GRAS Notice for *Butyrivibrio fibrisolvens* ASCUSDY19 for Use as a Direct Fed Microbial in Dairy Cattle

December 30, 2020

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LIST OF ABBREVIATIONS

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

NOMENCLATURE

The notified substance is *Butyrivibrio fibrisolvens* ASCUSDY19 and is deposited in the NRRL as B-67347. The microbial strain may be encapsulated with hydrogenated glycerides for use in direct fed microbial products for dairy cattle which is referred to as 'fat encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19'.

The microbial strain *Butyrivibrio fibrisolvens* ASCUSDY19 is often referred to in some appended reports as 'Dairy-19' or 'DY19' which are the internal research name for *Butyrivibrio fibrisolvens* ASCUSDY19.

GRAS Notice for *Butyrivibrio fibrisolvens* ASCUSDY19 for Use as a Direct Fed Microbial in Dairy Cattle

PART 1 – SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §570 Subpart E consisting of §570.203 to 280, Native Microbials, Inc. hereby informs the U.S. Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for *Butyrivibrio fibrisolvens* ASCUSDY19.

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 *Butyrivibrio fibrisolvens* ASCUSDY19 (microbial strain). It is manufactured as a freeze-dried milled product which is further standardized and stabilized by encapsulation in fat for use in direct fed microbial products for dairy cattle. The standardized product is referred to as 'fat encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19' or '*Butyrivibrio fibrisolvens* ASCUSDY19 encapsulated'. In addition, a number of the appended reports refer to *Butyrivibrio fibrisolvens* ASCUSDY19 or the fat encapsulated product under the internal research name, Dairy-19.

1.3 Intended Conditions of Use

B. fibrisolvens ASCUSDY19 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. The intended purpose of supplementation of the microorganism is to augment the digestion of feed in the rumen. The microbial strain will be delivered in the fat encapsulated form to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing fat encapsulated *B. fibrisolvens* ASCUSDY19 may be incorporated into the diet of dairy cattle include as part of the total mixed ration (TMR), as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. It is anticipated that *B. fibrisolvens* ASCUSDY19 will be incorporated into feed at a recommended level of $1x10^8$ CFU/cow/day.

1.4 Statutory Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §570.30(a) and (b), *B. fibrisolvens* ASCUSDY19 manufactured by Native Microbials, has been concluded to have GRAS status for use as a direct fed microbial in dairy cattle, as described in Part 1.3, on the basis of scientific procedures.

1.5 Premarket Exception Status

Native Microbials hereby informs the U.S. FDA of the view that *B. fibrisolvens* ASCUSDY19 is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on Native Microbials conclusion that the notified substance is GRAS under the conditions of intended use as described in Part 1.3 above.

1.6 Availability of Information

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

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

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

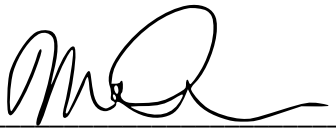
1.7 Freedom of Information Act, 5 U.S.C. 552

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

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 *Butyrivibrio fibrisolvens* ASCUSDY19.

Signed,



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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, *B*. *fibrisolvens* ASCUSDY19, is provided in Table 2.1. *B. fibrisolvens* ASCUSDY19 is a prominent anaerobic, non-spore-forming, member of the ruminant gut microbiome (Bryant and Small 1956). Recent whole genome assemblies have revealed that, in at least some strains, the genome consists of 2 chromosomes (Rodríguez Hernáez et al. 2018). In the rumen, the species degrades fibrous plant material and ferments polysaccharides (Hespell, Wolf, and Bothast 1987). While the genus name implies that the genera produces butyrate, fermentation products within the *B. fibrisolvens* species are strain specific. The notified strain predominant fermentation products are acetate and butyrate (Table 2.3). Many strains of *B. fibrisolvens* produce butyrate as a major product while others favor lactate, formate, or acetate (Paillard et al. 2007). The species has also been shown to play a role in biohydrogenation of fatty acids, both *in vitro* and *in vivo* (Shivani et al. 2016; Maia et al. 2010). Specifically, *B. fibrisolvens* converts linoleic acid to cis-9 trans-11 conjugated linoleic acid (CLA) (McKain, Shingfield, and Wallace 2010). Some CLA isomers have been implicated with milk fat depression, specifically trans-10 cis-12, which are not produced by *B. fibrisolvens* (Baumgard et al. 2000).

Table 2.1: Taxonomic Classification of *B. fibrisolvens*

Kingdom	**Bacteria**
Phylum	**Firmicutes**
Class	**Clostridia**
Order	**Clostridiales**
Family	***Lachnospiraceae***
Genus	***Butyrivibrio***
Species	***Fibrisolvens***

2.1.2 Source of the Microorganism

B. fibrisolvens ASCUSDY19 was identified and isolated to axenicity from the rumen contents of a healthy, mid-lactation Holstein cow rumen obtained via cannula. The sample was received and isolated by Native Microbials (Native Microbials, 10255 Science Center Dr, San Diego, 92121). The isolate was deposited in the NRRL, Agricultural Research Service Culture Collection, and referenced as NRRL B-67347. Proof of deposit can be found in Appendix 001.

2.1.3 Description of the Microorganism

B. fibrisolvens ASCUSDY19 is a small rod, typically 2-5 µm in length, normally found as single cells or in chains (Figure 2.1). Cells are motile and stain gram-negative (Figure 2.2). When cultured on tryptic soy agar, *B. fibrisolvens* ASCUSDY19 forms circular, slightly umbonate colonies with a spreading edge and opaque center (Figure 2.3).

Figure 2.1: Methylene Blue Stain for *B. fibrisolvens* ASCUSDY19 after 48 hours of incubation (1000x magnification)

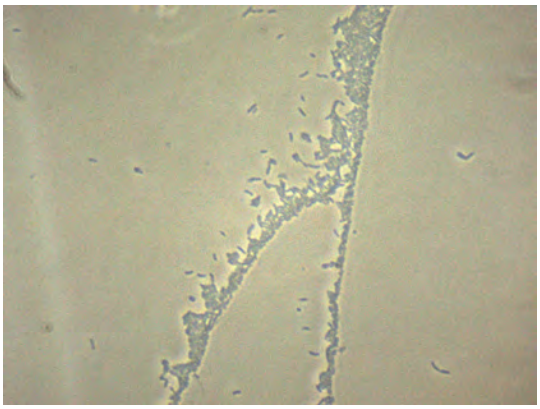


Figure 2.2: Gram Stain for *B. fibrisolvens* ASCUSDY19 after 7 days of incubation (stationary phase, 400x magnification)

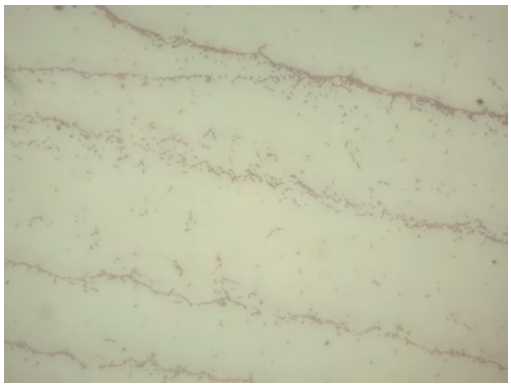
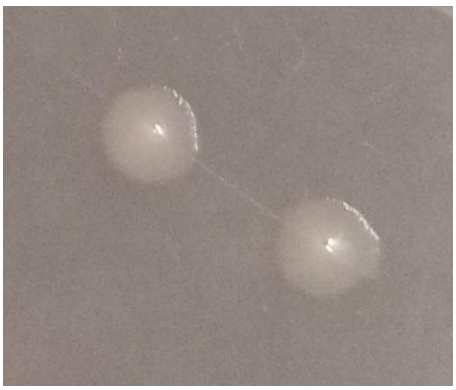


Figure 2.3: *B. fibrisolvens* ASCUSDY19 Colonies on Tryptic Soy Agar (4x magnification)



In vitro assays demonstrate that *B*. *fibrisolvens* ASCUSDY19 grows on a variety of substrates including arabinose, xylose, glucose, fructose, rhamnose, esculin, salicin, cellobiose, melibiose, saccharose, raffinose, and starch. Full results can be found in Table 2.2. The fermentation profile of *B. fibrisolvens* ASCUSDY19 is similar to what has previously been reported for the species (M. A. Cotta 1988; Marounek and Petr 1995; Van Gylswyk, Hippe, and Rainey 1996).

Table 2.2: Growth of B. ***fibrisolvens*** **ASCUSDY19 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	No Growth
D-Ribose	No Growth	N-AcetylGlucosamine	No Growth
D-Xylose	Growth	Amygdalin	No Growth
L-Xylose	No Growth	Arbutin	No Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	Growth
Methyl-BD-xylopyranoside	No Growth	Salicin	Growth
D-Galactose	No Growth	D-Cellobiose	Growth
D-Glucose	Growth	D-Maltose	No Growth
D-Fructose	Growth	D-Lactose	No Growth
D-Mannose	No Growth	D-Melibiose	Growth
L-Sorbose	No Growth	D-Saccharose	Growth
L-Rhamnose	Growth	D-Trehalose	No Growth
Dulcitol	No Growth	Inulin	No Growth
D-Melezitose	No Growth	D-Tagatose	No Growth
D-Raffinose	Growth	D-Fucose	No Growth
Starch	Growth	L-Fucose	No Growth
Glycogen	No Growth	D-Arabitol	No Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	No Growth	Potassium Gluconate	No Growth
D-Turanose	No Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth		

Metabolite production of *B. fibrisolvens* ASCUSDY19 was measured at 52 hours elapsed fermentation time using an Agilent 1260 series HPLC with refractive index (RI) detector. The results are summarized in Table 2.3 and Appendix 002. Major fermentation products include acetate, butyrate, and lactate.

Table 2.3: Metabolite Production of *B. fibrisolvens* ASCUSDY19 on Complex Media with Glucose

Metabolite	Production (g/L)
Pyruvic acid	0
Succinic acid	0.08
Lactic acid	1.42
Glycerol	0.51
Acetic acid	2.56
Propionic acid	0.02
Butyric acid	3.83
Ethanol	0.09
1-Butanol	0

2.1.4 Identification of the Microorganism

2.1.4.1 16S rRNA Gene Sequencing

The 16S rRNA gene was amplified from the strain using 27F and 543R primers and paired end sequenced [2x300 base pairs (bp)] using an Illumina Miseq (Schumann 1991; Muyzer, de Waal, and Uitterlinden 1993). The resulting sequence was quality trimmed and compared to National Center for Biotechnology Information (NCBI) databases using the Basic Local Alignment Search Tool (BLAST) to establish the identity of the strain. Details of the analysis including the BLAST output are provided in Appendix 003A and 003B. Strains of *B. fibrisolvens* and unnamed rumen bacterium provided 16S rRNA sequence matches that fall within the minimum 98.7% sequence identity threshold typically used to define a species (Yarza et al. 2014). The best match was to *B. fibrisolvens* InBov1 at 99.7% sequence identity. Results can be found in Table 2.4.

Table 2.4: 16S rRNA alignment to *B. fibrisolvens* ASCUSDY19 16S rRNA by BLAST

Genus species (Genbank accession #)	Identity (%)	Coverage (%)
B. fibrisolvens InBov1 (JN642599)	99.7%	100%
Rumen Bacterium NK3B81 (GU324363)	99.7%	99%
Rumen Bacterium NK4A61 (GU324372)	99.3%	99%
Rumen Bacterium NK4A114 (GU324377)	98.9%	99%
B. fibrisolvens WV1 (AF396927)	98.3%	99%

2.1.4.2 Whole Genome Sequence Assembly and Annotation

Genomic DNA was isolated from a pure culture of *B. fibrisolvens* ASCUSDY19 and sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA). The resulting libraries were paired-end sequenced (1x300bp) on an Illumina Miseq and in parallel, long-read libraries were prepared from the same extracted DNA using SQK-RAD004 kit (Oxford NanoporeTechnologies, Oxford) following the protocol outlined by Jain *et al*. (2018) and 1D sequenced on the MinION (R9.4 flowcell; Oxford Nanopore, Oxford) (Jain et al. 2018). The genome was assembled through hybrid methods utilizing both short and long reads. Read quality and genome coverage was evaluated using FASTQC for Illumina data and NanoStat for the Oxford Nanopore reads. The *B. fibrisolvens* ASCUSDY19 genome was closed with no gaps and consisted of 2 chromosomes, a main chromosome (4,116,214 bp) and a chromid (336,856 bp). The presence of a chromid is consistent with previous observations of the species (Rodríguez Hernáez et al. 2018). The total length of the genome is 4,453,070 bp and a GC content of 39.9%. Assembly statistics can be found in Table 2.5. The full details of the assembly are provided in Appendix 003C.

Protein coding genes were predicted through GLIMMER2 and through an iterative process of annotating putative genes using the FIGfams database (Delcher 1999; Meyer, Overbeek, and Rodriguez 2009). To identify protein coding open reading frames of potential genes, contigs were first filtered of all potential tRNA coding genes (T. M. Lowe and Eddy 1997) and rRNA genes (Aziz et al. 2008).

The *B. fibrisolvens* ASCUSDY19 genome contains 3,867 coding sequences which were subsequently built into a metabolic reconstruction describing 235 functional subsystems (DeJongh et al. 2007; Becker and Palsson 2005). These subsystems include larger metabolic groups describing metabolism, virulence, plasmids, disease, defense metabolic products, stress response and dormancy.

The assembled genome has been deposited at NCBI under accession number CP065800 for the main chromosome and CP065801 for the chromid.

Table 2.5: Assembly Statistics for *B. fibrisolvens* ASCUSDY19

# of Contigs	2
# of Contigs ≥ 5,000 bp	2
Longest Contig (bp)	4,116,214
Assembly Length	4,453,070
N50	4,116,214
N75	4,116,214
GC%	39.91

2.1.4.3 Whole Genome Sequence Comparison

To determine relatedness of *B. fibrisolvens* ASCUSDY19 to other closely related species at a higher resolution, whole genomes were compared using ANI. Candidate genomes for genome-genome comparison to *B. fibrisolvens* ASCUSDY19 were selected by full length 16S rRNA similarity and downloaded from the NCBI database. MUMmer was used to generate the alignments for ANI on the

basis that this software is adept at aligning highly similar sequences and is more stringent than most other aligners such as BLAST (Kurtz et al. 2004). Results for the MUMmer alignment can be found in Table 2.6.

The only ANI matches to *B. fibrisolvens* ASCUSDY19 above the 95% ANI cutoff to be considered the same species were two strains of *B. fibrisolvens* (Richter and Rosselló-Móra 2009).

Table 2.6: Average Nucleotide Identity (ANI) of Related Species to *B. fibrisolvens* ASCUSDY19 by MUMmer

Genus species (assembly)	**ANI (%)**	**Coverage (%)**
B. fibrisolvens INBov1 (GCA_003175155)	97.6	72.1
B. fibrisolvens YRB2005 (GCA_000423985)	96.8	77.3
B. fibrisolvens DSM3071 (GCA_900129945)	89.2	34.8
Butyrivibrio proteoclasticus B316n (GCA_000145035)	86.4	3.69
Butyrivibrio proteoclasticus P6B7 (GCA_000622085)	85.5	2.8
Butyrivibrio hungatei NK4A153 (GCA_000424465)	84.8	2.6
Butyrivibrio hungatei MB2003 (GCA_001858005)	84.4	3.4

2.1.4.4 Summary and Conclusions

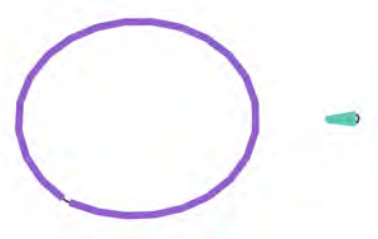
16S rRNA and whole genome analysis confirm that *B. fibrisolvens* ASCUSDY19 represents a member of the species *B. fibrisolvens.*

2.1.5 Plasmid Analysis

To confirm the presence/absence of plasmids, the assembly graph for the *B. fibrisolvens* ASCUSDY19 assembly was analyzed by Bandage (Wick et al. 2015). The assembly graph analysis confirmed that the *B. fibrisolvens* ASCUSDY19 was contained in 2 circular chromosomes with no unincorporated fragments, verifying the completeness of the assembly. Image of the assembly graph can be found in Figure 2.4.

