridium acetobutylicum	Adherence factors (2 searches due to too many outputs)	search 1	"Clostridium acetobutylicum"+"cbpA" OR "CD0873" OR "CD2831" OR "CD3246" OR "cna" OR "cwp66" OR "cwp84" OR "cwpV"	520	514	28	285	124	13	2
		search 2	"Clostridium acetobutylicum"+"fbpA" OR "fbp68" OR "groEL" OR "slpA"	934	910	28				
	Exoenzyme factors		"Clostridium acetobutylicum"+"zmp1"	7	7	0				
	Regulation factors		"Clostridium acetobutylicum"+"virS" OR "virR"	126	116	0				
	Toxin factors (2 searches due to terms were too long)	search 1	"Clostridium acetobutylicum"+"cloSI" OR "plc" OR "cpb2" OR "atx" OR "tcnA" OR "cpe" OR "cdtA" OR "cdtB" OR "entA" OR "entC" OR "entD" OR "entB"	725	719	174				
		search 2	"Clostridium acetobutylicum"+"Hemolysin" OR "colA" OR "nagH" OR "nagI" OR "nagJ" OR "nagK" OR "nagL" OR "pfoA" OR "nanH" OR "nanI" OR "nanJ" OR "tetX" OR "toxA" OR "toxB"	740	690	6				
	neurotoxin		"Clostridium acetobutylicum"+"neurotoxin" OR "neurotoxins"	408	380	105				
Clostridium butylicum	Adherence factors		"Clostridium butylicum"+"cbpA" OR "CD0873" OR "CD2831" OR "CD3246" OR "cna" OR "cwp66" OR "cwp84" OR "cwpV" OR "fbpA" OR "fbp68" OR "groEL" OR "slpA"	25	25	1	14	4	0	0
	Exoenzyme factors		"Clostridium butylicum"+"zmp1"	0	0	0				
	Regulation factors		"Clostridium butylicum"+"virS" OR "virR"	1	1	0				
	Toxin factors (2 searches due to terms were too long)	search 1	"Clostridium butylicum"+"cloSI" OR "plc" OR "cpb2" OR "atx" OR "tcnA" OR "cpe" OR "cdtA" OR "cdtB" OR "entA" OR "entC" OR "entD" OR "entB" OR "Hemolysin"	35	35	9				
		search 2	"Clostridium butylicum"+"colA" OR "nagH" OR "nagI" OR "nagJ" OR "nagK" OR "nagL" OR "pfoA" OR "nanH" OR "nanI" OR "nanJ" OR "tetX" OR "toxA" OR "toxB"	17	18	0				
	neurotoxin		"Clostridium butylicum"+"neurotoxin" OR "neurotoxins"	25	14	4				
Clostridium butyricum	Adherence factors		"Clostridium butyricum"+"cbpA" OR "CD0873" OR "CD2831" OR "CD3246" OR "cna" OR "cwp66" OR "cwp84" OR "cwpV" OR "fbpA" OR "fbp68" OR "groEL" OR "slpA"	335	330	27	292	157	88	35
	Exoenzyme factors		"Clostridium butyricum"+"zmp1"	2	0	0				
	Regulation factors		"Clostridium butyricum"+"virS" OR "virR"	71	61					
	Toxin factors (2 searches due to terms were too long)	search 1	"Clostridium butyricum"+"cloSI" OR "plc" OR "cpb2" OR "atx" OR "tcnA" OR "cpe" OR "cdtA" OR "cdtB" OR "entA" OR "entC" OR "entD" OR "entB" OR "Hemolysin"	836	810	189				
		search 2	"Clostridium butyricum"+"colA" OR "nagH" OR "nagI" OR "nagJ" OR "nagK" OR "nagL" OR "pfoA" OR "nanH" OR "nanI" OR "nanJ" OR "tetX" OR "toxA" OR "toxB"	435	390	3				
	neurotoxin*		"Clostridium butyricum"+"neurotoxin" OR "neurotoxins"+"broiler" OR "broilers" OR "chicken" OR "chickens" OR "avian" OR "avians" OR "poultry" OR "turkey" OR "turkeys" OR "bird" OR "birds"	608	638	101				

* Search "Clostridium butyricum"+"neurotoxin" alone produced 1340 results. Google Scholar does not allow the download beyond 1000 results. Therefore the search terms were altered for poultry and bird specific.

❖Number of results displayed on the first page under the search field. It is a number of estimation.

◆The retrievable counts are the number of results that were actually obtained in pdf or electronic form. Only results in English (English title at the very minimum) are retrieved.

₸ Must contain the second half of the species names (eg., beijerinckii) and at least one of the conditional terms connected with "OR".

A. pathogen keywords are any words containing 'pathogen', 'tox', 'infect', 'diseas', 'bacteremia'.

B. animal keywords are any words containing 'homo', 'patient', 'man', 'woman', 'men', 'women', 'male', 'female', 'child', 'infant' , 'animal', 'mammal', 'broiler', 'chicken', 'turkey', 'poultry', 'bird', and 'avian'.

Table 4. Specific search and evaluation on *C. beijerinckii* and relatives in eggs.

Species	Input terms in search box	Displayed Counts❖	Retrievable Counts◆	With Combination of the Keywords Ŧ	Combined (dereplicated by titles)	Results containing pathogen keywords [A]	Results containing pathogen keywords and animal keywords [B]	Final relevant results
Clostridium beijerinckii	"Clostridium beijerinckii"+"egg"	394	377	99	115	6	3	0
	"Clostridium beijerinckii"+"eggs"	178	163	34				
Clostridium acetobutylicum	"Clostridium acetobutylicum"+"egg"	962	935	281	356	28	5	1
	"Clostridium acetobutylicum"+"eggs"	404	410	130				
Clostridium butylicum	"Clostridium butylicum"+"egg"	81	69	34	44	1	0	0
	"Clostridium butylicum"+"eggs"	42	32	18				
*Clostridium butyricum**	"Clostridium butyricum"+"egg"+"chicken"	871	848	261	395	80	76	9
	"Clostridium butyricum"+"egg"+"poultry"	801	778	240				
	Clostridium butyricum+"eggs"+"chicken" OR "poultry"	656	630	154				

* Search "Clostridium butyricum"+"egg" alone produced 2050 results. Google Scholar does not allow the download beyond 1000 results. Therefore the search terms were altered for poultry and chicken specific.

❖Number of results displayed on the first page under the search field. It is a number of estimation.

◆The retrievable counts are the number of results that were actually obtained in pdf or electronic form. Only results in English (English title at the very minimum) are retrieved.

Ŧ Must contain the second half of the species names (eg., beijerinckii), the term connected with "+", and at least one of the conditional terms connected with "OR".

A. pathogen keywords are any words containing 'pathogen', 'tox', 'infect', 'diseas', 'bacteremia'.

B. animal keywords are any words containing 'homo', 'patient', 'man', 'woman', 'men', 'women', 'male', 'female', 'child', 'infant' , 'animal', 'mammal', 'broiler', 'chicken', 'turkey', 'poultry', 'bird', and 'avian'.

Microbiome Safety for *Clostridium beijerinckii* ASCUSBR67

Objectives

The objective of this review is to:

a) Demonstrate that the typical microbial composition and diversity of the gastrointestinal microbial community of poultry 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 *Clostridium beijerinckii* ASCUSBR67 does not increase its own abundance nor the overall composition of the microbiome beyond typically observed ranges.

Robust Nature of the Chicken Gastrointestinal Microbiome

Native Microbials Animal Experiments: A series of experiments were conducted in order to obtain a representative sampling of the gastrointestinal (GI) microbiome composition of poultry. These samples were used to determine the typical ranges of abundances of poultry GI microorganisms under normal, farm-like conditions.

Microbiome Survey : Two survey experiments were conducted to identify the ileal microbial composition of birds at 21 days of age. In each survey, 120 newly hatched chicks were left to grow in floor pens for 14 days. Following day 14, 96 of these birds were placed in individual cages and were monitored for weight gain and feed efficiency until day 21, upon which they were sacrificed for sampling.

> **Findings**: The results of the survey experiments are summarized in Table 1, showing the average chicken GI 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 *C. beijerinckii*, specifically, in the GI tract based on Native Microbials survey was found to be 0.0183% on average (ranged from 0.044% to 0.1105%) of the GI bacterial population.

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

	Average Abundance (%)	Abundance Range (%)
Firmicutes	94.12	24.56 - 99.96
Actinobacteria	3.1	0 - 56.59
Cyanobacteria/Chloroplast	1.76	0.0131 - 16.88
Proteobacteria	0.9106	0 - 13.93
Verrucomicrobia	0.0321	0 - 0.1857
Synergistetes	0.0227	0 - 0.6985
Tenericutes	0.0172	0 - 0.4139
Aquificae	0.0145	0 - 0.2048
Deinococcus-Thermus	0.0101	0 - 0.3233
Bacteroidetes	0.0027	0 - 0.1432
Candidatus_Saccharibacteria	0.0004	0 - 0.0212
Acidobacteria	0.0001	0 - 0.0067

Product Studies:

Study 1: In this study, two native poultry GI microorganisms (*C. beijerinckii* ASCUSBR67 and *C. beijerinkii* ASCUSBR67) were administered in the daily feed of 12 pens containing 26 birds in each, in order to determine the effect of microorganism supplementation on bird performance and health. The trial was conducted at a facility located in (b)(6). Birds were fed a corn/soy-based mash feed representative of commercial diets. An additional 12 pens were provided a calcium carbonate base powder as a control. Birds were followed for 42 days from hatching to monitor performance, with ileal content sampling occuring on days 17, 21, 28 and 42.

> **Findings:** In this administration experiment, it can be seen that addition of *C. beijerinckii* ASCUSBR67 and *C. beijerinckii* ASCUSBR67 did not significantly alter the chicken GI bacteria microbiome composition when compared to the control group. Abundances of all bacterial phyla are within standard ranges observed in animals not fed native GI microbes. The average abundance of each phylum tended to be similar across experimental groups.