As noted in Part 2.1.4.2, the presence of a smaller, circular second replicon (chromid) is consistent with other assemblies of the species. The annotated features on the putative chromid are associated with general housekeeping and metabolic functions, which is consistent with gene composition of chromids (Harrison et al. 2010). No genes encoding virulence factors, toxins, antimicrobial resistance, or transposable elements were found on the chromid.

Figure 2.4: ***B. fibrisolvens*** **ASCUSDY19 Assembly Graph as Generated by Bandage**



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

Phenotypic testing was conducted on *B. fibrisolvens* ASCUSDY19 to determine the minimum inhibitory concentrations (MICs) against a selected group of antimicrobials of relevance to human and veterinary medicine. The full study report is provided in Appendix 004 and results can be found in Table 2.7. The results were evaluated against the resistant breakpoints set by the European Food Safety Authority (EFSA) for "other gram positive bacteria", the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for "gram positive anaerobes" and the Clinical and Laboratory Standards Institute (CLSI) for "anaerobes" (where available). The MIC values reported for *B. fibrisolvens* ASCUSDY19 were equal, or lower than, the cut-off values and break-points established by EFSA, EUCAST and/or CLSI for chloramphenicol, and ampicillin. The isolate would be considered susceptible to Vancomycin and Clindamycin according to EFSA and EUCAST breakpoints but considered intermediately sensitive to Clindamycin per CLSI MIC values were also considered to be in the intermediate range established by CLSI for tetracycline. MIC values reported for *B. fibrisolvens* ASCUSDY19 were higher than the cutoff values and break-points established by EFSA for tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin.

It should be noted that susceptibility to aminoglycosides (gentamicin, kanamycin, streptomycin) and macrolides (erythromycin) decrease significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al. 2016). As such, classifications set forth by EFSA are for general gram-positive organisms and should be carefully applied to *Butyrivibrio fibrisolvens* due to its anaerobic nature. CLSI and EUCAST refrain from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. Tetracycline resistance was indicated by values above the EFSA breakpoint and in the intermediate range by CLSI breakpoint. Tetracycline resistance is not uncommon among ruminal derived organisms. Among 68 livestock derived *Clostridium* strains analyzed by Dutta et al. (1983) 17/68 (25%) strains displaying MIC values above the EFSA microbiological cut-off value. More recent studies have shown that tetracycline resistance is widespread amongst diverse taxa in the rumen (Dutta, Devriese, and Van Assche 1983). Sabino et al. (2019) found that 69% of the ruminal isolates they screened contained tetracycline resistance genes, which were not only expressed, but also reflected in a resistant phenotype (Y. N. V. Sabino et al. 2019).

Table 2.7:	***B. fibrisolvens* ASCUSDY19 Antimicrobial Susceptibility in Relation to EFSA, EUCAST, and CLSI breakpoints**				
Antimicrobial	**Tested Range (ug/mL)**	**MIC (ug/mL) of *B. fibrisolvens* ASCUSDY19**	**2018 EFSA Microbiology Cut-off Values (ug/mL) for Other Gram +**	**EUCAST Resistant Breakpoints (ug/mL) Gram + Anaerobes**	**CLSI Resistant Breakpoints (ug/mL) Anaerobes***
Ampicillin	0.5-128	< 0.5	1	8	≥ 2 (R) ≥ 1 (I)
Chloramphenicol	0.5-64	4	4	8	≥ 32 (R) ≥ 16 (I)
Clindamycin	0.03-32	4	4	4	≥ 8 (R) ≥ 4 (I)
Erythromycin	0.5-16	4	1	Not available	Not available
Gentamicin	0.5-32	8	4	Not available	Not available
Kanamycin	0.5-64	> 64	16	Not available	Not available
Streptomycin	0.5-64	16	8	Not available	Not available
Tetracycline	0.0625-64	8	4	Not available	≥ 16 (R) ≥ 8 (I)
Vancomycin	0.125-32	0.25	1	2	Not available

*R = Resistant Breakpoint; I = Intermediate Sensitivity / Susceptible, Increased Exposure. A microorganism is categorized as "I" when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.

To evaluate the presence of antimicrobial resistance genes in the *B. fibrisolvens* ASCUSDY19 genome, amino acid sequences from coding regions identified in Part 2.1.4.3 were aligned to the PATRIC database. Included in the PATRIC database is the Comprehensive Antibiotics Resistance Database (CARD) and NCBI's National Database of Antibiotic Resistant Organisms (NDARO) for assessing antimicrobial resistance. In addition to the protein sequences from the databases, PATRIC has compiled protein hits to CARD and NDARO from 331,756 bacterial genomes and included those as redundant gene entries as a means to understand the global distribution of antimicrobial resistance proteins across diverse taxa isolated from a wide range of environments and hosts. Antimicrobial resistance was further explored using the ResFinder web server (Zankari et al. 2012) and BLASTp alignment to the NCBI AMR database as used by AMRFinder (Note: this database differs from NARDO used by PATRIC) (Feldgarden et al. 2019). Between these databases there are a total of 30,748 protein sequences, characteristics of each database can be found in Table 2.8.

Table 2.8: Characteristics of Databases Used to Assess Antimicrobial Resistance				
Database Name	**Number of Entries**	**Number of *Butyrivibrio* Entries**	***B. fibrisolvens* Entries**	**Contains Redundant Entries**
CARD (PATRIC)	17,559 (2,227 non redundant proteins	0	0	Yes
NDARO (PATRIC)	5,138 (4,004 non redundant proteins)	0	0	Yes
ResFinder	3,105	0	0	No
AMRFinder Plus	6,946	0	0	No

To ensure no hits were missed due to codon bias or sequencing error, protein alignments were considered a hit if they have greater than 80% identity over more than 70% query coverage. Results can be found in Tables 2.9 to 2.11.

Genetic analysis of *B. fibrisolvens* ASCUSDY19 identified one possible resistance gene (see Tables 2.9 to 2.11).

- The antimicrobial gene in question is a 100% match to the tetracycline resistance gene, tetW, in both the ResFinder and NCBI AMR databases and a 99% match to the same gene in the Card and NDARO databases. TetW confers resistance to tetracycline through ribosomal protection (Aminov, Garrigues-Jeanjean, and Mackie 2001). The tet(W) gene is a ubiquitous gene in the bacterial population of ruminants, humans, and other farm animals (Pal et al. 2016; Joyce et al. 2019; Y. Sabino et al. 2019).

Table 2.9: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by PATRIC								
Source	**Source Organism**	**Gene**	**Product**	**Function**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
CARD/ NDARO	*Bifidobacterium longum*	tetW	Tetracycline resistance, ribosomal protection	MULTISPECIES: tetracycline resistance ribosomal protection protein Tet(W)	100	100	99	0.0

Table 2.10: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by ResFinder				
Gene	**Identity**	**Query Coverage**	**Function**	**Accession number**
tetW	99.9	100% (1920/1920)	Tetracycline Resistance	AJ427422

Table 2.11: *B. fibrisolvens* ASCUSDY19 Antimicrobial Resistance by NCBI AMR BLASTp				
Gene	**e-value**	**Percent Identity**	**Query Coverage**	**Subject Coverage**
tet(W)	0	99.8	100	100

2.1.6.1 Section Summary

In vitro testing demonstrated that *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin. Resistance to aminoglycosides and macrolides such as gentamicin, kanamycin, streptomycin, and erythromycin is reflective of *B. fibrisolvens* ASCUSDY19 being anaerobic rather than any specific resistance mechanism or genotype. *In silico* analyses revealed the presence of tetW, a gene implicated in tetracycline resistance. This finding is consistent with the tetracycline resistant phenotype observed in the MIC testing. *B. fibrisolvens* ASCUSDY19 is susceptible to chloramphenicol, vancomycin, and ampicillin and therefore could easily be controlled with readily available antibiotics.

2.1.7 Antimicrobial Production

Butyrivibrio fibrisolvens ASCUSDY19 supernatant obtained post fermentation was tested for inhibitory activity against reference strains known to be susceptible to a range of antibiotics. No zones of inhibition were observed indicating that the strain is not an antimicrobial producer. Further details of the study are provided in Appendix 005.

2.1.8 Toxigenicity and Pathogenicity

To assess the presence of virulent and pathogenic genes, amino acid sequences from coding regions identified in Part 2.1.4.3 were aligned to several databases. All applicable, publicly available databases were used to identify potential pathogenic genes. The characteristics of these databases are described in Table 2.12. The PATRIC database has compiled relevant genes from external databases including Victors, Virulence Factors Database (VFDB), and the PATRIC_VF database. These genes represent 331,756 bacterial genomes. Redundant gene entries (e.g. the same toxin showing up in multiple microbial species) are included as a means to understand the global distribution of pathogenicity and virulence associated proteins across diverse taxa isolated from a wide range of environments and hosts. PathogenFInder and IslandViewer web servers (Cosentino et al. 2013; Bertelli et al. 2017) as well as BLASTp alignment to the Pathogen-Host Interaction Database (Phi-BASE) (Urban et al. 2015) were also utilized to assess the pathogenicity and virulence of *B. fibrisolvens* ASCUSDY19. The total number of sequences in the PATRIC and Phi-BASE databases is 134,396 and includes no sequences from *Butyrivibrio.* IslandViewer contains 4,065 pathogenicity islands including 4 from *Butyrivibrio* species. The analysis in PathogenFinder is database independent and uses a model trained with protein sequences from 886 whole genome sequences. The PathogenFinder model predicts pathogenicity based on matches to proteins found differentially in pathogenic and non-pathogenic bacteria regardless of their annotated function. Therefore, a single hit to a protein found in pathogenic species does not necessarily suggest the query organism is virulent or pathogenic, but a collection of hits to proteins uniquely found in pathogens could be enough for PathogenFinder to deem the organism pathogenic, even if the proteins are not traditionally implicated in virulence or pathogenicity. The program allows the organism to be evaluated more holistically and enables the evaluation of proteins that are potentially involved in virulence and pathogenicity beyond well annotated virulence factors such as toxins.

Table 2.12: Characteristics of Databases Used to Assess Virulence and Pathogenicity				
Database Name	**Number of Entries**	**Number of *Butyrivibrio* Entries**	***B. fibrisolvens* Entries**	**Contains Redundant Protein ID entries**
Victors (PATRIC)	67,914 (4,950 non-redundant proteins)	0	0	Yes
VFDB (PATRIC)	20,911 (2,595 non-redundant proteins)	1	1	Yes
PATRIC_VF	38,791(1,570 non-redundant proteins)	0	0	Yes
Phi-Base	6,780	0	0	No
IslandViewer4	4,065 pathogenicity islands	4	0	No
PathogenFInder	N/A	N/A	N/A	N/A

The alignment process compares all identified *B. fibrisolvens* ASCUSDY19 genes against all known pathogen-related genes that have been identified across the Bacterial and Fungal kingdoms. To ensure no hits are missed due to codon bias or sequencing error, protein alignments are considered a hit if they have greater than 80% identity over more than 70% query coverage.

No genes involved in toxin synthesis, pathogenicity, or virulence were identified in the VFDB, PATRIC_VF, or Phi-Base databases. Additionally, no hits to pathogenicity islands were identified by IslandViewer. A site specific recombinase was identified as a potential virulence factor by both Victors and PathogenFInder. While the annotation, protein sequence, and source organism slightly differs between the two databases, the protein in question in the *B. fibrisolvens* ASCUSDY19 genome is the same. The recombinase is homologous to a recombinase found in pathogenic *Streptococcus pneumoniae.* Phage derived site-directed recombinases have been known to excise and insert pathogenic elements in *Streptococcus* species (Carroll et al. 1995). However, excision and insertion of genetic material by the recombinase requires other phage encoded proteins which are not present in the *B. fibrisolvens* ASCUSDY19 genome. Homologues of the recombinase were found to be one of 337 genes necessary to cause lung infections by *S. pneumoniae* in mice, though there was no indication that the recombinase itself was sufficient to cause pathogenicity (Hava and Camilli 2002). There is some evidence that recombinases might play a role in regulation of surface protein production in Streptococci as part of the evolution from commesal to pathogen (Holden et al. 2009). However, there is no evidence linking the recombinase encoded by the *B. fibrisolvens* ASCUSDY19 genome to this type of activity. A global search of the organisms in the PATRIC database was conducted to assess the global distribution of similar site-directed recombinases. The search returned 134,507 unique protein hits between diverse taxa including pathogenic and non-pathogenic species. Alignment of the recombinase protein identified in the *B. fibrisolvens* ASCUSDY19 genome yielded hits in pathogenic Streptococci and in non pathogenic commensals alike, suggesting that the recombinase does not solely cause pathogenicity or virulence. Results for these analyses can be found in Tables 2.13 to 2.17.

Table 2.13:	**Significant Alignments Between Virulence Databases and *B. fibrisolvens* ASCUSDY19**					
Organism	**Protein Hits to Victors**	**Protein Hits to VFDB**	**Protein Hits to PATRIC_VF**	**Protein Hits to Phi-Base**	**Pathogenicity Island Hits in IslandViewer**	**Hits to Proteins from Pathogens in PathogenFinder**
B. fibrisolvens ASCUSDY19	1	0	0	0	0	1

Table 2.14:	***B. fibrisolvens* ASCUSDY19 Hits to Pathogenic Genes in Victors**							
Source	**Source Organism**	**Gene**	**Product**	**Function**	**Subject Coverage**	**Query Coverage**	**identity**	**E-Value**
Victors	*Streptococcus pneumoniae TIGR4*	SP_1040	Site-specific recombinase	Phage Integration	12	100	88	2e-27

Table 2.15*:*	**PathogenFinder Results *B. fibrisolvens* ASCUSDY19**		
Gene Matches	**Proteins from Pathogens Matched**	**Proteins from Non-Pathogens Matched**	**Predicted as Human Pathogen?**
9	1	8	No

Table 2.16*:*	***B. fibrisolvens* ASCUSDY19 Hits to Pathogenic Genes in PathogenFinder**		
Gene	**Genbank Accession Number**	**Source Organism**	**Percent Identity**
Lactobacillales site-specific recombinase	CAW99778	*Streptococcus equi subsp. zooepidemicus H70*	90.1

Table 2.17*:* **Top BLASTp Hits to Site-specific Recombinase found in *B. fibrisolvens* ASCUSDY19 (excluding hits to organisms without standing nomenclature)**					
Organism	**Protein Name**	**Genbank Accession Number**	**Percent Identity**	**Query Coverage**	**Known Pathogen?**
Pseudobutyrivibrio xylanivorans	recombinase family protein	WP_072915090	100	100	no
Enterococcus cecorum	Hypothetical protein	KLO65182	99	100	Rare opportunistic pathogen
Peptoanaerobacter stomatis	hypothetical protein	EHL18418	92	100	yes, periodontal disease
Streptococcus pneumoniae	site-specific DNA recombinase	CVU12401	90	100	yes
Coprococcus comes	recombinase family protein	WP_147357729	89	100	no
Eubacterium rectale	recombinase family protein	WP_138305609	89	100	no

2.1.8.1 Section Summary

No genes directly involved in pathogenesis or toxin production were identified.

All publicly available pathogen and virulence-related databases were queried to determine the pathogenic potential of *B. fibrisolvens* ASCUSDY19. In total, these databases encompass 138,461 known pathogen-related genes spanning all microbial taxonomies. Comprehensive alignment of the *B. fibrisolvens* ASCUSDY19 genome to these databases yielded 1 hit above the 80% identity, 70% query coverage threshold. The single hit was to a site-specific recombinase that does not confer pathogenicity alone, and is found in pathogenic and non-pathogenic species alike. The analysis also included a search of 4,065 pathogenicity islands, 4 of which originated from *Butyrivibrio* species by the IsandViewer web interface. Additionally, database independent analysis using the PathogenFinder web interface was conducted. IslandViewer did not identify any pathogenicity islands. The same site-specific recombinase identified in the database alignment was also identified by PathogenFinder. Ultimately, PathogenFinder deemed that *B. fibrisolvens* ASCUSDY19 is not a pathogen.