Table 2. Abundance of Chicken GI Bacterial Phyla from Native Microbials Product Study 1, Reported as a Percent

Phylum	Control Group	Experimental Group: Two Microbes
Firmicutes	91.74	93.91
Proteobacteria	6.23	3.78
Actinobacteria	1.61	2.03
Cyanobacteria/Chloroplast	0.3285	0.2321
Candidatus_Saccharibacteria	0.0322	0.0117
Synergistetes	0.0243	0.0161
Bacteroidetes	0.0191	0.0065
Tenericutes	0.004	0.0011
Verrucomicrobia	0.0038	0.0027
Chrysiogenetes	0.0023	0.0045
Deinococcus-Thermus	0.0018	0.0013
Acidobacteria	0.0006	0.0005
Fusobacteria	0.0004	0.0012
Armatimonadetes	0.0003	0.0001

Study 2: In this study, two native poultry GI microorganisms were administered in the daily feed of 24 pens containing 35 birds each in order to determine the effect of microorganism supplementation on bird performance and health at different doses. The trial was conducted at a facility located in (b)(6) Birds were fed a corn/soy-based pelleted feed representative of commercial diets. An additional 12 pens were provided non-medicated feed, serving as a control. Birds were followed for 42 days from hatching to monitor performance, with ileal content sampling occuring on days 14, 19, 23, 29 and 42.

Findings: In this administration experiment, it can be seen that addition of *C. beijerinckii* ASCUSBR67 and *C. beijerinckii* ASCUSBR67 to pelleted feed did not significantly alter the chicken GI 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 GI microbes. The average abundance of each phylum did not differ significantly across experimental groups.

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

Phylum	Control Group	Experimental Group: Two Microbes
Firmicutes	97.66	97.37
Proteobacteria	1.13	1.91
Actinobacteria	0.8996	0.4189
Bacteroidetes	0.2205	0.0853
Cyanobacteria/Chloroplast	0.0467	0.1367
Synergistetes	0.0215	0.0519
Candidatus_Saccharibacteria	0.0146	0.0146
Tenericutes	0.0055	0.0073
Chrysiogenetes	0.0035	0.0051
Acidobacteria	0.0005	0.0001
Chloroflexi	0.0002	0
Armatimonadetes	0.0002	0.0006
Verrucomicrobia	0.0001	0

Study 3: In this study, two native poultry GI microorganisms were administered in the daily feed of 12 pens containing 28 birds each in order to determine the effect of microorganism supplementation on bird performance and health. The trial was conducted at a facility located in [redacted]. Birds were fed a corn/soy-based mash feed representative of commercial diets. An additional 12 pens were provided non-medicated feed, serving as a control. Birds were followed for 42 days from hatching to monitor performance, with ileal content sampling occuring on days 16, 21, 28 and 42.

Findings: In this administration experiment, it can be seen that addition of *C. beijerinckii* ASCUSBR67 and *C. beijerinckii* ASCUSBR67 to mash feed did not significantly alter the chicken GI 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 microbes. The average abundance of each phylum tended to be similar across experimental groups.

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

Phylum	Control	Two Microbes
Firmicutes	94.89	95.91
Proteobacteria	4.48	3.15
Actinobacteria	0.3936	0.7199
Bacteroidetes	0.1807	0.1861
Fusobacteria	0.0456	0.0187
Cyanobacteria/Chloroplast	0.0104	0.0094
SR1	0.002	0.0021
Synergistetes	0.0012	0.0016
Candidatus_Saccharibacteria	0.001	0.0015
Tenericutes	0.0006	0.0015
Spirochaetes	0.0004	0.0009
Verrucomicrobia	0.0002	0.0002

Animal Experiments from Peer-Reviewed Literature: Peer reviewed manuscripts describing the bacterial GI tract community using high-throughput, comprehensive bacterial community analyses were collected for further comparative analysis to establish the microbial composition of the “typical” poultry GI tract. Several bacterial analyses conducted by academic institutions were found for chickens: Paul et al. 2021; Fidler et al. 2020; Oakley and Kogut 2016; Awad et al. 2016; Wang et al. 2018; and van der Hoeven-Hangoor et al. 2013.

These manuscripts were selected based on the marker selected for microbiome analysis (e.g. to maintain compatibility and consistency to internal analyses) and the breadth of growth conditions and sampling regions represented in the analyses:

a) Paul et al. 2021 studied the core-microbiome of chickens by comparing the microbiome differences among breeds. The study selected the commercial broiler chicken breed and three indegenous Indian breeds based on their geographic locations. Eight healthy male birds from each breed were harvested during the finishing stage and luminal contents from duodenum to cloaca including caeca were collected for microbiome analysis. The luminal microbiomes across all 4 breeds are reported below.

b) Fidler et al. 2020 evaluated the effect of different DNA extraction methods on the observed broiler chicken fecal microbiomes. Chicken fecal samples were collected from 4 healthy female and 4 healthy male birds each per poultry facility and 8 randomly selected poultry facilities.

Different sample homogenization, cell lysing, and DNA purification methods were applied. Targeted gene (16S rRNA gene) sequencing was used to evaluate the chicken fecal microbiome variations due to sample processes. The fecal microbiome profile is reported below.

c) Oakley and Kogut 2016 examined the spatial and temporal variations of broiler chicken gastrointestinal (GI) microbiomes. The authors collected fecal samples and cecal contents from 20 healthy broiler chickens aged 1, 3, and 6 weeks old. Microbiomes from different GI locations are reported below

d) Awad et al. 2016 also examined the spatial and temporal variations of broiler chicken GI microbiomes. Jejunum and cecum contents were collected from 5 healthy birds at age 7, 14, 21, and 28 days old. The microbiome from each GI location is reported below.

e) Wang et al. 2018 studied the impact of temperature on broiler chicken GI microbiomes. The birds (96 total) were split into two groups at 21-day of age, the control group at ambient temperature and the group exposed to 31°C (6 cages and 8 birds each per group). Ileal contents were collected at day 42 from 6 randomly selected healthy birds. The ileal content microbiome profile is reported below.

f) van der Hoeven-Hangoor et al. 2013 evaluated the broiler ileal content microbiome from birds fed with various commercial diets. A total 5 different diets were used in the study. The study involved a total of 540 male Ross birds (30 cages with 18 birds per cage). At 34-day of age, three healthy birds per cage were randomly selected and the ileal contents were collected. Although their microbiome analyses were not robust enough to include in the analysis here, their results are consistent with others' and Native Microbicrobials' findings.

Findings:

i) The chicken GI 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, sampling location, and season. Additionally, there are groups of microorganisms that are unique to particular breeds of poultry, regions, ages, and individual animals that further increase the inherent complexity of the microbial community native to the GI tract. Despite this variability, there is a core microbiome that appears in the majority of poultry. 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 birds (see Table 5).

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

	Paul et al. 2021	Fidler et al. 2020	Oakley and Kogut 2016		Awad et al. 2016		Wang et al. 2018
Bacterial phyla	**Luminal (duodenum to cloaca including caeca) contents**	**Fecal materials**	**Cecal contents**	**Fecal materials**	**Jejunum contents**	**Cecum contents**	**Ileal contents**
Bacteroidetes	6-62	5-45	0-50	0-34	0-0.02	0-0.94	0-30
Firmicutes	24-81	30-95	35-99	22-99	63.5-94.58	44-93.14	43-98
Proteobacteria	4-8	0-42	0-22	1-88	5.2-32.68	4.42-55.34	0-27
Actinobacteria	0-1	0-5	0-10	0-5	0.05-1.46	0.01-0.03	< 1
Cyanobacteria	0.8-6	0-10		0-5	0-0.02	< 0.05	0-4
Verrucomicrobia	0-2	0-5	0-3	<1	< 0.05	< 0.05	< 1
Fusobacteria	0-5		< 1				< 1
Tenericutes		0-5	< 1	0-10	0.1-3.29	0-22.61	< 1

ii) The poultry GI tract microbiome is very plastic and highly responsive to external variables. Because of this, defining a “normal healthy” microbiome is challenging. High-throughput bacterial community analyses were found for different poultry GI tract regions collected at different ages under the influence of different diets and growth conditions Paul et al. 2021; Fidler et al. 2020; Oakley and Kogut 2016; Awad et al. 2016; Wang et al. 2018; van der Hoeven-Hangoor et al. 2013. These manuscripts were further investigated to determine prevalence of the overall bacterial taxonomic composition of the typical GI microbiome. These studies showed that although the GI tract region influences the observed chicken GI microbiomes, there is an overall consistent healthy GI microbiome regardless of breed, diet, and age.

iii) Cumulatively, these independent studies investigated the poultry GI microbial community across a variety of breeds, diets, age, feed management regimes, and sampling regions. Table 5 (above) summarizes the findings from Paul et al. 2021; Fidler et al. 2020; Oakley and Kogut 2016; Awad et al. 2016; Wang et al. 2018 at the phylum level. Overall, Firmicutes tended to consistently dominate the chicken GI bacterial community. Proteobacteria and Bacteroidetes were also largely abundant but with a greater variation among samples. As can be seen from this data, there is a broad range of abundances. *C. beijerinckii* ASCUSBR67 falls into the Firmicutes phylum, which was found to comprise 22% - 99% of the GI microbial community.

iv) Despite the high variability in abundance, there does seem to be a typical range for the most predominant phyla. Overall, the observed abundance of Firmicutes within this group of healthy chicken ranged from 22% - 99%, while the observed abundance of Proteobacteria ranged from 0% - 88%. Bacteroidetes ranged from 0% - 62%. Other phyla did appear, but often represented less than 10% of the total bacterial population. These ranges were utilized to describe the “average” chicken GI microbiome in comparative analyses.