2.1.9 Summary of Organism Safety Based on Genomics

B. fibrisolvens ASCUSDY19 was identified as a strain of *B. fibrisolvens* by 16S rRNA and whole genome analysis. *In vitro* antimicrobial susceptibility testing revealed *B. fibrisolvens* ASCUSDY19 is resistant to tetracycline, gentamicin, kanamycin, streptomycin, and erythromycin. The strain is susceptible to chloramphenicol, vancomycin, and ampicillin. Consistent with the *in vitro* antimicrobial resistance

data, *in silico* analyses revealed one antimicrobial resistance gene in the genome that plays a role in tetracycline resistance. Phenotypic testing confirmed that no antimicrobials were produced by *B. fibrisolvens* ASCUSDY19 during fermentation. Comparison of the *B. fibrisolvens* ASCUSDY19 genome to several databases containing known pathogenic-related genes revealed one protein hit. However, the identified recombinase does confer pathogenicity alone. Homologues of the recombinase are found in pathogens as well as non-pathogens indicating that the feature is not solely responsible for pathogenicity or virulence. Based on these analyses, *B. fibrisolvens* ASCUSDY19 is safe for use as a direct fed microbial.

2.2 Method of Manufacture

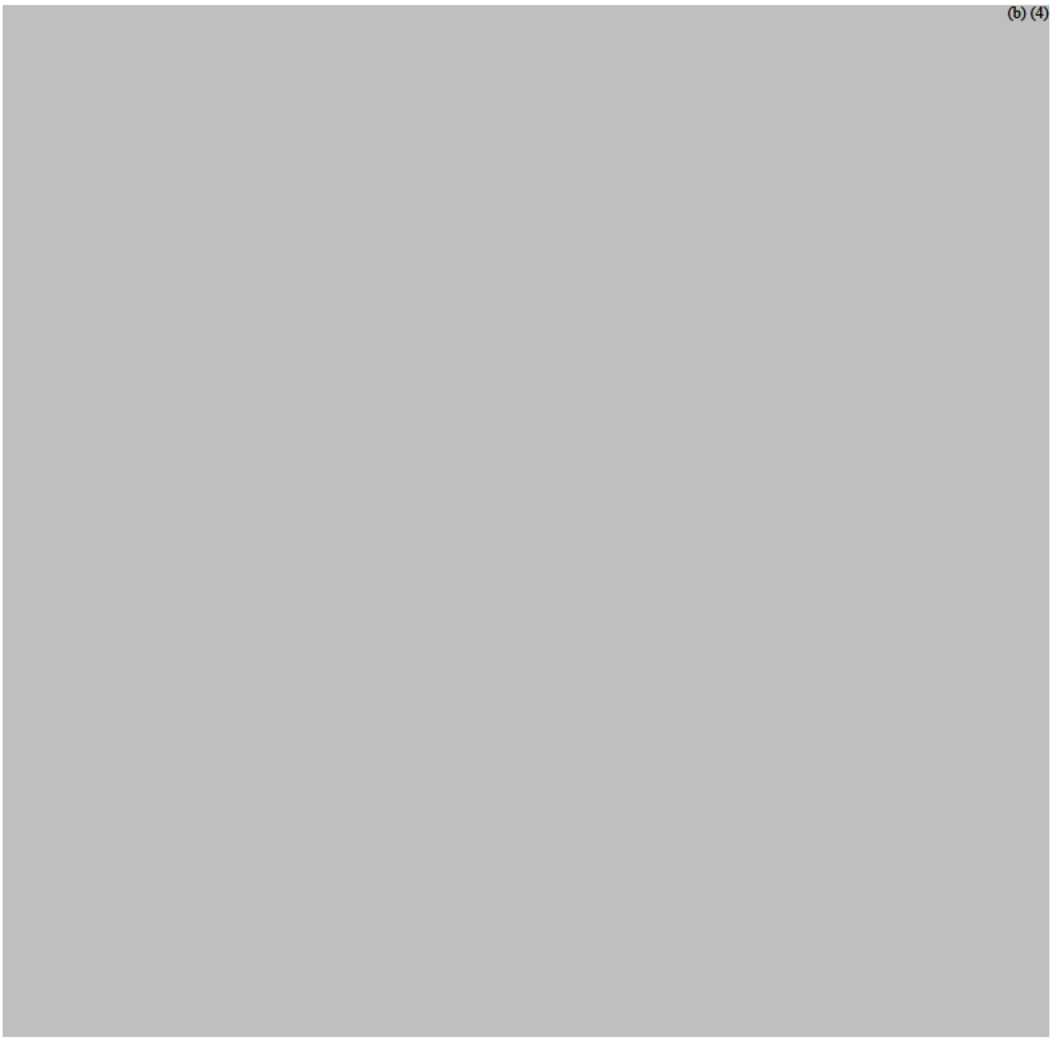
2.2.1 Raw Materials and Processing Aids

The raw materials and processing aids used in the manufacture of fat encapsulated *Butyrivibrio fibrisolvens* ASCUSDY19 are listed in Appendix 10. All raw materials used in the manufacture of *B. fibrisolvens* ASCUSDY19 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 *B. fibrisolvens* ASCUSDY19 is provided in Figure 2.5. *B. fibrisolvens* ASCUSDY19 is produced through a standard anaerobic dextrose fed-batch fermentation process. A working cell culture stock is maintained by Native Microbials and used for the seed fermentation. (b) (4) Details on the manufacturing process are provided in Appendix 10.

Figure 2.5: Schematic Overview of the Manufacturing Process



2.2.3 Production Controls

Commercial manufacture of *B. fibrisolvens* ASCUSDY19 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 §117 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

Appropriate feed-grade specifications have been established for the *B. fibrisolvens* ASCUSDY19 cell concentrate and are presented in Table 2.18. Copies of the methods of analysis are provided in Appendices 07 and 12.

Table 2.18: *B. fibrisolvens* ASCUSDY19 Concentrate Specifications		
Botulinum toxins	(b) (4)	**FDA BAM**

Abbreviations: BAM = Bacteriological Analytical Manual

2.3.2 Batch Analyses for the Cell Concentrate

Three batches of *B. fibrisolvens* ASCUSDY19 cell concentrate 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 2.19 and the Certificates of Analysis are provided in Appendix 13. No botulinum toxins were identified in any of the batches (Appendix 008).

Table 2.19: Analytical Results for 3 Batches of *B. fibrisolvens* ASCUSDY19 Concentrate					
			Analytical Results		
Parameter	**Unit**	**Specification**	**Lot 1801.2033**	**Lot 1801.2035**	**Lot 1801.2037**
Botulinum toxins*	**Per 2 g**	**Negative**	(b) (4)		

* Testing done at end of fermentation process

2.3.3 Proposed Product Specifications for the *B. fibrisolvens* ASCUSDY19 Freeze-dried Powder

Appropriate feed-grade specifications have been established for *B. fibrisolvens* ASCUSDY19 manufactured as a freeze-dried powder and are presented in Table 2.20. Copies of the methods of analysis are provided in Appendices 07 and 12.

Table 2.20: *B. fibrisolvens* ASCUSDY19 Freeze-dried Powder Specifications		
Parameter	**Specification Limits**	**Analytical Method**
Viable cell count	(b) (4)	**Internal Method**

Abbreviations: CFU = colony forming units. Internal Method Appendix 12C

2.3.4 Batch Analyses for *B. fibrisolvens* ASCUSDY19 Freeze-dried Powder

Three batches of *B. fibrisolvens* ASCUSDY19 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.21 and the Certificates of Analysis are provided in Appendix 013.

Table 2.21: Analytical Results for 3 Batches of *B. fibrisolvens* ASCUSDY19 Freeze-dried Powder

Parameter	Unit	Specification	Analytical Results		
			Lot 1801.2033	Lot 1801.2035	Lot 1801.2037
Viable cell count	CFU/g	> 1 x 10^9 CFU/g	(b) (4)		

Abbreviations: CFU = colony forming units.

2.3.5 Proposed Product Specifications for the *B. fibrisolvens* ASCUSDY19 Fat Encapsulated Product

Appropriate feed-grade specifications have been established for *B. fibrisolvens* ASCUSDY19 manufactured as a fat encapsulate and are presented in Table 2.22. Copies of the methods of analysis are provided in Appendices 07 and 12.

Table 2.22: *B. fibrisolvens* ASCUSDY19 Fat Encapsulate Product Specifications

Parameter	Specification Limits	Analytical Method
Viable cell count	(b) (4)	Internal Method
Coliform		AOAC 2018.13
E. coli		AOAC 2018.13
Salmonella		AOAC 2013.01
Listeria		AOAC 2013.10

Abbreviations: CFU = colony forming units; BAM = Bacteriological Analytical Manual; AOAC = Association of Official Analytical Chemists. Internal Method Appendix 12C

2.3.6 Batch Analyses for *B. fibrisolvens* ASCUSDY19 Fat Encapsulated Product

Three batches of *B. fibrisolvens* ASCUSDY19 representative of the commercial material were analyzed to verify that the manufacturing process produces a consistent product that complies with the proposed specifications. The results are summarized in Table 2.23 and the Certificates of Analysis are provided in Appendix 13.

Table 2.23: Analytical Results for 3 Batches of *B. fibrisolvens* ASCUSDY19 Fat Encapsulate					
			Analytical Results		
Parameter	Unit	Specification	Lot 1801.2033	Lot 1801.2035	Lot 1801.2037
Viable cell count	CFU/g	> 2 x 10^7 CFU/g	(b) (4)		
Coliform	CFU/g	<10			
E. coli	CFU/g	<10			
Salmonella	Per 25 g	Negative			
Listeria	Per 25 g	Negative			

Abbreviations: CFU = colony forming units.

2.3.7 Additional Analytical Data

The levels of heavy metals are also routinely monitored in batches of *B. fibrisolvens* ASCUSDY19. Three batches of *B. fibrisolvens* ASCUSDY19 representative of the commercial material were analyzed to verify that the levels of these contaminants fall within acceptable ranges. The results are summarized in Table 2.24 and the Certificates of Analysis from analytical laboratories are provided in Appendix 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 2.24: Further Analytical Results for 3 Batches of *B. fibrisolvens* ASCUSDY19					
		Analytical Results			Analytical Method
Parameter	Unit	Lot 1801.2033	Lot 1801.2035	Lot 1801.2037	
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 guarantee conformity of fat encapsulated *B. fibrisolvens* ASCUSDY19 to the product specification (see Table 2.22) for a minimum of 12 months when stored in the original, unopened packaging at refrigerated temperature (2 - 10°C). The proposed shelf life is supported through accelerated stability studies in which 3 batches of fat encapsulated *B. fibrisolvens* ASCUSDY19 representative of the commercial material were stored at 40°C, 50°C, and 60°C, respectively and analyzed through Arrhenius equation regression to represent real-time equivalents, using methods similar to those previously described (Wirunpan, Savedboworn, and Wanchaitanawong 2016; King, Lin, and Liu 1998) and generally accepted for accelerated shelf-life determination (Tang 2017). Packaging information is provided in Appendix 06.

2.4.1.1 Accelerated Stability Study at 40°C

The results of the stability study conducted at 40°C for 21 days on *B. fibrisolvens* ASCUSDY19 are summarized in Table 2.25 with decay rates plotted in Figure 2.6. The report is provided in Appendix 15.

Table 2.25: Results of a Stability Study on 3 Batches of *B. fibrisolvens* ASCUSDY19 Stored at 40°C

Time (Days)	Unit	Analytical Results					
		1801.2033		1801.2035		1801.2037	
		Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	(b) (4)					
3	CFU/g						
7	CFU/g						
14	CFU/g						
21	CFU/g						

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

2.4.1.2 Accelerated Stability Study at 50°C

The results of the stability study conducted at 50°C for 96 hours on *B. fibrisolvens* ASCUSDY19 are summarized in Table 2.26 with rates of decay plotted in Figure 2.6. The report is provided in Appendix 15.

Table 2.26: Results of a Stability Study on 3 Batches of *B. fibrisolvens* ASCUSDY19 Stored at 50°C							
		Analytical Results					
		1801.2033		1801.2035		1801.2037	
Time (Hours)	Unit	Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	(b) (4)					
8	CFU/g						
24	CFU/g						
48	CFU/g						
96	CFU/g						

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

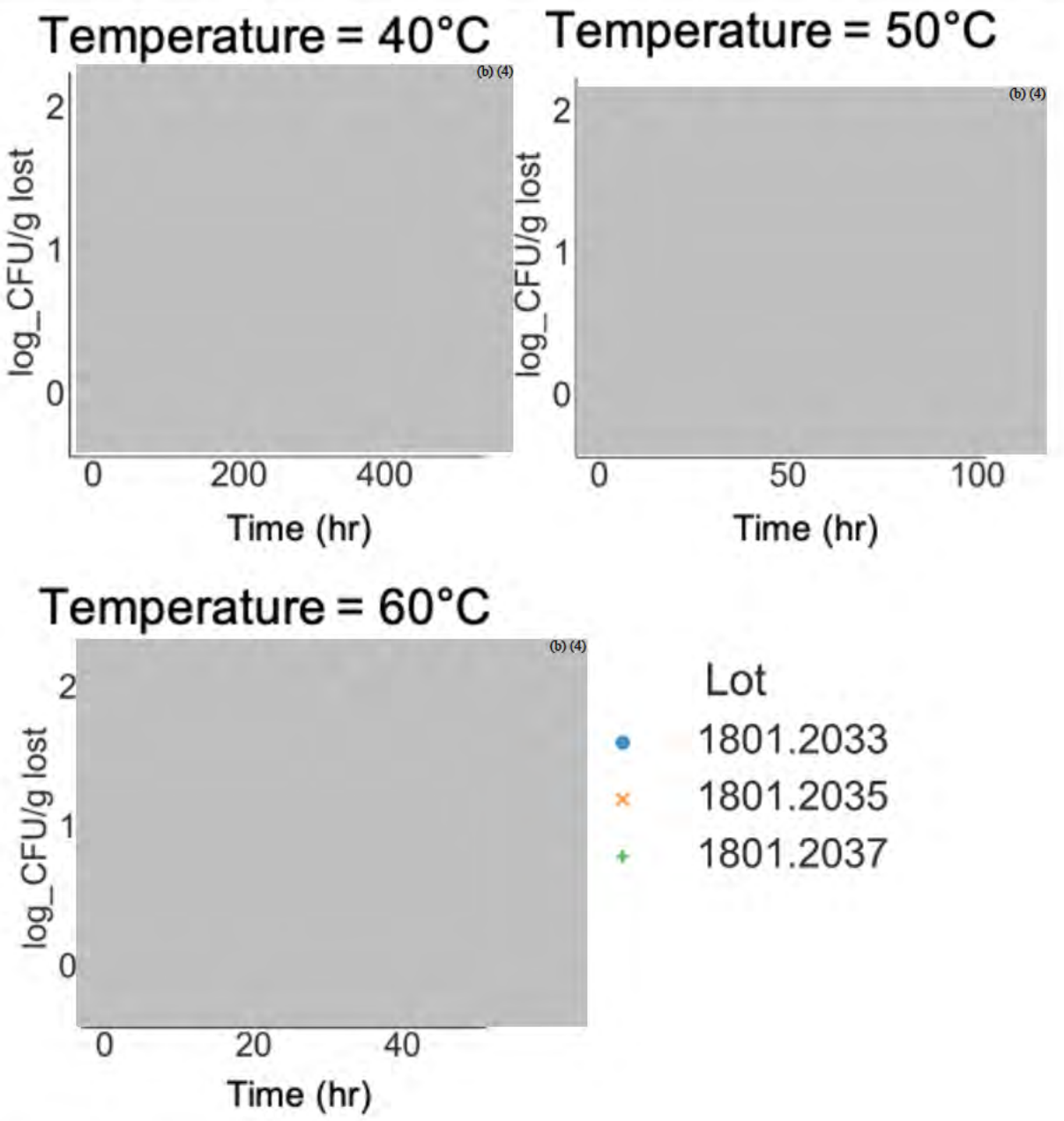
2.4.1.3 Accelerated Stability Study at 60°C

The results of the stability study conducted at 60°C for 48 hours on *B. fibrisolvens* ASCUSDY19 are summarized in Table 2.27 with decay rates plotted in Figure 2.6. The report is provided in Appendix 15.

Table 2.27: Results of a Stability Study on 3 Batches of *B. fibrisolvens* ASCUSDY19 Stored at 60°C							
		Analytical Results					
		1801.2033		1801.2035		1801.2037	
Time (Hours)	Unit	Viable Cells Count	SD	Viable Cells Count	SD	Viable Cells Count	SD
0	CFU/g	(b) (4)					
4	CFU/g						
8	CFU/g						
24	CFU/g						
48	CFU/g						

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

Figure 2.6 Rates of Decay at 40°C, 50°C, and 60°C. The decay over time is plotted for each lot at each temperature. A rate of decay was calculated from the slope of the regression, displayed as a dark line. The light shaded area represents the 95% confidence interval for the regression.



2.4.1.4 Shelf Life Prediction

Rates of decay for each lot at each temperature were calculated from the slope of decay over time. As described in the report (Appendix 15), the probability distributions of predicted rates of decay for the 3 batches at 40°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 at 10°C predicts a worst case of 2.48×10^{-6} Log CFU/day with

a minimum shelf life of 41,366 days among the 3 batches of *B. fibrisolvens* ASCUSDY19, confirming a one year shelf life based on accelerated data.

2.4.2 In-Feed Stability

As mentioned in Part 1, *B. fibrisolvens* ASCUSDY19 may be incorporated into the diet of dairy cattle as part of the TMR, as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. The strain is encapsulated with fat to generate a stable product suitable for handling under practical commercial farming conditions in the U.S. The dry matter intake of dairy cattle is optimized by feeding fresh TMR on a twice daily basis. The forage content is typically adjusted to meet the nutrient requirements of the animals on a pen basis. Under the conditions of intended use, *B. fibrisolvens* ASCUSDY19 may be mixed directly into the TMR or added as a top-dressing at the point of use. On this basis, long-term stability is not relevant, and an in-feed stability study was not conducted.

2.4.3 Homogeneity Data

Due to the highly similar manufacturing process and ensuing encapsulated cell size, the powder attributes, formula, particle size and moisture content (see Appendix 11) of the commercial offering of *B. fibrisolvens* ASCUSDY19 was noted to be nearly identical to that described in a recent prior submission (AGRN 38, 2020) and therefore a separate homogeneity study was deemed unnecessary.

2.4.4 Manufacturing Summary

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

2.5 Effect of the Notified Substance

This portion of the notice addresses the requirements specified in 21 CFR 570.230(d):

> (d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such effect.

The GRAS Final Rule (81 FR 54960) provides interpretation of this regulation specific to animal feed ingredients in response to comment 144: "We agree that data and information bearing on the physical or other technical effect the notified substance is intended to produce are only necessary when they bear on safety." A product like phytase would require data, however, the intended purpose of supplementation of *B. fibrisolvens* ASCUSDY19 is to augment normal rumen digestion. As described below, Native Microbials has determined that the technical effect of *B. fibrisolvens* ASCUSDY19 when fed to dairy cattle as a direct fed microbial under the conditions of intended use does not have a bearing on safety. Thus, data and information demonstrating the intended effect of *B. fibrisolvens* ASCUSDY19 in the feed of dairy cattle are not required as part of this GRAS notice.

The use of this organism is to facilitate the digestion of degraded fibrous plant material and ferments polysaccharides (Hespell, Wolf, and Bothast 1987). The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour *et al*., 2015), and is further described below in context of technical effect and animal safety (Part 6.4 of this notice).

Supplementation of dietary fibrolytic enzymes could improve DMI and milk production has also been reported (Rode et al., 1999). As a commensal microorganism, feeding *B. fibrisolvens* would have no impacts on animal health. Should *B. fibrisolvens* not degrade fibrous plant material and ferment polysaccharides, there would be no safety impact, as the other rumen microorganism will continue fermentation, and the feed was formulated to assure nutrient requirements were met without consideration of the potential for increased digestion of feed.

2.5.1 Rumen Microbiome

The most recent authoritative text on the nutrition of major ruminants (NRC, 2016), states that the rumen is a "complex dynamic anaerobic ecosystem." The dynamics of the microbial community arises from variability introduced by feed source, the environment, and physiological state impacts the microbiome (Xue et al. 2018). Experts (NRC, 2016) note that diurnal shifts of a full pH unit are not uncommon, and this can significantly impact the microbial population. The rumen microbial population is well adapted to these standard diurnal shifts in the rumen environment and continue to serve the function of digestion of feed despite these changes (NRC, 2016). This ability to rapidly adapt is due in part to the rumen microbiome's ability to utilize specialized enzymes and enzyme complexes to convert feed components to end products of digestion and microbial cells (NRC, 2016). It is this specific understanding that Native Microbials uses in their identification of existing, commensal microorganisms in the rumen of high producing ruminants. Particularly, understanding of their unique enzymatic properties and physiology support the selection and use of them as DFMs.

Several studies have linked the rumen microbiome profile to animal performance and digestibility (Lima et al. 2015; Jami et al. 2013; Kumar et al. 2015). The rumen microbiome is highly variable depending on several factors including age, breed, diet composition, time after feeding, season, stage of lactation, location, and farm management practices (Pitta et al. 2016; Furman et al. 2020; Henderson et al. 2015). Additionally, there are groups of microorganisms that are unique to particular breeds of cow (i.e., Jersey or Holstein), regions, and individual animals that further increase the inherent complexity of the microbial community native to the rumen. Diet, in particular, has been shown to be the main driver of microbiome composition (Ghaffari et al. 2014). To better study the microbiome in context of this variability, many studies have focused on identifying and characterizing the core rumen microbiome (Petri et al. 2013; Xue et al. 2018; Henderson et al. 2015; Furman et al. 2020; Kumar et al. 2015; Jami et al. 2013; Lima et al. 2015; Fouts et al. 2012). The concept of core microbiome, a common assemblage of microorganisms that exists in or is associated with a specific habitat, was first introduced and applied to differentiate human microbiomes associated with healthy and diseased conditions (Turnbaugh et al. 2009; Turnbaugh and Gordon 2009; Turnbaugh et al. 2007). Since then, core microbiomes have been identified in a broad spectrum of environments including agroecosystems, monogastric animals, and ruminants (Shade and Handelsman 2012; Yeoh et al. 2017; Toju et al. 2018; B. A. Lowe et al. 2012; Dougal et al. 2013).

There is a core microbiome that appears in the majority of dairy cows that provides the basal level of fermentation required for animal survival. Although the results are variable at times and defining a "normal healthy" rumen is challenging, there are several phyla that tend to appear across all ruminants. Henderson *et al*. (2015) reported 32 different species of ruminants globally shared a core assembly of rumen bacteria. Xue *et al*. (2018) demonstrates that individual animals within a large cohort of dairy cattle with similar genetics, diet, environment, and management can have significant differences in their rumen microbiome species. The core microbiome identified included microorganisms from over 391 genera covering 26 phyla. The microorganisms unique to individual animals (termed "pan microbiome") along with the core microbiome dictated the variability in rumen fermentation and production. Consistent with other studies (Jami et al. 2013; Jami and Mizrahi 2012; Lima et al. 2015; Deusch et al. 2017; Huws et al. 2018; Xue et al. 2018), members of Bacteroidetes, Firmicutes, Proteobacteria, and Fibrobacteres were among the topmost abundant bacteria identified regardless of animal origin and diet.

As more rumen microbiomes were studied, it became clear that diet was the major determinant of observed microbiome differences (Johnson and Johnson 1995; Brulc et al. 2009; Carberry et al. 2014; Deusch et al. 2017; Alejandro Belanche et al. 2019; Kumar et al. 2015; Mizrahi and Jami 2018). This indicates the direct impact of diet on rumen microbial populations. Hence, modifying either diet or microbiome could influence the rumen fermentation process (Moraïs and Mizrahi 2019; Furman et al. 2020; A. Belanche et al. 2012). *B. fibrisolvens* has been fed to ruminants as well as monogastrics. In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported. Furthermore, *B. fibrisolvens* has been utilized as a probiotic in mice, being analyzed for its CLA production (Fukuda et al. 2006) and potential for tumor reduction (Ohkawara et al. 2007) Both studies reported that *B. fibrisolvens* had positive impacts on the health of the mice in the studies and reported no adverse health effects of administration. A strain has also been tested as an aspect of a dietary study in rats to increase intestinal production of short-chain volatile fatty acids (Nielsen et al. 2016). Similarly, this study also did not report any adverse health impacts of *B. fibrisolvens.* Although this species is not commercially available and has not seen widespread application in feed, academic and scientific research has shown that there are no adverse effects when *B. fibrisolvens* is fed to animals, thus it is unlikely that this organism is dramatically altering rumen fermentation processes. The intent of feeding DFMs, particularly *B. fibrisolvens* ASCUSDY19, is to improve the nutrient availability from feed. Feeding *B. fibrisolvens* ASCUSDY19 to dairy cattle supplements the existing populations of *B. fibrisolvens* ASCUSDY19 in the rumen, and ultimately provides additional nutrient availability to the animal. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. This notice includes a more detailed discussion of the core microbiome and microbiome safety in Part 6.4 of this GRAS notice.

2.5.2 Impact of Failure of the Notified Substance

If this product fails, that is, the product fails to enhance feed digestibility in the rumen, there would not be a safety concern with respect to the animal's health or nutrition. The notified substance increases the digestion of carbohydrates by acting upon the existing feed within the rumen. The diet offered to the animal would be formulated to meet the existing nutritional needs of the animal (NRC, 2001). Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients.

Several published experiments have directly investigated the impacts of DFMs by comparing groups of animals receiving a "dead" microbial against a variety of treatment conditions. Cunha, *et al*. (2019) compared heifers fed a basal diet against heifers fed the same basal diet containing a live yeast or inactive yeast supplement (2 different doses) in a 5x5 Latin square experimental design with 15-day periods. Live and dead yeasts were administered to the appropriate animals after each feeding through infusion directly into the rumen. No differences in digestibility were observed between the control, live yeast, or either of the inactive yeast doses. No differences were observed in feed intake nor animal behavior. Hence the inactive yeast did not alter the overall digestion of the feed, nor impact the health of the animals. Feeding inactive yeast did not decrease rumen function.

Muscato, *et al*. (2002) evaluated the feeding of fresh and inactivated rumen fluid to calves in a series of four experiments. The animals were dosed daily with 8 mL of either fresh or inactivated rumen fluid obtained from a cannulated Holstein cow from 0-6 weeks of age. In the first experiment, calves were either fed a typical basal ration or the same basal ration supplemented with fresh rumen fluid. In the second experiment, calves were fed the basal ration with either the cell pellet of fresh rumen fluid, supernatant of fresh rumen fluid, or no addition. In the third experiment, calves were fed a basal ration, or a basal ration supplemented with autoclaved rumen fluid. Autoclaving rumen fluid ensures microbial death, thus inactivating the biological component. The fourth experiment had a similar set-up to the third experiment, but rumen fluid was only fed for 5 days rather than 6 weeks. In the studies that evaluated autoclaved rumen fluid, the number of days of scouring were significantly decreased compared to the control. Similarly, the calves receiving autoclaved rumen fluid experienced higher gains in the first two weeks, but by the end of the experimental period there was no impact on growth. There were no differences in the outcomes of calves receiving fresh rumen fluid as compared to calves receiving autoclaved rumen fluid. This study suggests that the feeding of inactivated microorganisms does not decrease rumen function or create a safety concern when fed to animals.

The contribution of members of *Butyrivibrio*, specifically, to the fermentation characteristics of the rumen has been evaluated in the published literature. In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly

to the high-grain diet and no negative health effects relating to microbial supplementation were reported.

Philippeau, *et al.* (2017) fed multiple DFM treatments to investigate the effects of DFM on rumen fermentation characteristics and digestibility. Animals were assigned one of four treatment groups: control (CON), *Propionibacterium* P63 (P63), *Propionibacterium* P63 and *Lactobacillus plantarum* 115 (P63+Lp), or *Propionibacterium* P63 and *Lactobacillus rhamnosus* 32 (P63+Lr). Each strain was administered at 10^{10} cfu/d. No change in ruminal VFA concentration was observed, and only P63 was found to impact the concentration of some milk fatty acids. pH increased on average 0.18 units in all DFM groups as compared to the control. Although the study did not demonstrate the positive response in performance as was expected, there was no negative change in the assessed parameters that may suggest a decrease in health. Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight, Linn, and Jung 2007; Abu-Tarboush, Al-Saiady, and Keir El-Din 1996; Higginbotham and Bath, 1992; McGilliard and Stallings 1998). In Weiss *et al*. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette *et al*. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette *et al*. (2007), *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman et al. 2011; Hagg et al. 2010; Zebeli et al. 2012; Kung and Hession 1995). A *Lactobacillus*-based probiotic fed alone and in combination with *S. cerevisiae* showed no change in milk production or efficiency in early-lactation dairy cows (Boga and Gorgulu 2007). In a meta-analysis conducted at INRA, 33 probiotic bacteria studies with or without yeast were evaluated for their impact on the production and health of dairy and beef cattle (Lettat et al. 2012). Variable performance and rument impacts were observed, however the study indicated no negative health consequences were reported. In the studies summarized above, even though the direct fed microbials did not achieve the performance response expected, there was no indication of a safety concern.

In these examples, failure of DFM supplementation or the DFM itself did not cause any harm to the fermentation characteristics of the rumen or animal well-being. In the case of *B. fibrisolvens* ASCUSDY19, if the DFM failed to provide improved digestibility, rumen fermentation of treated cows would be identical to rumen fermentation of untreated cows. Since no alterations are made to the standard feeding regime when using this product, the value of the feed that would be digested and utilized for the nutrients required to sustain life is identical between the control and treated group. Animals would be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001). Any non-performing *B. fibrisolvens* ASCUSDY19 or deceased *B. fibrisolvens* ASCUSDY19 would pass through the GI tract with the normal flow of digesta, providing nutrients for absorption by the animal (NRC, 2016).

In this respect, based on the results of published comparative studies, *B. fibrisolvens* ASCUSDY19 will act only to support normal ruminal function of digestion of animal feed. Like other DFMs, while *B. fibrisolvens* ASCUSDY19 may aid the digestion of feed, the effect is not required for the general well-being and normal performance of dairy cattle. Thus, the absence of the anticipated effect of *B. fibrisolvens* ASCUSDY19 on feed digestion by dairy cattle would not have an impact on safety. Native Microbials product labeling does not suggest a change in normal feeding regime, and its use would be specific for gaining additional nutritional value from a typical balanced ration. Animals would continue to be fed rations that meet established nutrient requirements as recommended by the NRC for dairy cattle (NRC, 2001).

2.5.3 Summary

In summary it is Native Microbials' understanding that the regulatory hurdle provided in §570.230(d), is not applicable to the conclusion of the generally recognized as safe substance *B. fibrisolvens* ASCUSDY19, that is "failure" of the intended use will not raise a safety concern, as the intended use is to provide increased nutritive value from nutritionally adequate feeds. As such, failure would result in typical nutrient availability of the diets, as they have been formulated to meet the nutritional requirements of the animal. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. Therefore, there is no regulatory requirement to provide specific utility data to support the intended use.

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, *B. fibrisolvens* ASCUSDY19 is intended for use as a source of viable microorganisms in feed for dairy cattle. The microbial strain will be delivered as a fat encapsulated direct fed microbial to dairy cattle either alone or in combination with other microbial strains. Examples of the conditions under which direct fed microbial products containing *B. fibrisolvens* ASCUSDY19 may be incorporated into the diet of dairy cattle include as part of the TMR, as top-dressing to individual feeds or the daily ration, and as a component of a feed supplement. The product will be incorporated into dairy cattle feed at the recommended use level of $1x10^8$ CFU of *B. fibrisolvens* ASCUSDY19/cow/day. As mentioned in Part 2.2, the fat encapsulated product is comprised of approximately 30% sodium sulfate, 50% hydrogenated glycerides and 20% freeze-dried *B. fibrisolvens* ASCUSDY19 powder. Thus, under the conditions of intended use, dairy cattle will be exposed to maximum 1 g of the *B. fibrisolvens* ASCUSDY19.