Conclusion

This summary covers the Native Microbial studies as well as published data to assess the potential risk of microbiome composition shifts in the poultry GI that may raise safety concerns. Information presented demonstrates that the normal microbial community in the chicken GI tract is robust and not adversely affected by the addition of native microorganisms, including *C. beijerinckii*. Hence, it is clear that the dietary addition of *C. beijerinckii* will not cause a safety concern based on changes in the microbiome**.**

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

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Attachments:

Sample Collection

On sampling days, chickens were sacrificed and ileal contents were collected. Samples were added to a 15-mL conical containing 3 mL stop solution consisting of 95% molecular grade 200 proof ethyl alcohol (b) (4)) and 5% TRI-Reagent (b) (4)) 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

Chicken GI contents 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)). 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) DNA Polymerase (b) (4)). Following amplification, PCR products were verified with a standard 2% agarose gel electrophoresis and purified using AMPure XP bead ((b) (4) The purified amplicon library was quantified and sequenced on the (b) (4) Platform (b) (4)) according to standard protocols using a 2x300 v3, 600-cycle kit. Raw fastq reads were de-multiplexed on the (b) (4) Platform (b) (4)). 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 USEARCH's UNOISE and SINTAX (v10.0.240) (Edgar 2016; Edgar and Flyvbjerg 2015; Edgar, n.d.) 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.

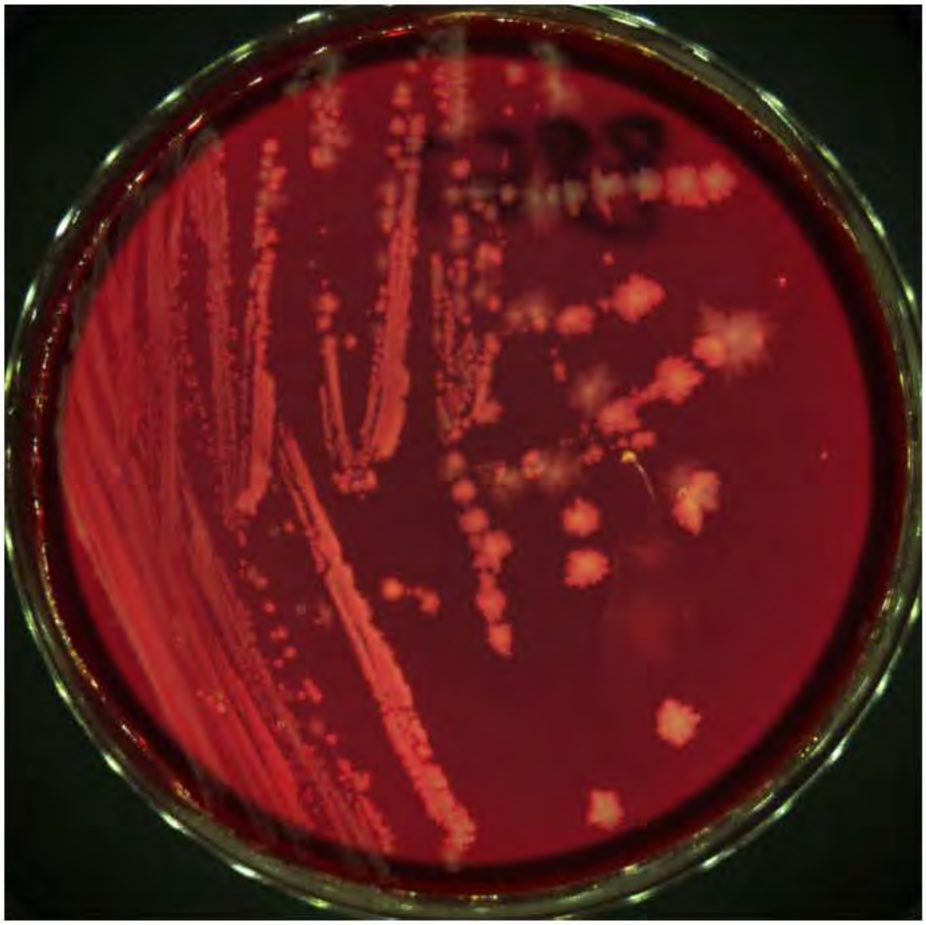
Appendix 019: Hemolysis Testing for *Clostridium beijerinckii* ASCUSBR67

(b)(6) 17May21

1. Purpose
   1.1. To determine BR 67's ability to induce hemolysis.
2. Methods
   2.1. Media Preparation
      2.1.1. Brucella Agar with (b) (4) Sheep Blood
      2.1.1.1. Components
         2.1.1.1.1. (b) (4)
         2.1.1.1.2. (b) (4).
   2.2. Inoculation
      2.2.1. Obtain a pure culture of BR 67 and cycle into an anaerobic chamber.
      2.2.2. Using a sterile loop, streak the culture onto Brucella Agar with (b) (4) Sheep Blood.
      2.2.3. Incubate for a minimum of (b) (4) or until colonies are of a size.
   2.3. Interpretation
      2.3.1. Examine the agar immediately around and below the BR 67 colonies for the following features:
      2.3.1.1. **Alpha Hemolysis** (α-hemolysis) – a dark or greenish discoloration of the media. Synonymous with incomplete or partial hemolysis. Caused by bacterium produced H_2O_2 oxidizing hemoglobin producing methemoglobin.
      2.3.1.2. **Beta Hemolysis** (β-hemolysis) – a lightened or translucent area on the media. Caused by the production of streptolysin, an enzyme produced by bacteria causing the complete lysis of red blood cells in the media.
      2.3.1.3. **Gamma Hemolysis** (γ-hemolysis) – the agar under and around the colony is unchanged. The organism does not induce hemolysis.

3. Results

3.1. BR 67 possesses gamma hemolysis. BR 67 lacks the ability to lyse red blood cells.



Signed: (b)(6)______________ Date: 17May21

(b) (4) RESEARCH FINAL REPORT

Effect of Novel Microbial Products Administered in Feed on the Immune Response, Performance, and Intestinal Morphology of Broilers with Necrotic Enteritis

(b) (4)

SPONSOR

ASCUS BIOSCIENCES, INC.
6450 Lusk Blvd., Suite E109
San Diego, CA 92121

TEST FACILITY

(b) (4)

June 2019

(b) (4) FINAL REPORT
(b) (4)

I. TITLE

Effect of Novel Microbial Products Administered in Feed on the Immune Response, Performance, and Intestinal Morphology of Broilers with Necrotic Enteritis

SPONSORS:

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I. OBJECTIVE

The objective of this study was to determine the effect of Ascus microbes on zootechnical performance, intestinal immune response, intestinal morphometrics, and necrotic enteritis lesion scores in broiler chickens orally challenged with *Clostridium perfringens*.

PROPOSED DATES:

Study Activity	Study Day	Calendar Date
Placed chicks, weighed birds by pen, issued starter feed.	D0	19MAR19 (Tuesday)
Placed 25 g (Days 0-7), or 250 g (Days 7-42) of premixed feed into each pen	D0-42	
Weighed and Sampled 1 bird per pen: jejunal collection; ileum content and swab; spleen/liver weights and samples.	D16	04APR19 (Thursday)
Weighed birds by pen, weighed back starter feed, and issued grower feed. Challenged groups 2,5,6, and 8 with *C. perfringens*	D17	05APR19 (Friday)
Weighed and Sampled 1 bird per pen: jejunal collection; ileum content and swab; blood; spleen/liver weights and samples. Lesion Scored 5 birds per pen (including 1 bird used for tissue samples; total of 5 birds/pen).	D21	09APR19 (Tuesday)
Weighed birds by pen, and weighed back grower feed Weighed and Sampled 1 bird/pen: jejunal collection; ileum content and swab; spleen/liver weights and samples. Lesion Scored 5 birds per pen (total of 6 birds/pen).	D28	16APR19 (Tuesday)
Weighed birds by pen, weighed back grower feed, and issued finisher feed	D35	23APR19 (Tuesday)
Weighed birds by pen, and weighed back finisher feed. Weighed and Sampled 1 bird per pen: jejunal collection; ileum content and swab; spleen/liver weights and samples. ENDED STUDY	D42	30APR19 (Tuesday)

II. MATERIALS AND METHODS

A. TESTING/SUPPORT FACILITIES

Facility	***Purpose***
(b) (4)	Investigator's office, diet formulation, test article storage, feed preparation, test animal housing, archives
Ascus Biosciences, Inc. 6450 Lusk Blvd., Suite E109 San Diego, CA 92121	Sponsor, test article source, feed sample analysis
(b) (4)	Proximate analysis
	Challenge preparation, enumeration
	Histology analysis
	Chick source

B. TEST & CONTROL ARTICLES

TEST ARTICLE

MICROBE COMPOSITION 1 (BR11)	Lot No/Exp: Not included in final report
Source	Ascus Biosciences, Inc.
Concentration	Included in study records
Inclusion Level	According to Experimental Design.
Method of Administration	Oral via complete feed, According to Experimental Design.
Duration	Day 0 through 42, per Experimental Design
Frequency of Administration	Daily in feed according to Experimental Design
Classification	Bacterial Microbe in Feed
Storage	Secured, temperature-monitored, dry area at ~ 4°C
Accounting	All quantities of test articles received and use in this study were documented and included in the study records.

MICROBE COMPOSITION 2 (BR21/67)	Lot No/Exp: Not included in final report
Source	Ascus Biosciences, Inc.
Concentration	Included in study records
Inclusion Level	According to Experimental Design.
Method of Administration	Oral via complete feed, According to Experimental Design.
Duration	Day 0 through 42, per Experimental Design
Frequency of Administration	Daily in feed according to Experimental Design
Classification	Bacterial Microbe in Feed
Storage	Secured, temperature-monitored, dry area at ~ 4°C
Accounting	All quantities of test articles received and use in this study were documented and included in the study records.