3.1.2 Exposure to the Other Components of the Fat Encapsulated Product

At the intended intake of $1x10^8$ CFU *B. fibrisolvens* ASCUSDY19/cow/day, the animal will be exposed to up to 5 g of the notified substance (min. $2x10^7$ CFU/g). The product is comprised of approximately 30% sodium sulfate, 50% hydrogenated glycerides and 20% freeze-dried *B. fibrisolvens* ASCUSDY19 powder (see Appendix 010). As mentioned in Part 2, the amount of hydrogenated glycerides, sodium sulfate, and freeze-dried *B. fibrisolvens* ASCUSDY19 powder is adjusted for each batch to standardize the viable cell count. These encapsulation ingredients are acceptable for use in dairy cattle feed and comply with the corresponding ingredient definitions in the AAFCO Official Publication (AAFCO 2020; ingredient definitions 33.19 and 57.106 - see Appendix 011). Under these conditions of use, the animal will be exposed up to a maximum of 2.5 g of hydrogenated glycerides and 1.5 g of sodium sulfate. Considering that the typical dry matter intake by the dairy cattle will be about 25 kg/cow/day, the contribution of hydrogenated glycerides to the dairy ration is expected to be no more than 0.006% DM. While the fat concentration of a typical dairy diet is reported to be relatively low (approximately 2.5% DM), supplemental fats can be added to achieve a total ration content of around 6% DM (MSD Veterinary Manual, 2019). On this basis, the use of hydrogenated glycerides or similar acceptable fat source as an encapsulating aid in the manufacture of fat encapsulated *B. fibrisolvens* ASCUSDY19 will have a negligible impact on the total fat intake by dairy cattle under the conditions of use. Similarly, an intake of 1 g/cow/day of sodium sulfate will provide dairy cattle with approximately 0.48 g of sodium/cow/day, representing less than 0.004% of the DM intake. The maximum tolerable levels of sodium chloride set by the National Research Council (NRC) for lactating cows is 3% of DM intake, equivalent to around 1% DM of sodium. Thus, the use of sodium sulfate as an encapsulating agent in the manufacture of fat encapsulated *B. fibrisolvens* ASCUSDY19 is not expected to have any significant impact on the overall sodium intake by dairy cattle under the intended conditions of use. Another element of interest is sulfur. The use of *B. fibrisolvens* ASCUSDY19 would provide approximately 1 g of sodium sulfate or 0.34 g of sulfur per day. The NRC (2005) has suggested that

Total Mixed rations (grain based) of cattle diets should be at a maximum tolerable level of 0.3% sulfur (75 g/cow/day), as such this ingredient would provide an insignificant amount of the total sulfur in the diet of the dairy cow.

3.1.3 Background Exposure to the Microorganism

As mentioned in Part 2, the strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and in this respect, *B. fibrisolvens* ASCUSDY19 will contribute to the native population of *Butyrivibio* species in the gut of the animal (see Part 6.4). *B. fibrisolvens* is part of the rumen microflora and is routinely isolated from livestock feces and rumen content (Henderson et al. 2015; Li et al. 2012; Petri et al. 2013; Asanuma, Kawato, and Hino 2001; Balamurugan et al. 2009; Vasta et al. 2010; Moore and Holdeman 1974; Cheng et al. 1969; Brown and Moore 1960; Bryant and Small 1956; Sundset et al. 2009; Forster et al. 1996). The species has also been isolated from bioreactors that were fed grasses that are commonly used as livestock feed (Sewell et al. 1988). Thus, while not present to a significant or intentional degree in feedstocks, background exposure by dairy cattle to *B. fibrisolvens* from the environment is likely to be significant.

3.2 Human Exposure

B. fibrisolvens ASCUSDY19 is intended for use as a supplemental source of viable microorganisms in the feed of dairy cattle. As mentioned in Part 2.1, the strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and in this respect, *B. fibrisolvens* ASCUSDY19 will contribute to the native ruminal population of *Butyrivibrio* species (see Part 6). No transfer of viable *B. fibrisolvens* ASCUSDY19 from the rumen to milk or other edible tissues is anticipated.

The strain has been unambiguously characterized as *B. fibrisolvens* and whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2.1). As a consequence, there should be no transfer of pathogenicity or toxigenicity to milk or edible tissues through the use of *B. fibrisolvens* ASCUSDY19 as a source of viable microorganisms in the feed of dairy cattle.

No withdrawal period is considered necessary on the basis that *B. fibrisolvens* ASCUSDY19 is native to the rumen of dairy cattle and as detailed in Part 6, and the strain has been shown to have no pathogenic or toxigenic properties.

PART 4 – SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with *B. fibrisolvens* ASCUSDY19.

PART 5 – EVIDENCE BASED ON COMMON USE BEFORE 1958

Not applicable.

PART 6 – NARRATIVE

The conclusion that *B. fibrisolvens* ASCUSDY19, as described herein, is GRAS under the conditions of intended use as a direct fed microbial in feed for dairy cattle is based on scientific procedures using product-specific characterization data on the microbial strain together with a body of published information on the prevalence and potential pathogenicity and toxigenicity of the *Butyrivibrio* species.

As mentioned in Part 1.3, fat encapsulated *B. fibrisolvens* ASCUSDY19 will be provided to dairy cattle either alone or in combination with other direct fed microbials. The strain was isolated from the rumen content of a healthy mid-lactation Holstein cow and is intended as a source of commensal microorganisms. In this respect, *B. fibrisolvens* ASCUSDY19 will contribute to the native microbial population in the rumen and the functionality of the direct fed microbial strain is considered in Part 6.1.

The safety of *B. fibrisolvens* ASCUSDY19 for use as a direct fed microbial for dairy cattle is evaluated according to the guidelines developed by (Pariza et al. 2015). These guidelines are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial cultures for consumption by humans and animals (AAFCO, 2019). In accordance with these guidelines, the safety of a microorganism without an extensive history of use in food or feed is primarily addressed by evaluating the pathogenic and toxigenic potential. In order to understand the pathogenic and toxigenic potential, the microbial strain must be fully characterized and the body of knowledge pertaining to safety based on its taxonomic unit considered. Full details of the characterization of *B. fibrisolvens* ASCUSDY19 are detailed in Part 2. The microorganism has been unambiguously characterized as *Butyrivibrio fibrisolvens* (see Part 2.1.4). Furthermore, whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2.1.8). Whole genome sequence analysis together with phenotypic testing indicate that *B. fibrisolvens* ASCUSDY19 is susceptible to antimicrobials and should not increase the risk of transfer of resistance to other microorganisms (see Part 2.1.5 and 2.1.6). Testing also confirms *B. fibrisolvens* ASCUSDY19 does not produce antimicrobial substances (see Part 2.1.7 and Appendix 005).

In addition to the characterization data, a body of information is available in the public domain pertaining to (a) the identity of *B. fibrisolvens* (see Part 6.2); (b) the history of exposure of the species by animals and humans (see Parts 6.4 and 6.5); and (c) the potential for toxigenicity and pathogenicity (see Part 6.6). Following the decision tree established by Pariza *et al*. (2015), these data are pivotal to the safety evaluation of *B. fibrisolvens* ASCUSDY19 and are summarized below. The Pariza *et al*. (2015) decision tree that outlines the safety evaluation is provided in Appendix 016.

6.1 Functionality

The microbial population of the rumen plays an important role in the utilization of feed by dairy cattle. Manipulation of rumen microbiota by dietary supplementation with sources of viable microorganisms is common practice in the dairy cattle industry in the U.S. in order to facilitate fermentation and contribute to the general digestive health of the animal (Yoon and Stern 1995; Chaucheyras-Durand and Durand 2010; Abd El-Tawab et al. 2016). The contribution of bacteria to the

fermentation characteristics of the rumen have been extensively evaluated in the published literature, with important functions reported to be stabilization of the rumen pH, increase in volatile fatty acid production, reduction in ammonia concentrations, improved microbial protein synthesis and fiber digestibility (*e.g.*, (McAllister et al. 2011; Nocek et al. 2002; Henning et al. 2010; Krehbiel et al., 2003; Qiao et al. 2010; Weinberg et al. 2007; Jeyanathan et al. 2019; Yoon and Stern 1995). As mentioned in Part 2, *B. fibrisolvens* ASCUSDY19 was isolated from the rumen content of a healthy mid-lactation Holstein and is expected to contribute in the same way as other bacteria to digestion and metabolism in the ruminal environment.

In particular, *B. fibrisolvens* was shown to degrade fibrous plant material and ferment polysaccharides (Hespell, Wolf, and Bothast 1987). It utilizes various carbon sources including simple carbohydrates (e.g., glucose and fructose), reducing sugars derived from plant materials such as xylose and cellobiose, glucosides derived from plant materials such as salicin and esculin, and starch (see Part 2.1). Similar phenotypes are reported in the published literature for other *B. fibrisolvens* strains (M. Cotta and Forster 2006; Hespell, Wolf, and Bothast 1987; Marounek and Petr 1995; M. A. Cotta 1992, 1988; VAN Gylswyk et al. 1996; Emerson and Weimer 2017). Additionally, many strains of the species degrade protein and pectin (M. A. Cotta and Hespell 1986; Sales, Lucas, and Blanchart 2000; M. Cotta and Forster 2006; Marounek and Duskova 1999; Gradel and Dehority 1972). Co-culture experiments have demonstrated that *B. fibrisolvens* is capable of degrading a variety of feedstuffs including barley, sorghum, wheat, lucerne, and cotton stalks (Ben-Ghedalia, Miron, and Solomon 1993; J. Miron and Ben-Ghedalia 1992; J. Miron 1991; J. Miron and Ben-Ghedalia 1993; Joshua Miron and Ben-Ghedalia 1993). Thus, the microorganism has the potential to support digestion by aiding fermentation of forages and partially degraded digesta in the rumen.

Similar to other *B. fibrisolvens* strains, *B. fibrisolvens* ASCUSDY19 has been shown to utilize a range of monosaccharides including glucose, fructose, and xylose to produce relatively high levels of butyrate with lower amounts of acetate, formate, and lactate (Emerson and Weimer 2017; Hespell, Wolf, and Bothast 1987). While butyrate is generally favored, there is some intraspecies heterogeneity and differing growth conditions may result in higher acetate or lactate production (Shane, Gouws, and Kistner 1969; Diez-Gonzalez et al. 1999; Hespell, Wolf, and Bothast 1987; Paillard et al. 2007). Fermentation of pectin by *B. fibrosolvens* generally yields higher proportions of acetate to butyrate/lactate (Marounek and Duskova 1999).

Volatile fatty acids (VFAs) are the main source of energy in ruminants and are produced predominantly through microbial fermentation of feed in the rumen (Bergman 1990; NRC 2007). The three major VFAs produced by anaerobic microbial fermentation in the rumen are acetate, propionate and butyrate with the relative ratios largely depending on the nature of the feed. The VFAs are readily absorbed and utilized by ruminants accounting for up to 80% of their maintenance energy requirements. Butyrate in particular acts as the major energy source for epithelial cells in ruminants and is recognized to play an important role in maintaining colonic health in the animal. Studies have also linked butyrate to the development of rumen papillary and calf gastrointestinal tracts (Weigand, Young, and McGilliard, 1974; Górka et al. 2018). Seymour, Campbell, and Johnson (2005) reviewed

the results of 20 studies evaluating the relationship between VFAs and production-related variables in Holstein cows. The strongest associations identified were between rumen butyrate concentration, which displayed a significant positive linear regression relationship with dry matter intake, and milk yield. The authors concluded that butyrate indirectly supports the milk yield and production although the relationship is complex. Similarly, a number of other studies in the published literature indicate that butyrate can support the general production performance of the animals (Rook and Balch 1961; Huhtanen, Miettinen, and Ylinen 1993; Miettinen and Huhtanen 1996). Similarly, acetate is considered essential for milk fat production and low levels of rumen acetate have been reported to lead to depressed milk fat content (Bergman 1990; Gäbel, Aschenbach, and Müller 2002; Aluwong, Kobo, and Abdullahi 2010). The role of VFAs as energy sources for dairy cattle also is supported by the existing food additive listing for the ammonium or calcium salts of isobutyric acid, iso-valeric acid, 2-methylbutyric acid and n-valeric acid as sources of energy in dairy cattle feeds under 21 CFR §573.914.

B. fibrisolvens is known to possess proteolytic activity (M. A. Cotta and Hespell 1986; Sales, Lucas, and Blanchart 2000). It is estimated that 30-50% of all ruminal isolates possess proteolytic capability (Fulghum and Moore 1963; Prins, van Rheenen, and van't Klooster 1983). Microbial protein degradation is an important ruminal process needed to break down proteins into smaller peptides and free amino acids that support the rumen microbiota and supply free amino acids to the host (Tamminga 1979; Bach, Calsamiglia, and Stern 2005). Upon degradation, microbes use the liberated amino acids for growth and subsequent VFA production or for protein synthesis (Bach, Calsamiglia, and Stern 2005; Argyle and Baldwin 1989; Regueira et al. 2020). Microbial protein synthesis in the rumen accounts for an estimated 50-80% of all absorbable protein supplied to the small intestine of dairy cows (Storm and Ørskov 1983; Clark, Klusmeyer, and Cameron 1992)

B. fibrisolvens has been reported to perform the metabolic hydrogenation of unsaturated fatty acids, also known as biohydrogenation (Polan, McNeill, and Tove 1964; Kepler et al. 1966; Maia et al. 2010; Shivani et al. 2016). Biohydrogenation is the process of bacteria converting unsaturated fatty acids into saturated fatty acids. One major metabolite that *B. fibrisolvens* produces from this process is *cis*-9 *trans*-11 conjugated lineoleic acid (CLA) (Kepler et al. 1966; McKain, Shingfield, and Wallace 2010). Some isomers of CLA have been implicated in the process of milk fat depression in dairy cows, specifially *trans*-10 *cis*-12 CLA, which is not produced by *B. fibrisolvens* (Baumgard et al. 2000). In humans, CLA is an FDA approved supplement that is considered to be an antioxidant and an aid to weight loss, though studies present conflicting results (den Hartigh 2019).

Taken together, these examples of the potential functionality of *B. fibrisolvens* in the rumen support the proposed role of *B. fibrisolvens* ASCUSDY19 as a source of viable microorganisms in the diet to positively influence the production of VFAs and general colonic health of the animals. While *B. fibrisolvens* ASCUSDY19 may contribute to the native population of *Butyrivibrio* species in the gut of the animal, the technical function has no bearing on the safety when used as a direct fed microbial in feed for dairy cattle. Should *B. fibrisolvens* ASCUSDY19 fail, other members of the existing rumen microbiome will continue to ferment feed, thus supplying the animal with sufficient nutrients. On this

basis, no further demonstration of the technical effect (utility) of *B. fibrisolvens* ASCUSDY19 was required for the safety evaluation (see Part 2.5).

6.2 Identity

The genus *Butyrivibrio* consists of motile, anaerobic curved rods that gram stain negative, but maintain gram positive structure. Members of the genus ferment glucose or maltose with butyrate as the major fermentation product (Anne Willems and Collins 2015). Heterogeneity exists amongst species in regards to fermentation of additional carbon sources, and fermentation products may differ due to species-specific metabolism, with some members of the genus favoring the production of lactate, acetate, or formate (M. Cotta and Forster 2006; Anne Willems and Collins 2015). 16S phylogeny has placed the genus in the *Clostridium* XIVa cluster. The genus is polyphyletic, with three distinct lineages and 12 identified rRNA subtypes (A. Willems, Amat-Marco, and Collins 1996; Anne Willems and Collins 2015; Forster et al. 1996).

B. fibrisolvens was the only species proposed at the time of the genus description, and while diversity was noted amongst isolates, many were identified as *B. fibrisolvens* based solely morphology and phenotype (Bryant and Small 1956). Due to initial phenotype based taxonomic classification, strains of *B. fibrisolvens* are more diverse genetically that what is typically seen between strains of a species, with G+C mol% between 39%-49.2% and 16S rRNA sequence similarity as low as 88% between strains (Mannarelli 1988; Mannarelli et al. 1991; Forster et al. 1996). As previously noted, the genus *Butyrivibrio* is polyphyletic, with species in the genus spread across three phylogenetically distinct clusters. The species *B. fibrisolvens* itself is split between two of the three clusters. *Butyrivibrio* group 1 consists of the *B. fibrisolvens* type strain, strains from *B. hungatei,* and other species from the genus *Clostridium.* Group 2, also known as the *Pseudobutyrivibrio* group consists of species of *B. fibrisolvens* and species from the genus *Pseudobutyrivibrio*. Group 3 consists of *B. crossotus* and similar species (Anne Willems and Collins 2015).

6.3 Literature Search

A comprehensive literature search was conducted in order to identify all publicly available information pertaining to the safety of *B. fibrisolvens* for the intended use as a source of viable cells for dairy cows. Results can be found in Appendix 17.