[redacted]

Calcium Carbonate	Lot No/Exp: Not included in final report
Source	Ascus Biosciences, Inc.
Concentration	NA
Inclusion Level	According to Experimental Design.
Method of Administration	Oral via complete feed, According to Experimental Design.
Duration	Day 0 through 42, per Experimental Design
Frequency of Administration	Daily in feed according to Experimental Design
Storage	Secured, temperature-monitored, dry area
Accounting	All quantities of test articles received and use in this study were documented and included in the study records.

Bacitracin Mehtylene Disalicylate (BMD)	Lot No/Exp: (b) (4)01DEC2019
Source	(b) (4)
Concentration	50g/ton
Inclusion Level	According to Experimental Design.
Method of Administration	Oral via complete feed, According to Experimental Design.
Duration	Day 0 through 42, per Experimental Design
Frequency of Administration	Daily in feed according to Experimental Design
Classification	In-Feed Antibiotic
Storage	Secured, temperature-monitored, dry area
Accounting	All quantities of test articles received and use in this study were documented and included in the study records.

Phytase 2500	Lot No: (b) (4) REC: 21DEC2018
Source	(b) (4)
Concentration	N/A
Inclusion Level	0.020%
Method of Administration	Oral via complete feed, According to Experimental Design.
Duration	Day 0 through 42, per Experimental Design
Frequency of Administration	Daily in feed
Storage	Secured, temperature-monitored, dry area
Accounting	All quantities of test articles received and use in this study were documented and included in the study records.

C. BASAL AND EXPERIMENTAL DIETS

The starter, grower, and finisher diets were a standard(b) (4)formulated corn/soy-based broiler diets that are representative of commercial broiler diets. The basal diets were manufactured at (b) (4) stored in bulk, mash form.

Final experimental diet mixing, was conducted at(b) (4)using a 25-lb (b) (4)mixer, 500-lb capacity vertical mixer, a 4000-lb capacity vertical mixer, or a 14,000-lb capacity horizontal mixer. Test article was added to the basal diets weekly, according to the Experimental Design. Feed was stored in 50-lb capacity feed sacks and/or bulk storage bins labeled with treatment code. Feed storage duration and feed disposition was documented and included in the final study records. Diet formulations was provided prior to the start study. When mixing treatments containing BMD (50g/ton) (TRT 7 and 8) a predetermined amount of basal feed was combined with BMD. The basal amount was added to the BMD container. The container was sealed and shaken or rotated as need to ensure homogenous mixture of BMD into basal feed. Once the BMD premix was made, it was slowly added back to the original amount of basal weighed out for that particular treatment, and placed into the mixer to assure a homogenous mix

When mixing treatments 1 through 6 with test article, a secondary premix was made to ensure an adequate mixture of all test article. An appropriate amount of basal feed was taken from the amount of basal weighed out for that particular treatment and was used to make the premix. The basal amount was added to the test article container with test article. The container was sealed and shaken or rotated as need to ensure homogenous mixture of test article into basal feed. Once test article premix was made, it was slowly added back to the original amount of basal weighed out for that particular treatment, and placed into the mixer for an appropriate amount of time to ensure homogenous mixture of test article premix with treatment basal feed. To avoid cross contamination between treatments, disinfecting was as follows in Appendix 2.

Once a week, a predetermined amount of mash feed was mixed with a predetermined amount of Ascus Microbe composition or calcium carbonate, as depicted in the approximate feed mixing schedule below. The treatment feed containing microbe compositions (TRT 3-6) was stored at 4°C.

Table 1. Approximate Feeding Schedule

Diet	Form	Period	~Lbs Feed Mixed per Treatment
Starter	Mash	Days 0 – 17	660
Grower	Mash	Days 17 – 35	1,610
Finisher	Mash	Days 35 – 42	730

Table 2. Approximate Feed Mixing Schedule (Premix)

Diet	Form	Period (Days)	~Total Feed mixed per Treatment (kg)	Product Added (g)	Trt Feed Location	~ Amount of Treatment feed added Pen/Day (g)
Starter	Mash	Days 0 – 6	2.6	111	Feeder Plate	25
Starter	Mash	Days 7 – 13	24.2	517	Feeder	250
Starter	Mash	Days 14 – 16	12.2	261	Feeder	250
Grower	Mash	Days 17- 20	15.2	325	Feeder	250
Grower	Mash	Days 21 – 27	24.2	517	Feeder	250
Grower	Mash	Days 28 – 34	24.2	517	Feeder	250
Finisher	Mash	Days 35 – 42	24.2	517	Feeder	250

D. SAMPLES AND ASSAYS

The basal feed and treatment diets was sampled in duplicate (~100 g of sample for Starter feed D0-6 and ~300 g sample size for all remaining diets). One sample of the basal and each treatment diet was submitted to the Sponsor for test article and control assay and one sample was retained by (b) (4) until study end. The basal retention samples were submitted to (b) (4) for proximate analysis, and the analysis was included within the original study records. All samples were labeled with the (b) (4) project number, treatment number, sample description and date of collection.

E. TEST SYSTEM

Species	Broiler Chicken
Strain	Commercial production
Breed/Cross	Cobb 500
Supplier	(b) (4)
Sex	Males
Age	~1 day of age upon receipt (Day 0) ~ 42 days at final weights
Identification	Pen cards
Number of birds/pen:	26 (D0); 25 (D16); 20 (D21); 14 (D28)
Number of treatments:	8
Number of pens/treatment:	12
Number of birds/treatment:	312 (D0); 300 (D16); 240 (D21); 168 (D28)
Total number of pens:	96
Total number of birds:	2,496 (D0); 2,400 (D16); 1,920 (D21); 1,344 (D28)

III. EXPERIMENTAL DESIGN

A. TEST GROUPS

The test facility was divided into 12 blocks of 8 pens. Treatments were assigned to the pens using a complete randomized block design. Birds were assigned to the pens randomly according to (b) (4)(Appendix 3). Specific treatment groups were as follows:

Trt	*Clostridium perfringens* Challenged	Day of Treatment Administration	Calcium Carbonate	Microbe 1 (BR11)	Microbe 2 (BR21/67)	BMD	No. of Birds/ Pen	No. of Pens	Number of Birds/ Treatment
1	No	0-42	See Mixing Schedule*				26	12	312
2	Yes	0-42	See Mixing Schedule*				26	12	312
3	No	0-42		See Mixing Schedule*			26	12	312
4	No	0-42			See Mixing Schedule*		26	12	312
5	Yes	0-42		See Mixing Schedule*			26	12	312
6	Yes	0-42			See Mixing Schedule*		26	12	312
7	No	0-42				See Mixing Schedule*	26	12	312
8	Yes	0-42				See Mixing Schedule*	26	12	312

*All treatments had daily administration of treatment feed (Microbes, calcium carbonate, or BMD mixed into feed, weekly) on feeder trays (Day 0-6), or on top of feed in feeder pans (Days 7-42). See Section C. Basal Feed and Experimental Diets, Table 2 for more details.

B. HOUSING AND MANAGEMENT

Housing

Assignment of treatments to pens was conducted using a computer program. The computer-generated assignment was as follows.

Block↓	Trt 1	Trt 2	Trt 3	Trt 4	Trt 5	Trt 6	Trt 7	Trt 8
B1	2	6	7, 8		3, 4		9	5
B2	100	101		98, 104		103, 105	102	99
B3	19	16	15, 18		13, 17		14	12
B4	93	92		91, 97		90, 95	94	96
B5	21	25	24, 26		20, 22		27	23
B6	83	82		84, 88		85, 86	89	87
B7	33	36	35, 37		31, 34		32	30
B8	81	78		74, 76		75, 79	80	77
B9	39	38	41, 44		42, 43		40	45
B10	68	66		69, 70		67, 71	72	73
B11	49	52	51, 53		54,55		48	50
B12	61	60		59, 64		57, 62	63	58

Birds were housed within an environmentally controlled facility ((b) (4) Appendix 1) in concrete floor pens (~ 4' × 4' minus 2.25 ft^2 feeder space) providing floor space & bird density of ~0.53 ft^2/bird (D0), ~0.55 ft^2/bird (D16), ~0.72 ft^2/bird (D21), ~1.06 ft^2/bird (D28). Temperature, humidity, lighting, feeder and water space was similar for all test groups. Birds were placed in clean pens containing an appropriate depth of used wood shavings top dressed with clean wood shavings to provide a comfortable environment for the chicks. Before placement three samples of litter throughout the house was taken and sent to the study sponsor. Additional shavings were added to pens if they became too damp for comfortable conditions for the test birds during the study. Were additional shavings needed at any time during the study, a note to file with the pen numbers was included in the study records. Lighting was via incandescent lights and a commercial lighting program was used as follows.

Approximate Bird Age (days)	Approximate Hours of Continuous Light per 24 hr period	~Light Intensity (foot candles)
0 – 5	24 hours	1.0 – 1.3
5 – 11	12 hours	1.0 – 1.3
11 – 19	12 hours	0.2 – 0.3
19 – 30	16 hours	0.2 – 0.3
30 – 37	18 hours	0.2 – 0.3
37 – 42	20 hours	0.2 – 0.3

Environmental conditions for the birds (i.e. bird density, temperature, lighting, feeder and water space) were similar for all treatment groups. Temperature logs were included in the final report. In order to minimize bird migration and bacterial spread from pen to pen, all birds were placed in solid plastic pens with walls approximately 48 inches in height between pens.

Vaccinations and Therapeutic Medication:

Birds were vaccinated for (b) (4) at the hatchery. Upon receipt (study day 0), birds were vaccinated for (b) (4) and Infectious Bronchitis ((½X dose; (b) (4)) Newcastle-Bronchitis Vaccine, B1 Type, B1 Strain, Massachusetts & Connecticut Types, Live Virus, Serial No. 311878, Expiration date 09FEB20), and (b) (4) (1X dose; (b) (4) Coccidiosis Vaccine Coccivac-B52, Live Oocysts, Serial No. 94320091, Expiration date 30MAY2019) by spray application using a spray cabinet. Documentation of vaccine lot number and expiration date were provided with the final report. No other vaccinations or medications (ionophore anticoccidials, etc.) were used unless approved by the Sponsor.