6.4 Natural Occurrence

6.4.1 Prevalence in Animals

B. fibrisolvens is ubiquitous in nature and has been isolated from rumen content of cattle, deer, sheep, goats, bison, camels, and giraffes, as well as fecal samples from horses, rabbits, dogs, cats, and humans (Asanuma, Kawato, and Hino 2001; Balamurugan et al. 2009; Vasta et al. 2010; Moore and Holdeman 1974; Cheng et al. 1969; Brown and Moore 1960; Bryant and Small 1956; Sundset et al. 2009; Forster et al. 1996; Henderson et al. 2015)

A total of 9 different strains of *B. fibrisolvens* have been isolated, sequenced, and analyzed in the JGI genome portal to date (https://genome.jgi.doe.gov/portal/), and 11 strains in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The Global Rumen Census found that the *Butyrivibrio* genus had a mean relative abundance of 3.4% in the rumen (Henderson et al. 2015),

while several other studies put the relative abundance of *Butyrivibrio fibrisolvens* near 1% (Li et al. 2012; Petri et al. 2013). Species in the *Butyrivibrio* genus were found in 100% of samples across 742 samples taken from 32 animal species in 35 countries (Henderson et al. 2015). Thus, *Butyrivibrio* and *B. fibrisolvens* are highly prevalent as commensal organisms of the rumen microbial ecosystem.

6.4.2 Microbiome Safety

The rumen microbiome is crucial for the digestion of feed and supplies necessary nutrients to ruminants (Faichney 1996; Huws et al. 2018). The rumen hosts a diverse group of microorganisms that work closely to degrade plant materials. The fermentation process converts nearly all dietary carbohydrates to volatile fatty acids (VFA), predominantly butyrate, acetate, and propionate. It has been widely recognized that the rumen VFAs are crucial for digestive system development and animal carbon and nitrogen needs (Storm and Ørskov 1983; Broudiscou and Jouany 1995; Weigand, Young, and McGilliard, 1974; Górka et al. 2018; Leng, Steel, and Luick 1967; Young 1977; Huws et al. 2018; Bach, Calsamiglia, and Stern 2005; Edwards et al. 2008; Wallace, Onodera, and Cotta 1997). Direction infusion of VFAs into the rumen can also improve animal performances. For example, direct infusion of butyrate into the rumen increased milk fat production without changing milk yield (Huhtanen, Miettinen, and Ylinen 1993) and direct infusion of propionate into the rumen increased milk protein production (Rook and Balch 1961).

The contribution of DFMs to the fermentation characteristics of the rumen has been extensively evaluated (Elghandour et al. 2015). Specific species within the genera *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Bacillus, Propionibacterium, Megasphaera and Prevotella* have been fed to animals (Nocek et al. 2002; Yoon and Stern 1995; Ghorbani et al. 2002; Stein et al. 2006; Yang and Beauchemin et al. 2004; Nagaraja et al. 1997; Chiquette, Allison, and Rasmussen 2008; Mohammed et al. 2012; Weiss, Wyatt, and McKelvey 2008; Aikman et al. 2011). There are several studies, for example, that describe the fermentation patterns and feed digestibility of ruminants fed a standard diet supplemented with a DFM compared to ruminants only on a standard diet. Feeding of *Lactobacillus plantarum* via silage in (Mohammed et al. 2012) showed no changes in production, but no deleterious effects on the animal. Similar results were observed in studies feeding *Lactobacillus acidophilus* (Raeth-Knight, Linn, and Jung 2007; Abu-Tarboush, Al-Saiady, and Keir El-Din 1996; Higginbotham and Bath, 1992; McGilliard and Stallings 1998). In Weiss, et al. (2008), dairy cows were supplemented with *Propionibacterium* P169 2 weeks before anticipated calving to 119 days in milk. Cows fed *Propionibacterium* P169 had lower concentrations of acetate and greater concentrations of propionate and butyrate compared to control cows. Treatment cows also produced similar amounts of milk with similar composition as cows fed the control diet and had similar body weights throughout the trial. Chiquette, et al. (2008) fed *Prevotella bryantii* 25A to dairy cows in early lactation, and found that administration did not change milk yield, but tended to increase milk fat. This is in alignment with the increased acetate and butyrate concentrations observed in the rumen of treatment animals. In Chiquette et al. 2007, *Ruminococcus flavefaciens* NJ was fed to non-lactating dairy cows on either a high concentrate or a high forage diet daily. Cows fed *R. flavefaciens* NJ exhibited improved *in sacco* digestibility of hay in the rumen when fed as part of a high concentrate diet. Several experiments have fed *Megasphaera elsdenii* with various results on digestibility and performance, but no deleterious impacts were observed (Aikman et al. 2011; Hagg et al. 2010; Zebeli et al. 2012; Kung and Hession 1995).

Bacteria catabolism also plays an important role in animal nutrient cycling. Hoogenraad et al. (1970) studied how model organisms of gram-negative bacterium (*Escherichia coli*) and gram-positive

bacterium (*Bacillus subtilis*) were utilized in adult sheep digestive tract. The study found that the freeze-dried whole cells of either bacteria were quickly digested by rumen microbiome and cell carbons were incorporated into VFAs. A large amount of the bacterial carbon (70%) was captured by the host animal. Bacterial whole cells and cell components such as cell wall and content were also readily digested and metabolized in abomasum. Despite the common belief that gram-positive cells are more difficult to metabolize due to the presence of peptidoglycan, 73-86% of *B. subtilis* cell and cell component carbon was captured by the animal through lower gut digestion. In contrast, a smaller portion (66-78%) of *E. coli* carbon was captured by the host animal. Notably, although *B. subtilis* cells contain a greater amount of glucose than *E. coli*, a much greater amount of *E. coli* carbon was incorporated into the lower gut glucose pool. The findings suggest that bacteria turnover in ruminant digestive tract is an important process and supplying building blocks to support the host metabolism.

The rumen microbiome is dynamic. Moraïs and Mizrahi (2019) summarized that multiple microbial community states exist within the rumen depending on the rumen metabolic needs. The flow of metabolites and energy were passed on from one functional group to the next rather than from one group to another. Thus, microbial interactions could drive larger changes in overall fermentation patterns and identifying the optimal microbial interactions could improve digestibility (Weimer 2015). Published studies showed that diet contributes to the greatest rumen microbiome shifts observed (Kumar et al. 2015; Deusch et al. 2017; Mizrahi and Jami 2018; Alejandro Belanche et al. 2019; Johnson and Johnson 1995; Brulc et al. 2009; Carberry et al. 2014). Under the same diet, the addition of DFMs does not change the rumen microbiome significantly but can improve rumen digestibility. Westergaard (2015) fed a *Bacillus pumilus* DFM to 21 dairy cows and compared the composition of their rumen microbiomes to 22 control animals. The study reported an insignificant increase in Firmicutes from 14.1% to 15.8% and an insignificant decrease of Bacteroidetes from 64.1% to 62.3% in rumen fluid of animals received the DFM. Its companion study reported that the animals receiving the DFM were more efficient at feed conversion (ECM:DMI) than the control animals, although not significantly ($p = 0.06$) (Luan et al. 2015). Le et al. (2017) conducted a study comparing the growth performance of 4 week-old dairy calves with and without DFM *Bacillus amyloliquefaciens* in feed. *B. amyloliquefaciens* was administered daily for 9 weeks to 12 calves and another 12 calves were used as controls. The study found that dairy calves administered *B. amyloliquefaciens* gained 20% more weight and suffered less diarrhea than the control group. Notably, its companion study observed that *B. amyloliquefaciens* supplementation did not change the dairy calf rumen microbiomes significantly, despite confirmation of colonization of the DFM strain in rumen (Schofield et al. 2018). In another study, Fomenky et al. (2018) compared the rumen digesta microbiome of pre- (33 days old) and post-weaned calves (96 days old) fed with control diet alone and control diet supplemented with *S. cerevisiae* (SCB) or *L. acidophilus* (LA) (8 per treatment). The study found that supplementing DFMs did not significantly change the overall rumen microbial community structure, where the p-values for alpha diversity indices ranged from 0.051 to 0.992 and the p-value for beta diversity (PERMANOVA) was 0.512. The study also predicted that pathways involved in lipid and protein metabolism and cellular processes were more abundant in pre-weaned rumen administered DFMs. Once weaned, no predicted pathways in rumen digesta were significantly different between control and LA fed animals. Riboflavin metabolism was the only significantly more abundant pathway in SCB fed animal rumen digesta than control. These studies demonstrated that DFMs could promote better microbial interactions and improve the overall rumen feed digestibility without significantly changing microbial community structures.

The rumen bacterial population composition was investigated using internal animal survey experiments as well as external, peer-reviewed experiments (Appendix 18). Typical ranges of the

native bacteria phyla as well as the abundance of the native population of *B. fibrisolvens* were identified, demonstrating that *B. fibrisolvens* is a ubiquitous constituent of the dairy cow microbiome.

The use of *B. fibrisolvens* to facilitate the digestion of fibrous plant material and polysaccharides (Hespell, Wolf, and Bothast 1987) of animal feed within the rumen utilizes enzymes related to amylase, xylanase, and beta-glucanase. Studies conducted on *B. fibrisolvens* have revealed the presence and induction of a collection of xylanases and hemicellulolytic isoenzymes in response to xylan (Sechovcová et al. 2019; Emerson and Weimer 2017; Lin and Thomson 1991; Hespell, Wolf, and Bothast 1987). The species has a demonstrated ability to hydrolyze starch through the expression of extracellular and cell-associated alpha amylase (M. A. Cotta 1992; Rumbak et al. 1991; M. A. Cotta 1988; Ramsay et al. 2006). Furthermore, *B. fibrisolvens* produces beta-glucanase (Pierre van Rensburg, van Zyl, and Pretorius 1994), and when taken together these fibrolytic enzymes are major factors in the digestion of plant material (Rode, Yang, and Beauchemin, 1999; Beauchemin et al., 2003). *B. fibrisolvens* is frequently found in rumen content globally, across many species of ruminants (Bryant and Small 1956; Lee and Moore 1959; Brown and Moore 1960; Cheng et al. 1969; Forster et al. 1996; Sundset et al. 2008; Vasta et al. 2010; Henderson et al. 2015; Anne Willems and Collins 2015) and is also commonly found in monogastric animals (Moore and Holdeman 1974; Asanuma, Kawato, and Hino 2001; Balamurugan et al. 2009; Mi et al. 2018). *B. fibrisolvens* is a common commensal rumen microorganism that has been used previously in non-commercial, research settings as a DFM (see Part 6.5).

Native Microbials conducted a series of experiments in order to obtain a representative sampling of the rumen microbial community in dairy cows under farm-like conditions in the U.S. The full study report is provided in Appendix 018. In two general survey experiments, animals were cannulated and sampling conducted across the different regions of the rumen over a number of days. In all of the experiments, the typical abundance of *B. fibrisolvens* specifically, in the rumen of dairy cows was found to vary from approximately 0.0001% to 1% of the bacterial population. General observations indicated that all animals were in good health. Taken together, these studies provide corroborative experimental evidence that *B. fibrisolvens* is naturally abundant in the rumen of dairy cattle and not associated with any health concerns.

Hence the use of *B. fibrisolvens* as a source of live microorganisms, will have a beneficial effect on the available nutrition from a typical dairy ration. However, with understanding of the typical microbiome shifts as related to influencers such as dietary composition, physiological changes and environmental impacts, the notified substance will not make marked or detrimental changes on the rumen microbiome.

6.4.3 Section Summary

B. fibrisolvens occurs in a wide range of animals, including essentially all ruminants, as a commensal organism in the gastrointestinal tract. Dietary supplementation of *B. fibrisolvens* will not negatively impact the function of the rumen or the well-being of the animal.

6.5 History of Use in Manufacture of Food and Feed Ingredients

In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B.*

fibrisolvens favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported. Furthermore, *B. fibrisolvens* has been utilized as a probiotic in mice, being analyzed for its CLA production (Fukuda et al. 2006) and potential for tumor reduction (Ohkawara et al. 2007). Both studies reported that *B. fibrisolvens* had positive impacts on the health of the mice in the studies and reported no adverse health effects of administration. A strain has also been tested as an aspect of a dietary study in rats to increase intestinal production of short-chain volatile fatty acids (Nielsen et al. 2016). Similarly, this study also did not report any adverse health impacts of *B. fibrisolvens.* Although this species is not commercially available and has not seen widespread application in feed, academic and scientific research has shown that there are no adverse effects when *B. fibrisolvens* is fed to animals.

Several other applications of this microorganism have been researched. Due to the high level of production of extracellular polysaccharides similar to xanthan gum, a particular strain *B. fibrisolvens* has been proposed for use as an industrial source of this biopolymer (Wachenheim and Patterson 1992). Some research regarding applications of the genome of *B. fibrisolvens* has been completed. Specifically, genes coding for xylan-degrading enzymes (Sewell et al. 1989; Utt et al. 1991), cinnamoyl ester hydrolase (Dalrymple and Swadling 1997), glucanase (Pierre van Rensburg, van Zyl, and Pretorius 1994; P. van Rensburg, van Zyl, and Pretorius 1997, 1996), glutamine synthase (Goodman and Woods 1993), and cellodextrinase (Berger et al. 1990) from *B. fibrisolvens* have been used in transformation of other bacteria. While these studies focus on a range of different enzymes and transform several species of bacteria, the core intent of all of these studies is to improve the digestive functionality of the transformed bacteria with enzymes from *B. fibrisolvens*.

6.6 Toxigenicity and Pathogenicity

Butyrivibrio species are largely considered to be non-pathogenic commensals and have not commonly been identified as opportunistic pathogens. The American Type Culture Collection (ATCC) lists *B. fibrisolvens* as BSL-1, indicating that it is a low-risk microorganism that poses little to no threat of infection in healthy humans and animals. DSMZ also classifies *B. fibrisolvens* as BSL-1.

Butyrivbrio have been cited in a small number of opportunistic infections since the 1970s. The first suspected infection in animals or humans by *Butyrivbrio* was reported in a farmer who suffered an eye injury from barbed wire in a cattle enclosure. Infection of the eye followed the injury, and *B. fibrisolvens* was suspected as the causative agent (Wahl 1974). *Butyrivbrio* like organisms have been isolated from both liver abscesses and gastrointestinal infections (Chow, Ota, and Guze 1976; Thadepalli et al. 1978; George et al. 1981). In all the cited cases of suspected *Butyrivbrio* infection, identification of the causative organism was based on morphology, metabolism, and antimicrobial susceptibility profiles and no infections have been confirmed using unambiguous molecular methods.

As noted in Part 2.1.5, the *B. fibrisolvens* ASCUSDY19 genome assembly contains a chromid. The presence of plasmids, mega-plasmids, and chromids are common in *Butyrivbrio* genomes and the presence of more than two extrachromosomal replicons have been observed in some cases (Palevich et al. 2017; Yeoman et al. 2011; Teather 1982; Rodríguez Hernáez et al. 2018; Palevich et al. 2019). Plasmids from *B. fibrisolvens* are not known to carry pathogenic genes, though a small collection of plasmids from the species have been characterized with the hope of developing vector systems to transform ruminal microbes (Anne Willems and Collins 2015; Hefford et al. 1997; Beard et al. 1995). The high rate of megaplasmids and chromids within the genus is believed to help lend a competitive advantage over other ruminal organisms by enhancing growth rate and cellular efficiency through copy number increase of key metabolic genes (Palevich et al. 2019; Morrison 1996), rather than bestow pathogenic ability. This hypothesis is at least in part supported by the gene composition observed in the only sequenced *B. fibrisolvens* chromid, which largely consists of genes which encode for carbohydrate degradation enzymes many of which are also encoded by the main chromosome (Rodríguez Hernáez et al. 2018).

6.6.1 Summary

Overall, the available information indicates that *B. fibrisolvens* is a prevalent organism in the gastrointestinal microbiome of animals, including humans. Few instances of infection have been attributed to the genus *Butyrivbrio* or the species *B. fibrisolvens* and no infections have been documented since the wide acceptance and implementation of molecular techniques that allow for unambiguous microbial identification. As indicated in Part 2.1.8, interrogation of the whole genome sequence of *B. fibrisolvens* ASCUSDY19 did not reveal the presence of any protein toxins and the single virulence factor identified is not solely responsible for pathogenicity or virulence.

6.7 Studies in Target Animals

The determination that *B. fibrisolvens* ASCUSDY19 is GRAS under the intended conditions is based on product-specific characterization data together with the body of information in the published literature. The organism is a commensal rumen organism.

In ruminants, *B. fibrisolvens* has been administered to goats, increasing the amount of CLA present in their rumens and milk (Shivani et al. 2016). These authors found that supplementation of *B. fibrisolvens* favorably altered the fatty acid composition of the milk, and reported no adverse health effects on the goats. This species has also been administered to cattle as a test of ruminal colonization alongside several other bacteria (Klieve et al. 2003). This study actively supplemented cattle being fed a high-grain diet with *B. fibrisolvens* and two other bacteria, and while the authors were not able to establish a new population of *B. fibrisolvens* in the rumen, the authors did note that most of the cattle adjusted unexpectedly quickly to the high-grain diet and no negative health effects relating to microbial supplementation were reported. Although this species is not commercially available and has not seen widespread application in feed, academic and scientific research has shown that there are no adverse effects when *B. fibrisolvens* is fed to ruminants.

6.8 Summary and Critical Evaluation of Target Animal Safety

B. fibrisolvens is a common commensal bacteria in the gut of humans and animals. No reports of toxigenicity or pathogenicity associated with *B. fibrisolvens* were identified in the published literature. Native Microbials has conducted an assessment of *B. fibrisolvens* ASCUSDY19 and confirmed the absence of any genes encoding for toxin production or other virulence factors known to be associated with pathogenicity (see Part 2.1.8). Furthermore, the susceptibility of *B. fibrisolvens* ASCUSDY19 strains to antibiotics of veterinary and pharmaceutical relevance, and the absence of antimicrobial production has been demonstrated (see Parts 2.1.6 and 2.1.7, and Appendices 004 and 005). Collectively, these data indicate that *B. fibrisolvens* ASCUSDY19 (the notified substance) should not be associated with any safety concerns for dairy cattle under the intended conditions of use as a direct fed microbial.

6.9 Summary and Critical Evaluation of Human Food Safety

As mentioned in Part 3.2, no transfer of viable *B. fibrisolvens* ASCUSDY19 from the rumen to milk or other edible species is anticipated under the conditions of intended use as a direct fed microbial in the feed of dairy cattle. Furthermore, the strain has been unambiguously characterized as *B. fibrisolvens* and whole genome sequence analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins (see Part 2.1.8). The absence of pathogenicity or toxigenicity is supported by the ubiquitous nature of *B. fibrisolvens* and its natural occurrence in the rumen and gastrointestinal tract of animals. Taken together, these data indicate that *B. fibrisolvens* ASCUSDY19 should not be associated with any human food safety concerns under the intended conditions of use as a direct fed microbial in the feed of dairy cattle.

In this safety assessment we identified, discussed and placed into context data and information that are, or may appear to be inconsistent with the GRAS status (21 CFR 570.250(c)(1)). Based on the preponderance of evidence, Native Microbials' conclusion of safety is scientifically justified.

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

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16–25.

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

INTERNATIONAL FORM

TO
Patent Dept.
Ascus BioSciences
6450 Lusk Blvd. E109/E209
San Diego, CA 92121

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

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Butyrivibrio sp. *Ascusb_826*	NRRL B-67347

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 December 15, 2016 (date of the original deposit)[2]

IV. RECEIPT OF REQUEST FOR CONVERSION

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

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:	Agricultural Research Culture Collection (NRRL) International Depositary Authority	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address:	1815 N. University Street Peoria, Illinois 61604 U.S.A.	Date: December 21, 2016

[1] Mark with a cross the applicable box.

[2] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

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

INTERNATIONAL FORM

TO

Patent Dept.
Ascus BioSciences
6450 Lusk Blvd. E109/E209
San Diego, CA 92121

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

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
Name: Dr. Adam Taylor Ascus Biosciences 6450 Lusk Blvd. E109/E209 Address: San Diego, CA 92121	Depositor's taxonomic designation and accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY *Butyrivibrio sp.* NRRL B-67347 Date of : December 15, 2016 ☑ Original Deposit ☐ New Deposit ☐ Repropagation of Original Deposit

III. (a) VIABILITY STATEMENT

Deposit was found:
☑ [2]Viable ☐ [2]Nonviable on December 17, 2016 (Date)
International Depositary Authority's preparation was found viable on December 23, 2016 (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): Date: December 23, 2016

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

Appendix 002: Supplementary Methods and Results for *B. fibrisolvens* ASCUSDY19 In Vitro Biochemical Assays

Objectives:

The objective of this work was to assess the carbohydrate fermentation capabilities and metabolite production of *B. fibrisolvens* ASCUSDY19 through in vitro assays.

Methods:

Carbohydrate fermentation of *B. fibrisolvens* ASCUSDY19 was qualitatively measured using the API 50CH carbon panel (BioMérieux, Marcy-l'Étoile, France). Results can be found in Table 1. *B. fibrisolvens* ASCUSDY19 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) bromocresol purple added as a pH indicator for acidification of carbohydrates (Avgustin et al. 1997).

Metabolite production of *B. fibrisolvens* ASCUSDY19 fermentation run 1801.2033 was measured at 8.2, 16.1, 28, 32.2, 40, 48, 52 hours using an Agilent 1260 series with RI detector operated at 35°C. The column used was a Bio-Rad Aminex HPX-87H #1250140 with Bio-Rad Cation H+ guard #1250129 operated at 60°C. The mobile phase was (b) (4) N Sulfuric Acid (b) (4) concentrated sulfuric to (b) (4) at a flow rate of (b) (4) /min. Pure standards were used at varying concentrations to generate a standard curve.

Results:

B. fibrisolvens ASCUSDY19 was assessed for fermentation of 50 carbon sources. Carbon source fermentation data is shown below in table 1. Metabolite production at each fermentation time point can be found in table 2.

Table 1. Carbon Source Fermentation by *B. fibrisolvens* ASCUSDY19

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	No Growth
D-Ribose	No Growth	N-AcetylGlucosamine	No Growth
D-Xylose	Growth	Amygdalin	No Growth
L-Xylose	No Growth	Arbutin	No Growth
D-Adonitol	No Growth	Esculin/Ferric Citrate	Growth

Methyl-BD-xylopyranoside	No Growth	Salicin	Growth
D-Galactose	No Growth	D-Cellobiose	Growth
D-Glucose	Growth	D-Maltose	No Growth
D-Fructose	Growth	D-Lactose	No Growth
D-Mannose	No Growth	D-Melibiose	Growth
L-Sorbose	No Growth	D-Saccharose	Growth
L-Rhamnose	Growth	D-Trehalose	No Growth
Dulcitol	No Growth	Inulin	No Growth
D-Melezitose	No Growth	D-Tagatose	No Growth
D-Raffinose	Growth	D-Fucose	No Growth
Starch	Growth	L-Fucose	No Growth
Glycogen	No Growth	D-Arabitol	No Growth
Xylitol	No Growth	L-Arabitol	No Growth
Gentiobiose	No Growth	Potassium Gluconate	No Growth
D-Turanose	No Growth	Potassium 2-KetoGluconate	No Growth
D-Lyxose	No Growth		

Table 2. Metabolite Production by *B. fibrisolvens* ASCUSDY19 on Complex Media with Glucose

	Glucose	Pyruvic Acid	Succinic Acid	Lactic Acid	Glycerol	Acetic Acid	Propionic Acid	Butyric Acid	1-Butanol	Ethanol
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										
16.1										
28.0										
32.2										
40.0										
48.0										
52.0										

(b) (4)

Conclusions:

In vitro assays demonstrate that B. *fibrisolvens* ASCUSDY19 grows on a variety of substrates including L-arabinose, D-xylose, glucose, fructose, rhamnose, esculin, salicin, cellobiose, melibiose, saccharose, raffinose, and starch. When grown on glucose B. *fibrisolvens* ASCUSDY19 produces lactate, acetate and butyrate as major fermentation products.

Signed: (b) (4), (b) (6) _____ Date: _____

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Appendix 003C: Supplementary Genome Comparison Data for *B. fibrisolvens* ASCUSDY19

Objectives

The objective of this work was to determine the identity of *Butyrivibrio fibrisolvens* ASCUSDY19 using genomic methods.

Methods

For 16S sequence analysis, the 16S gene was amplified from *Butyrivibrio fibrisolvens* ASCUSDY19 the 27F/534R primers and sequenced using an Illumina Miseq (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 November 23, 2020.

Genomic DNA was isolated from a pure culture of *B. fibrisolvens* ASCUSDY19 by a modified Sambrook phenol-chloroform extraction/purification protocol (Jain et al. 2018). Short read sequencing libraries were prepared using the Nextera XT kit (Illumina, San Diego, CA) by manufacturer's recommended protocol and the resulting libraries were sequenced (1x300bp) on an Illumina Miseq. In parallel, long read libraries were prepared from the same extracted DNA using the SQK-RAD004 kit (Oxford Nanopore Technologies, Oxford, UK) using a modified version of the protocol outlined by (Jain et al. 2018) and 1D sequenced on the MinION (R9.4 flowcell) by Oxford Nanopore. 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 analysis of *B. fibrisolvens* ASCUSDY19

Genus species (GenBank accession #)	Percent Match	Percent Coverage
B. fibrisolvens InBov1 (JN642599)	99.7%	100%
Rumen Bacterium NK3B81 (GU324363)	99.7%	99%
Rumen Bacterium NK4A61 (GU324372)	99.3%	99%
Rumen Bacterium NK4A114 (GU324377)	98.9%	99%
B. fibrisolvens WV1 (AF396927)	98.3%	99%

Whole genome average nucleotide identity (ANI) was used to confirm the 16S identification. Genomes from multiple strains from *B. fibrisolvens* as well as other *Butyrivibrio* were compared to *B. fibrisolvens* ASCUSDY19 by ANI (% identity and coverage). As shown in Table 2, the *B. fibrisolvens* ASCUSDY19 genome most closely matched *B. fibrisolvens.*

Table 2: Whole Genome ANI analysis of *B. fibrisolvens* ASCUSDY19

Genus species (GenBank accession #)	**ANI (%)**	**Coverage (%)**
B. fibrisolvens INBov1 (GCA_003175155)	97.6	72.1
B. fibrisolvens YRB2005 (GCA_000423985)	96.8	77.3
B. fibrisolvens DSM3071 (GCA_900129945)	89.2	34.8
Butyrivibrio proteoclasticus B316n (GCA_000145035)	86.4	3.69
Butyrivibrio proteoclasticus P6B7 (GCA_000622085)	85.5	2.8
Butyrivibrio hungatei NK4A153 (GCA_000424465)	84.8	2.6
Butyrivibrio hungatei MB2003 (GCA_001858005)	84.4	3.4

Conclusions

B. fibrisolvens ASCUSDY19 most closely matched the whole genome assemblies of *B. fibrisolvens* strains by ANI. The genomic data in this Appendix should be used along with the phenotypic data from Appendix 2 to confirm the identity as *B. fibrisolvens.*

Documentation

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

(b) (4)

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

(b) (4)

Signed: [redacted] (b) (4) _____ Date: _____

References

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Yarza, Pablo, Pelin Yilmaz, Elmar Pruesse, Frank Oliver Glöckner, Wolfgang Ludwig, Karl-Heinz Schleifer, William B. Whitman, Jean Euzéby, Rudolf Amann, and Ramon Rosselló-Móra. 2014. "Uniting the Classification of Cultured and Uncultured Bacteria and Archaea Using 16S rRNA Gene Sequences." *Nature Reviews. Microbiology* 12 (9): 635–45.

RID: VSDH46A7013
Job Title:Nucleotide Sequence
Program: BLASTN
Query: None ID: lcl|Query_56439(dna) Length: 299
Database: nt Nucleotide collection (nt)

Sequences producing significant alignments:

Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query cover	E Value	Per. Ident	Acc. Len	Accession

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Appendix 003C: Supplementary Whole Genome Analysis Methods and Read QualityMetrics for *B. fibrisolvens* ASCUSDY19

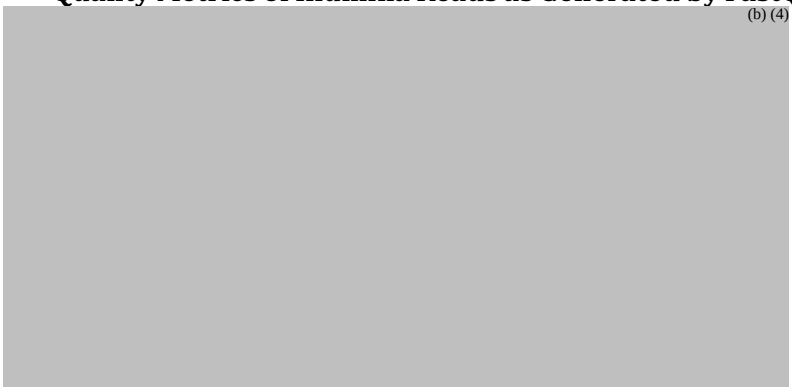
The *B. fibrisolvens* ASCUSDY19 genomic DNA was extracted and sequenced as described in the main text of the dossier. This appendix contains details about the assembly methods used, the protocol for NexteraXT library preparation, FastQC and NanoStat quality metrics for the Illumina and Oxford Nanopore reads respectively, metrics generated by Quast for the completed assembly, and a visualization of the assembly graph generated by Bandage.

Assembly Pipeline in Detail

NexteraXT Protocol as Provided by the Manufacturer

Full Protocol: NexteraXT

Quality Metrics of Illumina Reads as Generated by FastQC



Read distribution as related to quality score

Metrics for *B. fibrisolvens* ASCUSDY19 Oxford Nanopore reads as generated byNanoStat.

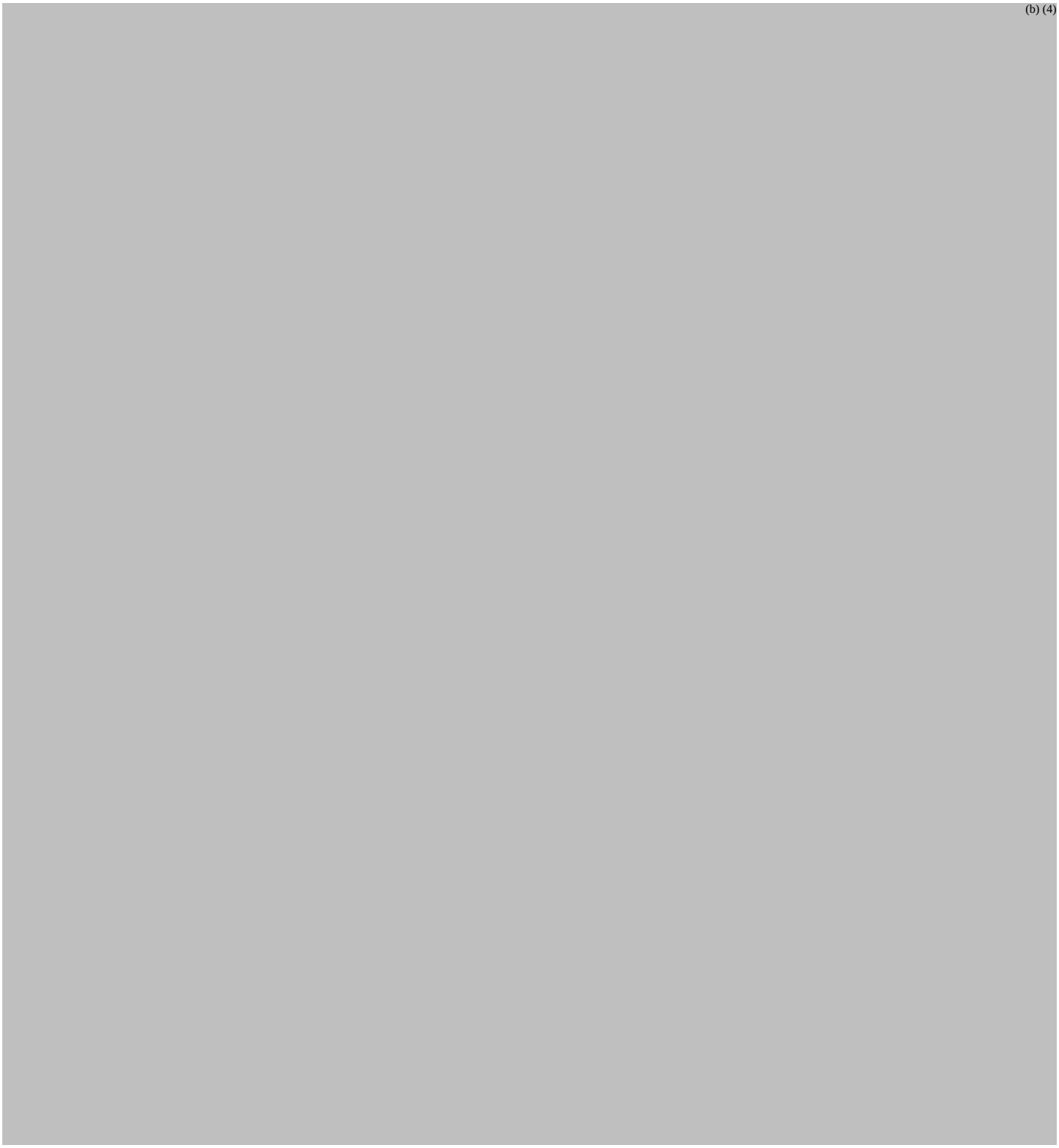
General Summary

Number, Percentage, and Megabases of Reads Above Quality Cutoffs

Longest Reads in Base Pairs (bp)

Assembly Statistics as reported by Quast

(b) (4)



Assembly Graph as Visualized by Bandage.