Water:

Water was provided *ad libitum* throughout the study via one (b) (4) bell drinker per pen. Drinkers were checked twice daily and cleaned as needed to assure a clean and constant water supply to the birds.

Feed:

Feed was provided *ad libitum* throughout the study via one hanging, ~17-inch diameter tube feeder per pen. A chick feeder tray was placed in each pen for approximately the first 7 days. Birds were placed on basal diets upon receipt (day 0) with treatment diets added daily according to the Experimental Design. Feed added and removed from pens from day 0 to study end was weighed and recorded.

Birds in treatment groups 1-6 consumed the same, untreated mash feed during the study, except for when treatment feed was placed on top of the feed in the feeder pan daily, according to Section C. Basal and Experimental Diets Table 2. Birds in treatment groups 7 and 8 consumed the same basal feed as the other treatments but containing bacitracin methylenedisalicylate (BMD). Feeders were not shaken prior to feeding of treated feed in order to assure that added treatment feed was being eaten. If feeders were shaken, they were done at the end of the day when all treatment feed had been consumed.

For the first 7 days, all treatments had 25g of premixed feed administered to each pen on a feeding tray. For the remainder of the trial, all treatments had 250 g of premixed feed administered to each pen in the feeder ring. Treatments groups 1 and 2 received feed mixed with a control, and treatment groups 3-6 received feed mixed with test article (Microbe Composition 1 and 2).

Daily Observations:

The test facility, pens and birds were observed at least twice daily for general flock condition, lighting, water, feed, ventilation and unanticipated events. If abnormal conditions or abnormal behavior was noted at any of the twice-daily observations, they were documented and documentation was included with the study records. The minimum-maximum temperature and humidity of the test facility was recorded once daily.

Pen Cards:

There were 2 cards attached to each pen. One card identified the pen number and the second identified the treatment number.

Animal Handling

The animals were kept under ideal conditions for livability. The animals were handled in such a manner as to reduce injuries and unnecessary stress. Humane measures were strictly enforced.

Veterinary Care, Intervention and Euthanasia

Birds that develop clinically significant concurrent disease unrelated to the test procedures may, at the discretion of the Study Investigator, or a designee, were removed from the study and euthanized in accordance with site SOPs. In addition, moribund or injured birds were also euthanized upon authority of a Site Veterinarian or a qualified technician. The reason for withdrawal was documented. If an animal died, or was removed and euthanized for humane reasons, it was recorded on the mortality sheet for the pen and a necropsy performed and was filed to document the reason for removal. If euthanasia was deemed necessary by the Study Investigator, animals were euthanized by cervical dislocation.

Mortality and Culls:

Starting on study day 0, any bird found dead or was removed and sacrificed was weighed and necropsied. Birds found dead or removal were not replaced after study day 0. Cull birds that are unable to reach feed or water were sacrificed, weighed, and documented. The weight and probable cause of death and necropsy findings were recorded on the pen mortality record.

Body Weights and Feed Intake:

Birds were weighed by pen on study days 0, 17, 28, 35, and 42. The feed remaining in each pen was weighed and recorded on study days 17, 28, 35 and 42. The feed intake during days 0 - 17, 0 - 28, 17 - 28, 0 – 35, 28 – 35, 0 – 42, and 35 - 42 was calculated.

Scales:

Scales used in weighing of feed, feed additives and birds were licensed by the State of (b) (4) At each use the scales were checked using standard weights according to (b) (4) Standard Operating Procedures. Scale certification certificates were provided in final study data.

C. *CLOSTRIDIUM PERFRINGENS* CHALLENGE

Challenge Material Preparation

The *Clostridium perfringens* culture were obtained from (b) (4). Administration of the *Clostridium perfringens* (CL-15, Type A, α and β2 toxins) cultures in this study were via the feed according to the Experimental Design. CL-15 was a field strain of *Clostridium perfringens* from a broiler outbreak in Colorado. The *Clostridium perfringens* culture (CL-15) was grown ~ (b) (4) (b) (4)

Quantitation was conducted by (b) (4) on the culture and results were documented in the study records.

Challenge Material Administration

Birds in treatment groups 2, 5, 6, and 8 were challenged with *Clostridium perfringens* on study day 17 via feed, according to the experimental design. Feed from each pen's feeder was used to mix with the culture. Prior to placing the cultures in the pens, the treatment feed was removed from the birds for approximately 4 – 8 hours. For each pen of birds, a fixed amount (~1.5-2 ml/bird) of the broth culture at a concentration of approximately $2.0 - 9.0 \times 10^8$ cfu/ml was mixed with a fixed amount of feed (~25 g/bird) in the feeder tray and all challenged pens were challenged at the same time. So that birds in all treatments are treated similar, the groups that are not challenged also had the feed removed during the same time period as the challenged groups. Most of the culture-feed was consumed within 1 – 2 hours. 5 - 12% NE mortality was the goal, this was attempted with the lower dose of *Clostridium perfringens* and controlled environmental factors.

D. LESION SCORING

On approximately study day 21±1, 5 birds, including the 1 bird already sacrificed for sampling, was randomly selected from each pen (by first bird caught), sacrificed and evaluated for jejunal lesion score for necrotic enteritis. This lesion scoring procedure was also performed on study day 28±1, but the 5 birds scored was in addition to the single bird sampled for organs. The number of birds scored for each pen was decreased and replaced by the number of birds that died from necrotic enteritis (NE) prior to lesion scoring up to 5 birds per pen. Lesions were scored as follows:

0 = **normal**: no NE lesions, small intestine has normal elasticity (rolls back to normal position after being opened)

1 = **mild**: small intestinal wall is thin and flaccid (remains flat when opened and doesn't roll back into normal position after being opened); excess mucus covering mucus membrane

2 = **moderate**: noticeable reddening and swelling of the intestinal wall; minor ulceration and necrosis of the intestine membrane; excess mucus

3 = **severe**: extensive area(s) of necrosis and ulceration of the small intestinal membrane; significant hemorrhage; layer of fibrin and necrotic debris on the mucus membrane (Turkish towel appearance)

4 = **dead or moribund**: bird that would likely die within 24 hours and has NE lesion score of 2 or more; or birds that died due to necrotic enteritis (NEM).

E. SAMPLING

Sampling Method

On study days 16, 21, 28, and 42, one (1) bird from each pen was removed, weighed individually, and recorded on the mortality sheet. The bird was euthanized and sampled for ileal swabs and collection of jejunum, spleen, and liver. On study day 21 the bird selected for sampling was from a bird already selected for lesion score. On study day 28, the bird selected for sampling was in addition to the 5 birds for lesion scoring.

For collection of ileal content, the ileum was removed and squeezed into tubes containing ~ 150 μL of stop solution and closed carefully. Precaution was taken not to touch the underside of the sample tube lid.

To collect the sterile ileal swabs, the outside of the intestine was first sterilized with 70% ethanol on a clean piece of butcher paper, and then cut open down the side of the organ with a new scalpel blade for each bird. The organ was allowed to unfold without touching it. A new sterile swab was used to vigorously rub the inside of the organ, and care was taken to avoid contaminating the swab with bacteria from the outside of the organ, as well as leftover fecal matter. The swab was placed into the sample tube containing ~ 600 μL of 25% stop solution, 75% PBS mixture removed from the sample tube, and returned to the original swab container. Precaution was taken not to touch the underside of the sample tube lid.

For collection of jejunum, a ~1.0 inch section of jejunum was removed and placed in a jar containing 10% neutral buffered formalin. A second section of jejunum (~1cm in length) was placed in an Eppendorf tube containing 1 ml of RNAlater.

Spleen and liver samples were placed on individual, a clean weighing cup per treatment and weighed using an analytical scale. Following collection of weights, the spleen was cut in half and a section of the right liver lobe was removed. Both tissue sections were placed in individual Eppendorf tubes containing 1 ml of RNAlater. All tubes containing RNAlater were maintained on wet ice during the sampling period. After sampling was completed, all samples in RNAlater were maintained at -80°C until shipped to Ascus Biosciences, Inc.

Additionally, on day 21, the bird already selected for sampling had blood drawn. Approximately 0.5-1 ml of blood was collected from the right brachial vein. The blood was centrifuged at 10,000 x g for separation of plasma. Plasma was then collected and shipped overnight in an insulated cooler with ice packs to Ascus Biosciences, Inc.

Shipping Samples

Ileal content, ileal swabs, and plasma samples were shipped overnight in an insulated cooler with ice packs or dry ice. Ileal mucosal swab, liver, and spleen samples were shipped overnight in an insulated cooler with dry ice. All samples were shipped the same day of sampling, or the following business day. If samples are collected on a Friday, or was not able to be shipped until a Friday, samples were held at -20 °C or -80°C until the following business day.

Samples were shipped to:

Ascus Biosciences, Inc.
6450 Lusk Blvd. Suite E109
San Diego, CA 92121

Samples placed in 10% neutral buffered formalin were shipped to:

(b) (4)

IV. DATA COLLECTED

- Bird weights by pen day 0, 17, 28, 35 and 42.
- Feed amounts added and removed from each pen from day 0 to study end.
- Mortality: sex, weight and probable cause of death day 0 to study end.
- Removed birds: reason for culling/removal, sex and weight, day 0 to study end.
- Daily observation of facility and birds, daily facility temperature.
- Samples from 1 bird per pen Day 16, 21, 28, and 42.
- Blood Collection from 1 bird Day 21.
- Lesion scores from 5 birds per pen Day 21 and 28.
- Three litter samples prior to placement.

V. DISPOSITIONS

Excess Test Articles

An accounting was maintained of the test articles received and used for this study. Excess test articles were dispositioned or returned to the Sponsor. Documentation was provided with the study records.

Feed

An accounting was maintained of all treatment diets. The amount mixed, used and discarded was documented. The sponsor requested that the unused feed be stored until approved notification to discard. Unused feed and retention feed samples was discarded to the landfill after receiving sponsor approval. Disposition was documented in the study records.

Test Animals

An accounting was maintained of all birds received for the study. Disposal of all mortalities and birds sacrificed during the study was by dumpster and landfill or by composting at (b) (4) Documentation of disposition was provided in the study records.

Animal Care and Use Statement

This study complied with all applicable animal welfare regulations related to the humane care and use of animals. All animal work was conducted under the direction of the (b) (4) (b) (4) Ethical Review Board. Documentation of the review was placed in the study file. All animal work was conducted in accordance with local, state, and national regulations.

VI. RECORDS AND REPORT

A final report and the original study records was provided to the Sponsor after the completion of live phase of the study. Proximate feed analysis was included, sponsor feed assay results and statistical analysis was not included. The Sponsor was provided with an electronic copy of the data. A copy of the report, data and study records were kept in (b) (4) archives for a period of 3 years.

VII. PERSONNEL

Key personnel involved in this study was as follows:

Sponsor	Kevin Bolek, PhD
Investigator	(b)(6)
Executive Vice President	(b)(6)
Research Manager	(b)(6)
Data Manager	
Vice President of Operations	
Feed Mill Manager	
Farm Technician	

II. INVESTIGATOR'S STATEMENT

There were no known circumstances that may have affected the data quality or integrity during this study. Summary tables and graphs of bird performance and mortality (Tables 1 - 38 & Graphs 1 - 14) have been prepared and are attached to this report. Due to the large amount of bird handling (sampling days, lesion scores, etc.) performance differences may be difficult to determine. All differences discussed in this statement have not been statistically analyzed and are numeric only.

Mortality was at expected levels to slightly higher than expected ranging from 4.167% (Trt 8) to 23.397% (Trt 2) based on the study conditions. Only treatments 2, 5, 6 and 8 were challenged with clostridium. Mortality attributed to necrotic enteritis in the challenged groups was 18.269%, 11.859%, 17.628% and 0.0% for treatments 2, 5, 6 and 8, respectively.

The bird performance and CVs for weight gain and feed conversion stayed within expected limits over the course of this study based on study conditions. Body weights ranged from 2.545 Kg (Trt 2) to 2.624 Kg (Trt 4) and adjusted feed conversion ranged from 1.590 (Trt 4) to 1.684 (Trt 2) at 42 days of age. From 0-17D and 0-28D treatment 7 had the highest average bird weight gain. Following the clostridium challenge, treatment 2 had the lowest average body weight gain and highest adjusted feed conversion compared to other treatments. During the challenge period (17-28D), treatment 5 had better performance than treatment 2. Treatment 8 had the best performance of all challenged treatments throughout the study.

Average NE lesion scores ranged from 0.000 (Trt 3, 4, and 7) to 2.017 (Trt 2) and 0.000 (Trt 4) to 1.950 (Trt 2) for 21D and 28D, respectively.

The average spleen weight (g) for 16D ranged from 0.43 (Trt 4 & 5) to 0.55 (Trt 8). The average spleen weight for 21D ranged from 0.68 (Trt 7) to 0.87 (Trt 4). The average spleen weight for 28D ranged from 1.42 (Trt 4) to 1.75 (Trt 8). The average spleen weight for 42D ranged from 3.12 (Trt 8) to 3.67 (Trt 2).

The average liver weight (g) for 16D ranged from 13.52 (Trt 1) to 17.60 (Trt 6). The average liver weight for 21D ranged from 24.25 (Trt 7) to 26.83 (Trt 2). The average liver weight for 28D ranged from 36.82 (Trt 7) to 41.21 (Trt 8). The average liver weight for 42D ranged from 61.62 (Trt 5) to 65.13 (Trt 3).

The report and data herein submitted to the Sponsor fo (b) (4) are accurate in that they represent the actual results of the study, were collected in a manner which did not misrepresent the true effects of the test articles, and were complete in that all data obtained in this study was submitted to the Sponsor.

(b)(6)

Study Investigator
(b)(6)

30JUL19
Date

Tables of Contents

(b) (4)

Tables (1-38):

Graphs (1-14):

LIST OF REPORT APPENDICES

Building Diagram
Disinfectant Procedure
(b) (4)
Body Weights, Feed, & Mortality
Challenge Information
Diet Formulas, Feed Preparation, Accounting, & Disposition
Test Article, Feed Additives, & Samples
Bird Receipt, Accounting, Vaccination, & Disposition
Daily Logs, House Observations, Scale Checks, & Notes to File
Personnel & Protocol
Correspondence

Building Diagram

(b) (4)

N ←

(b) (4)

Disinfectant Procedure

1. Prior to mixing, generously spray the mixer with (b) (4). Allow for 75 minutes to elapse.
2. Once 75 minutes has elapsed spray with 70% Ethanol, allow to sit for 15 [redacted] minutes.
3. Wipe clean, flush with corn
4. Start with mixing controls (Group 1 and 2)
    a. Flush with corn, then mix group three (3)
    b. After group three (3) has been mixed flush with corn again then mix group five (5).
    c. Once group five (5) has been mixed, repeat steps one, two, and three. Continue corn flush process with groups four (4) and six (6).
    d. Repeat steps one and two when finished

[redacted]

[redacted]

[redacted]

[redacted]

Appendix 3 - (b) (4)

(b) (4)

STANDARD OPERATING PROCEDURE
(SOP)

RANDOMIZATION

Effective Date:	19 MAY 14
Supersedes:	(b) (4)
References & Resources.	
Author Name:	(b)(6)
Approval Date:	23 APR 14
Approved By:	(b)(6)
Approval Date:	23APR14
Approved By:	(b)(6)

REASON FOR REVISION

- New SOP format
- Merged the following SOPs into one procedure: (b) (4) (b) (4)

AREAS INVOLVED

This SOP is applicable to all (b) (4) personnel with responsibilities for the randomization of research study animals

PURPOSE

The purpose of this SOP is to provide guidance on acceptable methods for the randomization of animals to a research study

SAFETY

Follow safety rules as described in (b) (4) Employee Manual

CONFIDENTIAL

(b) (4)

STANDARD OPERATING PROCEDURE
(SOP)

PROCEDURAL DESCRIPTION

1.0 RANDOMIZATION OF TREATMENTS TO PENS

(b) (4)

2.0 RANDOMIZATION OF BIRDS TO FLOOR PENS/BATTERY CAGES

(b) (4)

STANDARD OPERATING PROCEDURE
(SOP)

3.0 RANDOMIZATION OF BIRDS IF STRATIFIED BY INDIVIDUAL WEIGHT

(b) (4)

(b) (4)

STANDARD OPERATING PROCEDURE
(SOP)

4.0 RANDOMIZATION OF BIRDS GROWN IN STARTING BATTERIES INTO GROWING BATTERIES

(b)(4)

(b) (4)

(b) (4)

STANDARD OPERATING PROCEDURE
(SOP)

CONFIDENTIAL

REVIEW

Reviewed by: (b)(6)	Date: 10JUN14
Reviewed by:	Date: 02AUG16
Reviewed by:	Date: 19DEC17
Reviewed by:	Date:
Reviewed by:	Date:
Reviewed by:	Date:
Reviewed by:	Date:

From: Kristi Smedley
To: Adams, Carissa
Subject: [EXTERNAL] RE: Native Microbials GRAS Submission Clostridium beijerinckii for poultry
Date: Tuesday, September 14, 2021 5:59:35 PM
Attachments: image001.png
App_003D_C.beijerinckii_ASCUSBR67_Housekeeping_gene_comparison.pdf

---

Carissa:

I have attached the missing Appendix App_003D. I apologize it was not posted to the share drive, hence it was not provided.

A Native Microbial scientists is reviewing the situation with the references, and we should have an explanation, in the next 24 hours.

We are also looking into providing a more detailed description of attachments, than that provided in the table of contents.

And we will definitely take this request for future submissions.

It is my hope you will have something on your desk

Kristi O. Smedley, Ph.D.

RECEIVED DATE
SEP 30, 2021

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: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]
Sent: Tuesday, September 14, 2021 12:27 PM
To: Kristi Smedley
Subject: Native Microbials GRAS Submission Clostridium beijerinckii for poultry

Dr. Smedley,

We are in the prefiling review process for the GRAS submission on behalf of Native Microbials, Inc. regarding *Clostridium beijerinckii* as a direct fed microorganism for use in poultry. I am reaching out for confirmation on documents provided on the CD.

In a cursory review it appears the following document is missing from the submission: Appendix 003D.

Is it possible to provide a list of all of the appendix documents that should be on the CD in the "Appendices" folder so that we can confirm we have all the files?

There are 318 files in the "References" folder on the CD, but there are 349 references cited in Part 7 of the submission. It appears that 43 reference files missing in total to those cited in Part 7. There were 12 reference files provided that I was unable to find the citation for in Part 7. Could you confirm the list of reference files that were provided on the CD to those cited in Part 7?

Please send us the pdf files of any of the missing materials.

Sincerely,

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

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

FDA U.S. FOOD & DRUG
ADMINISTRATION

Appendix 003D: Housekeeping Gene Comparison for *Clostridium beijerinckii* ASCUSBR67

To further elucidate the taxonomy of *C. beijerinckii* ASCUSBR67, DNA sequence comparison between four housekeeping genes was conducted for the related species. The four genes in the comparison were ribonuclease P RNA (rnpB), ATP synthase alpha subunit (atpA), RNA polymerase alpha subunit (rpoA), and phenylalanyl-tRNA synthetase (pheS). Gene sequences were downloaded from the NCBI database. BLAST was used to generate the sequence alignments for the gene comparison through the pyANI wrapper script (Pritchard et al. 2016). The results are summarized in Table 1.

Table 1. Comparison of Housekeeping Genes is *C. beijerinckii* ASCUSBR67 and Related Clostridia Species

	Housekeeping Genes							
Related Species	pheS Identity	Coverage	rnpB Identity	Coverage	rpoA Identity	Coverage	atpA Identity	Coverage
C. beijerinckii	98.6	100	100	100	100	100	99.8	100
C. diolis	98.5	100	100	100	99.8	100	99.7	100
C. butyricum	85.6	99.7	84.1	100	91.9	100	82.6	99.9

Comparison of all four housekeeping genes gave the best match for *C. beijerinckii*, with greater than 98.6% identity for all the housekeeping genes. Close matches were also obtained for *Clostridium diolis*, which based on the limited evidence may be a strain of *C. beijerinckii.*

References

Pritchard, Leighton, Rachel H. Glover, Sonia Humphris, John G. Elphinstone, and Ian K. Toth. 2016. "Genomics and Taxonomy in Diagnostics for Food Security: Soft-Rotting Enterobacterial Plant Pathogens." *Analytical Methods*. https://doi.org/10.1039/c5ay02550h.

From: Kristi Smedley
To: Adams, Carissa
Subject: [EXTERNAL] RE: Native Microbials GRAS Submission Clostridium beijerinckii for poultry
Date: Wednesday, September 15, 2021 2:21:37 PM
Attachments: image001.png
BR67 Appendix List of Contents.pdf

I have attached a full listing of the appendices, as you requested. We will follow this model in the future.

Thank you for your query.

We are still working to give you an understanding of the submitted refernces.

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: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]
Sent: Tuesday, September 14, 2021 12:27 PM
To: Kristi Smedley
Subject: Native Microbials GRAS Submission Clostridium beijerinckii for poultry

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Sincerely,

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

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

Clostridium beijerinckii ASCUSBR67 GRAS Dossier Appendices

Appendix	Appendix Name
1	Certificate of deposit
2	Biochemical Results
3	Taxonomic Identification Using Genomics
3A	16S and WGS Genomic Identification
3B	BLAST Alignment Data
3C	Supplementary Whole Genome Analysis Methods and Read Quality Metrics
3D	Housekeeping Gene Comparison
4	Antimicrobial Susceptibility report
5	Antimicrobial Production report
6	Example of packaging
7	Methods of contamination
7A	AOAC Petrifilm E.coli and coliform method
7B	FDA BAM Ch 17: Clostridium botulinum method
7C	FDA BAM Ch 3: Aerobic Plate Count Method
7D	AOAC 2015:01 Heavy Metals Method ICP-MS
7E	Anresco Lab Heavy Metals Method
7F	AOAC 2013.01 Salmonella Method
7G	AOAC 2013.10 Listeria Method
8	Botulinum Toxin Testing Results
9	Specifications of Manufacturing Ingredients
9A	Ammonium Hydroxide
9B	Block co-polymer (Anti Foam)
9Ba	Anti Foam ETA Letter
9C	Cysteine Hydrochloride Monohydrate
9D	Dibasic Potassium Phosphate, anhydrous
9E	Potassium Phosphate Monobasic
9F	Sodium Acetate, Anhydrous
9G	Sodium Hydroxide
9H	Ammonium Chloride
9I	Biotin
9J	Corn Steep Solulys 095
9K	Dextrose Monohydrate
9L	Ferrous Sulfate, Heptahydrate
9M	Magnesium Sulfate Heptahydrate
9N	Manganese Sulfate, Monohydrate
9O	Phosphoric Acid
9P	Sodium Chloride
9Q	Thiamine Dydrochloride
9R	Procelys NuCel 581
9S	Yeast Extract Amberex 1003
9T	Starch, CAPSUL
10	Manufacturing Process

11	Homogneity Studies
	Lot 1102C Mash Homogeneity Protocol
	Lot 1102D Mash Homogeneity Protocol
	Lot 1102F Mash Homogeneity Protocol
	Lot 1102C Mash Homogeneity Protocol
	Protocol #1113 Generic Mash Homogeneity Protocol
	BR67 Homogeneity Protocol Executed (Raw Data from execution)
	BR67 Homogeneity Summary Report
12	Analytical methods and validation reports
	BR67 Liquid Intemediate Microbe Enumeration Method
	BR67 Liquid Intemediate Validation Executed
	BR67 Liquid Intemediate Validation Summary
	BR67 Pellet/Mash Method Validation Protocol
	BR67 Pellet/Mash Method Summary Report
	BR67 Pellet/Mash Method Microbe Enumeration
	BR67 SDP Microbe Enumeration Method
	BR67 SDP Validation Protocol Executed
	BR67 SDP Validation Summary
13	Certificates of Analysis
	BR67 SDP CofA Lot 20-0202-049-P6
	BR67 SDP CofA Lot 20-0202-049-P7
	BR67 SDP CofA Lot 20-0202-049-P12A
14	Heavy Metals and Microbial Contamination Report
15	Stability Data
	BR67 Mash Stability Protocol
	BR67 Mash Stability Protocol Amended
	BR67 Pellet Stability Protocol
	BR67 Pellet Stability Protocol Amended
	BR67 25C Stability Protocol
	BR67 25C Stability Protocol Amended
	BR67 50C Stability Protocol
	BR67 50C Stability Protocol Amended
	BR67 60C Stability Protocol
	BR67 60C Stability Protocol Amended
	BR67 70C Stability Protocol
	BR67 70C Stability Protocol Amended
	BR67 Accelerated Stability Report
	Stability Report BR67 Mash Final
	Stability Report BR67 Pellet Final
16	Appendix Intentially Blank
17	Literature Search Stategy
18	Microbiome Safety
19	Hemolysin Testing Report
20	Target Animal Study 1

21	Target Animal Study 2
22	Target Animal Study 3

From: Kristi Smedley
To: Adams, Carissa
Subject: RE: [EXTERNAL] RE: Native Microbials GRAS Submission Clostridium beijerinckii for poultry
Date: Tuesday, September 21, 2021 10:27:40 AM
Attachments: image001.png
BR67 Appendix List of Contents 9-20-2021.pdf

Carissa:

The protocol amendment on the 25 c was inadvertently listed. That protocol was not used and an accelerated study was done it its place. As such it should not have been listed or provided.

It was an error to list the Lot 1102C Mash Homogeneity protocol twice.

The list of appendices has been corrected, and attached.

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: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]
Sent: Monday, September 20, 2021 5:10 PM
To: Kristi Smedley
Subject: RE: [EXTERNAL] RE: Native Microbials GRAS Submission Clostridium beijerinckii for poultry

Hi Kristi,

I have went through the table of the full listing of appendices, it was very helpful.

It looks like we do not have "(b) (4)" (within Appendix 15), but we do have the amended version. Please send us the pdf file of "(b) (4)."

For Appendix 11, "Lot 1102C Mash Homogeneity Protocol" is listed twice in the table you provided. Can you confirm if it was listed twice in error? We have one file under Appendix 11 with the file name "1102C Protocol Mash Homogeneity BR67 signature page.docx.pdf."

Thanks,

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

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

---

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Wednesday, September 15, 2021 2:20 PM
To: Adams, Carissa <Carissa.Adams@fda.hhs.gov>
Subject: [EXTERNAL] RE: Native Microbials GRAS Submission Clostridium beijerinckii for poultry

I have attached a full listing of the appendices, as you requested. We will follow this model in the future.

Thank you for your query.

We are still working to give you an understanding of the submitted refernces.

Kristi O. Smedley, Ph.D.

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

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Cell (b)(6)
Fax 703-580-8637

From: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]
Sent: Tuesday, September 14, 2021 12:27 PM
To: Kristi Smedley

Subject: Native Microbials GRAS Submission Clostridium beijerinckii for poultry

Dr. Smedley,

We are in the prefiling review process for the GRAS submission on behalf of Native Microbials, Inc. regarding *Clostridium beijerinckii* as a direct fed microorganism for use in poultry. I am reaching out for confirmation on documents provided on the CD.

In a cursory review it appears the following document is missing from the submission: Appendix 003D.

Is it possible to provide a list of all of the appendix documents that should be on the CD in the “Appendices” folder so that we can confirm we have all the files?

There are 318 files in the “References” folder on the CD, but there are 349 references cited in Part 7 of the submission. It appears that 43 reference files missing in total to those cited in Part 7. There were 12 reference files provided that I was unable to find the citation for in Part 7. Could you confirm the list of reference files that were provided on the CD to those cited in Part 7?

Please send us the pdf files of any of the missing materials.

Sincerely,

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

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration
Tel: 240-402-6283
Personal e-mail address: carissa.adams@fda.hhs.gov
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FDA U.S. FOOD & DRUG
ADMINISTRATION

Clostridium beijerinckii ASCUSBR67 GRAS Dossier Appendices

Appendix	Appendix Name
1	Certificate of deposit
2	Biochemical Results
3	Taxonomic Identification Using Genomics
3A	16S and WGS Genomic Identification
3B	BLAST Alignment Data
3C	Supplementary Whole Genome Analysis Methods and Read Quality Metrics
3D	Housekeeping Gene Comparison
4	Antimicrobial Susceptibility report
5	Antimicrobial Production report
6	Example of packaging
7	Methods of contamination
7A	AOAC Petrifilm E.coli and coliform method
7B	FDA BAM Ch 17: Clostridium botulinum method
7C	FDA BAM Ch 3: Aerobic Plate Count Method
7D	AOAC 2015:01 Heavy Metals Method ICP-MS
7E	Anresco Lab Heavy Metals Method
7F	AOAC 2013.01 Salmonella Method
7G	AOAC 2013.10 Listeria Method
8	Botulinum Toxin Testing Results
9	Specifications of Manufacturing Ingredients
9A	Ammonium Hydroxide
9B	Block co-polymer (Anti Foam)
9Ba	Anti Foam ETA Letter
9C	Cysteine Hydrochloride Monohydrate
9D	Dibasic Potassium Phosphate, anhydrous
9E	Potassium Phosphate Monobasic
9F	Sodium Acetate, Anhydrous
9G	Sodium Hydroxide
9H	Ammonium Chloride
9I	Biotin
9J	Corn Steep Solulys 095
9K	Dextrose Monohydrate
9L	Ferrous Sulfate, Heptahydrate
9M	Magnesium Sulfate Heptahydrate
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18	Microbiome Safety
19	Hemolysin Testing Report
20	Target Animal Study 1
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22	Target Animal Study 3

From: Kristi Smedley
To: Adams, Carissa
Subject: [EXTERNAL] RE: Native Microbials GRAS Submission Clostridium beijerinckii for poultry
Date: Thursday, September 16, 2021 6:46:34 PM
Attachments: image001.png
PART 7 (AMENDED) - LIST OF SUPPORTING DATA AND INFORMATION.pdf

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Carissa:

We have revised section 7 (it is attached). We removed a number of unused references.

I will need to send you a DVD of the reference file that has exactly 337 references on it.

Thank you for your diligence on this review.

Kristi O. Smedley, Ph.D.

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

Ph. 703-590-7337
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Fax 703-580-8637

From: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]
Sent: Tuesday, September 14, 2021 12:27 PM
To: Kristi Smedley
Subject: Native Microbials GRAS Submission Clostridium beijerinckii for poultry

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

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

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Sincerely,

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PART 7 (AMENDED) – LIST OF SUPPORTING DATA AND INFORMATION

1. Abd-Alla, M. H., Zohri, A. N. A., El-Enany, A. W. E., & Ali, S. M. (2015). Acetone-butanol-ethanol production from substandard and surplus dates by Egyptian native *Clostridium* strains. *Anaerobe*, *32*, 77–86. https://doi.org/10.1016/j.anaerobe.2014.12.008

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Clostridia Species Spores along Cheesemaking Streams of 5 Commercial Dairy Plants." *Journal of Dairy Science* 99 (5): 3358–66.

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Center for Regulatory Services, Inc.

consultants to the regulated industry

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5200 Wolf Run Shoals Road
Woodbridge, VA 22192-5755
703 590 7337 (Fax 703 580 8637)
Smedley@cfr-services.com

September 21, 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: References
DFM *Clostridium beijerinckii* ASUSBR67-for
Poultry
Notifier: Native Microbials, Inc.
10255 Science Center
Dr., Suite C2 6450 Lusk
Blvd Suite 209
San Diego, California 92121

Dear Dr. Edwards:

Based on a number of emails exchanged between Carissa Adams and myself, I have provided a new DVD, that includes the 337 references that support the AGRN for the use of DFM Clostridium beijerinckii ASUSBR67 as a DFM for poultry feed.

I note that it is not a regulatory requirement to provide cited reference material in support of GRAS notices, but it is certainly Native Microbials interest in providing all information so that the review can be as efficient as possible.

We look forward to the substantive review of this GRAS notice.

Sincerely,

(b)(6)

Consultant to Native Microbials, Inc.

ATTACHMENTS:

DVD of the referenced material

T-0003

From: Kristi Smedley
To: Animalfood-premarket
Cc: Edwards, David
Subject: RE: [EXTERNAL] RE: Status Update for GRAS Notice AGRN #49 Clostridium beijerinckii ASCUSBR67
Date: Monday, July 11, 2022 5:35:22 PM
Attachments: image019.png
image021.png
image023.png
image025.png
image027.png
image029.png
image030.png
image031.png
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RECEIVED DATE:
July 13, 2022

This is in response to the Division's email of May 23, 2022, giving Native Microbials the opportunity to withdraw AGRN 49 (Clostridium beijerinckii ASCUSBR67 for use as a viable microorganism in diets of poultry) , our response of May 31, 2022, and your follow up on June 10, 2022 , June 19, 2022 and our discussion on June 29, 2022.

On behalf of Native Microbial's I am requesting withdrawal of AGRN 49.

Native Microbial's generally recognized as safe conclusion is supported by significant published safety information and a complete genome assessment, we believe that this probiotic is safe for the intended use. Post your review of AGRN 41 (also a *Clostridium beijerinckii* strain), and expressed concerns, we completed additional assessment on both strains on the potential for hemolytic activity, beyond our initial studies. The results of this robust testing (multiple conditions, various types of blood, positive and negative controls, etc.), confirmed our initial testing results of no hemolytic activity. Furthermore, we found these same non-functioning hemolysis genes in various AAFCO approved species that are also known to be non-hemolytic. We continue to believe that *Clostridium beijerinckii* ASCUSBR67 is safe, and would have preferred the opportunity to share these results. However, without that opportunity, we will are withdrawing this submission so that we have the opportunity to provide this new information and additional genetic information in a new submission.

We are requesting a meeting to discuss the Division's evaluation of AGRN 49.

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: Monday, July 11, 2022 2:43 PM
To: Kristi Smedley
Cc: Animalfood-premarket; Edwards, David
Subject: RE: [EXTERNAL] RE: Status Update for GRAS Notice AGRN #49 Clostridium beijerinckii ASCUSBR67

Dr. Smedley,

Traditionally, we have asked for a formal request to withdraw a notice. If the notifier is requesting us to cease to evaluate the notice please clarify the request in a letter (or email) to animalfood-premarket@fda.hhs.gov.

We are also acknowledging your meeting request and I will reach out with potential dates to discuss our evaluation of the notice once it is completed.

Sincerely,

Carissa Adams, MPH
Biologist, Division of Animal Food Ingredients

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

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Edwards, David <David.Edwards@fda.hhs.gov>
Sent: Tuesday, July 5, 2022 10:04 AM
To: Kristi Smedley <smedley@cfr-services.com>; Adams, Carissa <Carissa.Adams@fda.hhs.gov>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Schell, Timothy <Timothy.Schell@fda.hhs.gov>
Subject: RE: [EXTERNAL] RE: Status Update for GRAS Notice AGRN #49 Clostridium beijerinckii

ASCUSBR67

Dr. Smedley,

Thank you for the requests. We will follow up to schedule our further conversations.

Best,

David Edwards, PhD
Director, Division of Animal Food Ingredients

Center for Veterinary Medicine
Office of Surveillance and Compliance
U.S. Food and Drug Administration
Tel: 240-402-6205
david.edwards@fda.hhs.gov
FDA U.S. FOOD & DRUG ADMINISTRATION

From: Kristi Smedley <smedley@cfr-services.com>
Sent: Thursday, June 30, 2022 8:46 PM
To: Adams, Carissa <Carissa.Adams@fda.hhs.gov>; Edwards, David <David.Edwards@fda.hhs.gov>
Cc: Animalfood-premarket <Animalfood-premarket@fda.hhs.gov>; Schell, Timothy <Timothy.Schell@fda.hhs.gov>
Subject: [EXTERNAL] RE: Status Update for GRAS Notice AGRN #49 Clostridium beijerinckii ASCUSBR67

Dr. Edwards:

This is in response to the Division's email of May 23, 2022, giving Native Microbials the opportunity to withdraw AGRN 49 (Clostridium beijerinckii ASCUSBR67 for use as a viable microorganism in diets of poultry) , our response of May 31, 2022, and your follow up on June 10, 2022 , June 19, 2022 and our discussion on June 29, 2022.

Native Microbial's generally recognized as safe conclusion is supported by significant published and genome assessment, we believe that this probiotic is safe for the intended use. Post your review of AGRN 41 (also a *Clostridium beijerinckii* strain) we completed additional assessment on both strains on the potential for hemolytic activity, beyond our initial studies. The results of this robust testing (multiple conditions, various types of blood, positive and negative controls, etc.), confirmed our initial testing results of no hemolytic activity. Furthermore, we found these same non-functioning hemolysis genes in various AAFCO approved species that are also known to be non-hemolytic. We continue to believe that *Clostridium beijerinckii* ASCUSBR67 is safe, and would have preferred the opportunity to share these results. However, without that opportunity, we will need to withdrawal this submission and resubmit with this study and additional genetic information.

We are requesting a meeting to discuss the Division's evaluation of AGRN 49.

Kristi O. Smedley, Ph.D.

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

Ph. 703-590-7337
Cell (b) (4)
Fax 703-580-8637

From: Adams, Carissa [mailto:Carissa.Adams@fda.hhs.gov]
Sent: Monday, May 23, 2022 1:44 PM
To: Kristi Smedley
Cc: Animalfood-premarket
Subject: Status Update for GRAS Notice AGRN #49 Clostridium beijerinckii ASCUSBR67

Good afternoon Dr. Smedley,

CVM is currently evaluating GRAS Notice AGRN #49 *Clostridium beijerinckii* ASCUSBR67, as a viable microorganism in diets of poultry, which was submitted by you on behalf of Native Microbials Inc.

At this time, CVM has questions on microbial safety of the notified substance. Specifically, we question the safety conclusion given that the strain's genome contains genetic elements that encode toxins or virulence factors. In addition we question the safety conclusion on the basis of the lack of suitability of the methodology utilized by the notifier to demonstrate strain ASCUSBR67 does not express toxins. We note that we are currently evaluating the notice and may identify additional questions.

CVM does not believe these questions can be answered in an amendment. Native Microbials has the opportunity to request that we cease to evaluate the GRAS notice. If the notifier intends to make such a request, it should send a letter via email to animalfood-premarket@fda.hhs.gov no later than two weeks from receiving this email, which is **June 7, 2022**. If no response is received, CVM will proceed with evaluation of the notice.

As always, Native Microbials may request a meeting with CVM once the evaluation of the notice is completed.

Sincerely,

Carissa Adams, MPH
Animal Scientist

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

FDA U.S. FOOD & DRUG
ADMINISTRATION