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References

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Characterization of Native Microbials *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19) Production Strain: Antibiotic Susceptibility Profile

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Table of Contents

Title: Characterization of Native Microbials *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19) Production Strain: Antibiotic Susceptibility Profile

1 OBJECTIVE

To determine the Susceptibility Profile of *Butyrivibrio fibrisolvens* (Dairy-19) production strain to European Food Safety Authority recommended antimicrobials.

2 STANDARDS OF COMPLIANCE

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

3 STUDY SITE

Antimicrobial susceptibility testing was performed at Native Microbials Inc.

4 MATERIALS AND METHODS

4.1 Isolate

A production strain of *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19) was procured from Commercial Working Cell Banks. The culture was streaked onto both Brucella agar and Mueller Hinton agar to verify that the organism is viable, pure, and morphologically typical of the purported species and to verify growth on the selected media.

4.2 Susceptibility Profile

4.2.1 Procedure

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

4.3 Media

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

4.4 Incubation and Interpretation of Susceptibility Tests

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

Sensitivities were compared to applicable values (Table 1) from EUCAST clinical breakpoints for gram positive anaerobes ("Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 10.0", 2020), CLSI breakpoints for anaerobic organisms (Clinical and Laboratory Standards Institute [CLSI], 2020), and EFSA breakpoints for gram-positive bacteria (Rychen et al., 2018).

4.5 Quality Control

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

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

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

	EFSA Gram-Positive		EUCAST Gram-Positive Anaerobes		CLSI Anaerobes		
Antibiotic	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**I**	**R >**
Ampicillin	1	1	4	8	0.5	1	2
Vancomycin	4	4	2	2			
Gentamicin	4	4	-	-			
Kanamycin	16	16					
Streptomycin	8	8					
Erythromycin	1	1	-	-			
Clindamycin	4	4	4	4	2	4	8
Tetracycline	2	2			4	8	16
Chloramphenicol	4	4	8	8	8	16	32

5 DISPOSITIONS

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

6 RESULTS

MIC results of the *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19) isolate and breakpoints interpretations are presented in Table 2. Photographs of agar dilution plates are shown in Appendix C. The isolate would be considered wild-type or susceptible according to all three criteria (EFSA, EUCAST, and CLSI) to Ampicillin and Chloramphenicol. The isolate would be considered susceptible to Vancomycin and Clindamycin according to EFSA and EUCAST breakpoints but would be considered intermediately sensitive to Clindamycin per CLSI. The isolate is considered intermediately sensitive to Tetracycline according to CLSI, although it would be considered resistant by EFSA. The isolate would be considered non-wildtype or non-susceptible against Gentamicin, Kanamycin, Streptomycin and Erythromycin to EFSA.

However, one must consider that some classifications set forth by EFSA are for general Gram-Positive organisms and are not applicable to *Butyrivibrio fibrisolvens* due to its anaerobic nature. EUCAST provides a breakpoint of "-" for Gentamicin and Erythromycin (Table 1) indicating that the species is a poor target for therapy with these antibiotics. CLSI refrains from providing a sensitivity for any aminoglycoside or macrolide class drugs for anaerobes. It is well documented that aminoglycosides are hindered by anaerobic growth. Active electron transport is required for aminoglycoside uptake into cells, so the class inherently lacks activity against anaerobic bacteria (Kislak, 1973; Martin, Gardner, and Washington, 1972; Ramirez and Tolmasky, 2010). Susceptibility to aminoglycosides and macrolides decreases significantly in anaerobic conditions when compared to aerobic conditions (DeMars et al., 2016).

Table 2. Minimal Inhibitory Concentrations for *Butyrivibrio fibrisolvens* and Sensitivity Interpretation

			Interpretation		
Antibiotic	Range Tested (ug/mL)	Butyrivibrio fibrisolvens	EFSA	EUCAST	CLSI
Ampicillin	0.5 - 128	< 0.5	S	S	S
Vancomycin	0.125 - 32	0.25	S	S	
Gentamicin	0.5 - 32	8	R	-	
Kanamycin	0.5 - 64	> 64	R		
Streptomycin	0.5 - 64	16	R		
Erythromycin	0.5 - 16	4	R	-	
Clindamycin	0.03 - 32	4	S	S	I
Tetracycline	0.0625 - 64	8	R		I
Chloramphenicol	0.5 - 64	4	S	S	S

MIC results of the QC strain ATCC 25922 *Escherichia coli* are presented in Table 3. ATCC 25922 performed within the expected range for Ampicillin, Tetracycline and Chloramphenicol. When compared to QC ranges for the aminoglycosides, Gentamicin, Kanamycin and Streptomycin, it appears to be out of specification. However, ATCC 25922 is a facultative anaerobe and in this testing, was grown in an anaerobic environment. The QC range provided by CLSI and EUCAST are for aerobic growth of ATCC 25922. For the reasoning provided above, these results are to be expected and are not indicative of a failure in the agar dilution plates.

The MIC results for the quality control organism is within the expected values, knowing that aminoglycosides (Gentamicin, Kanamycin and Streptomycin) and macrolides (Erythromycin) have reduced efficacy in anaerobic conditions.

Table 3. Minimal Inhibitory Concentrations for QC Strain ATCC 25922

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

7 REFERENCES

1. "Breakpoint Tables for Interpretation of MICs and Zone Diameters Version 10.0." 2020. The European Committee on Antimicrobial Susceptibility Testing. 2020. http://www.eucast.org.

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8. Rychen G, G Aquilina, G Azimonti, V Bampidis, M de Lourdes Bastos and G Bories, et al. 2018. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), “Guidance on the Characterisation of Microorganisms Used as Feed Additives or as Production Organisms.” *EFSA Journal. European Food Safety Authority* 16 (3): e05206.

Appendix A. Agar-Dilution Susceptibility Testing of Anaerobes

1 General Considerations

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

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

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

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

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

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

2 Media Preparation

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

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

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4 Inoculation of Plates

(b) (4)

5 Reading Results

(b) (4)

Appendix B. Raw Data

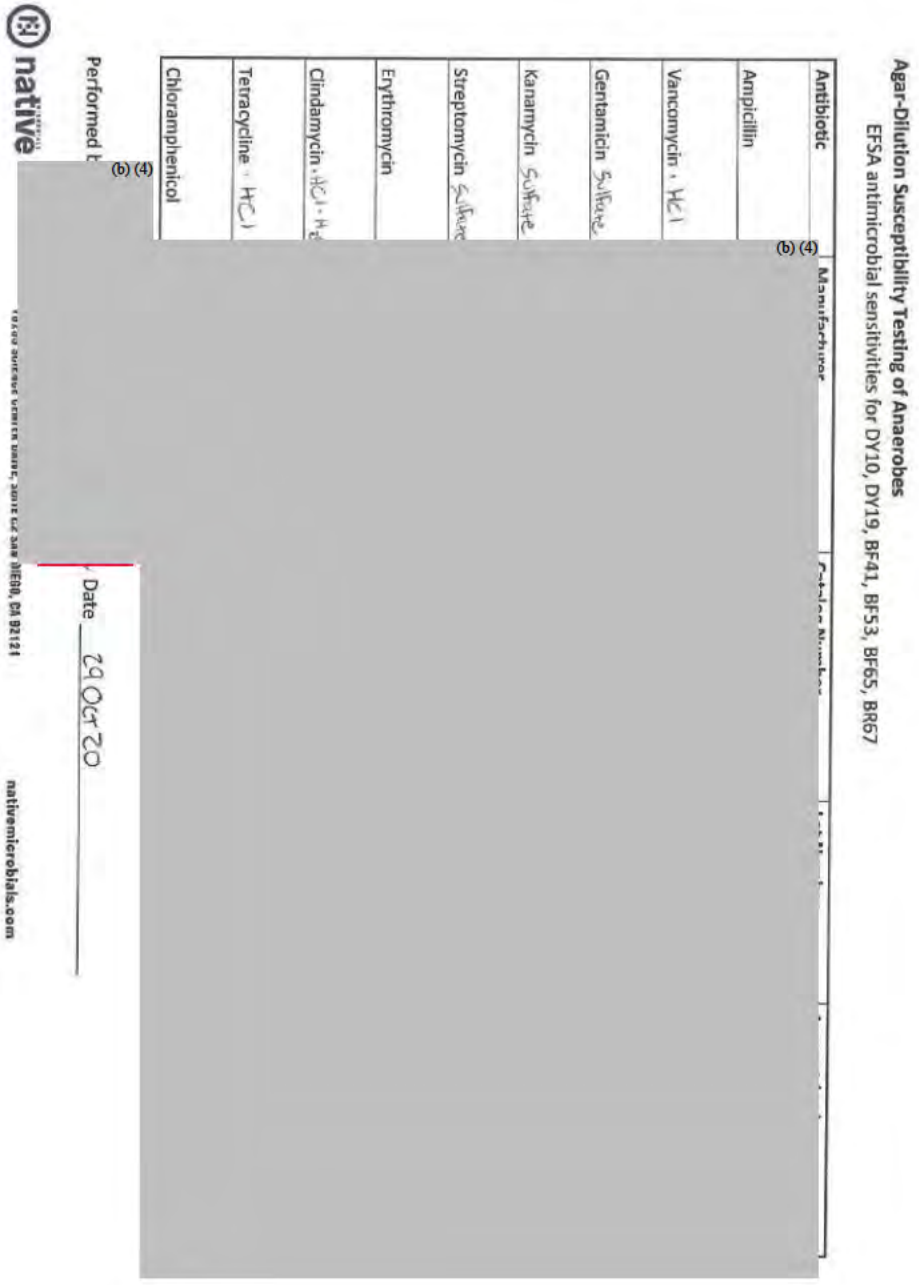
Brucella Agar

48 Project No. _____
Book No. _____ TITLE 29Oct20 Agar Dilution Media
From Page No. ____

(b) (4)

Recorded by: (b) (4) Date 29Oct20 (b) (4) Date 10/30/20

Antibiotic Sources



native MICROBIALS

Agar-Dilution Susceptibility Testing of Anaerobes

EFSA antimicrobial sensitivities for DY10, DY19, BF41, BF53, BF65, BR67

Antibiotic	Manufacturer	Catalog Number	Lot #	Concentration
Ampicillin	(b) (4)			
Vancomycin - HCl				
Gentamicin Sulfate				
Kanamycin Sulfate				
Streptomycin Sulfate				
Erythromycin				
Clindamycin - HCl - H2				
Tetracycline - HCl				
Chloramphenicol				

Performed b (b) (4)

Date 29 Oct 20

[redacted] San Diego, CA 92121

nativemicrobials.com

Strain Sources

Agar-Dilution Susceptibility Testing of Anaerobes

EFSA antimicrobial sensitivities for DY10, DY19, BF41, BF53, BF65, BR67

Strain (Genus species)	Strain #	Source	Inoculated (Y/N)
Ruminococcus bovis	(b) (4)		
Butyrivibrio fibrisolvens			
Prevotella albensis			
Succinivibrio dextrinosolvens			
Chaetomium cunicolorum			
Clostridium beijerinckii			
Escherichia coli			

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Performed by (b) (4)

Date 30 Oct 20

Antibiotic Concentration Calculations

Vancomycin

(b) (4)

Ampicillin

(b) (4)

Gentamycin

Kanamycin

Streptomycin

(b) (4)

Erythromycin

(b) (4)

Chloramphenicol

Clindamycin

Tetracycline

Appendix C. Agar Dilution Data and Photos

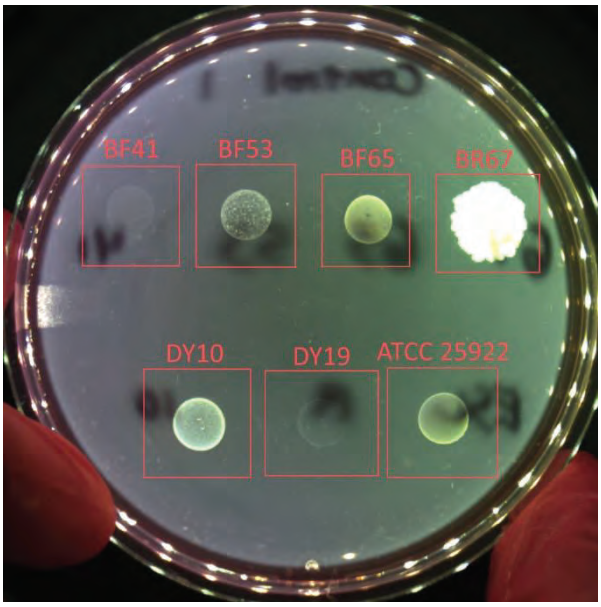
Table C-1. Agar Dilution Antibiotic Results and Susceptibility Photos: Ampicillin

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

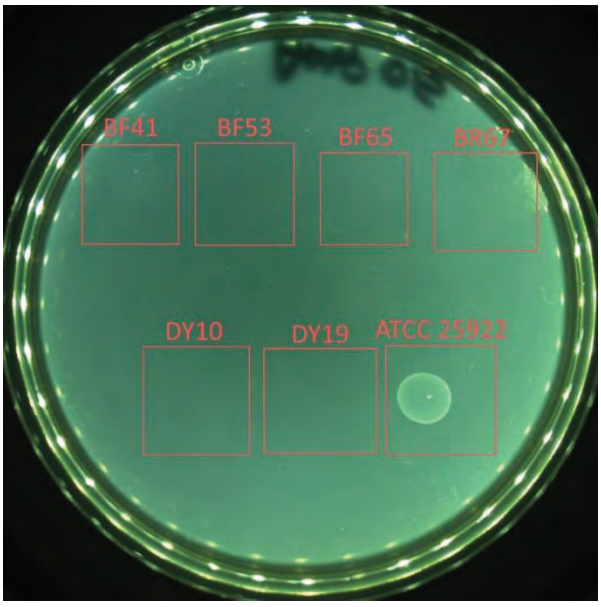
G = Growth

NG = No Growth

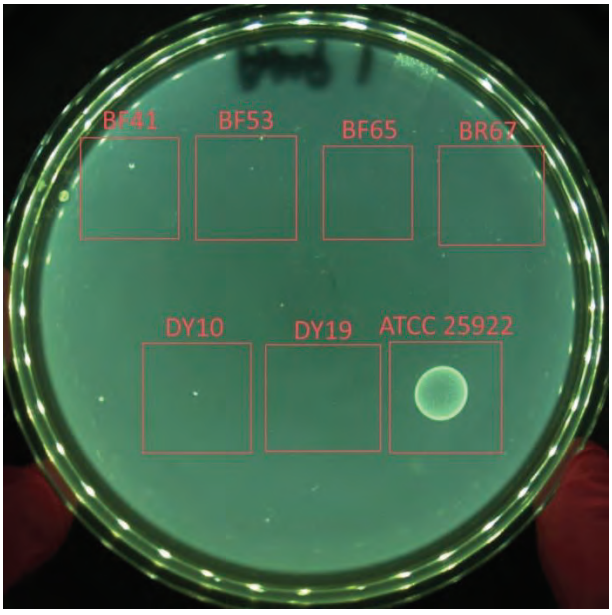
Agar Dilution Antibiotic Susceptibility Photos: Ampicillin



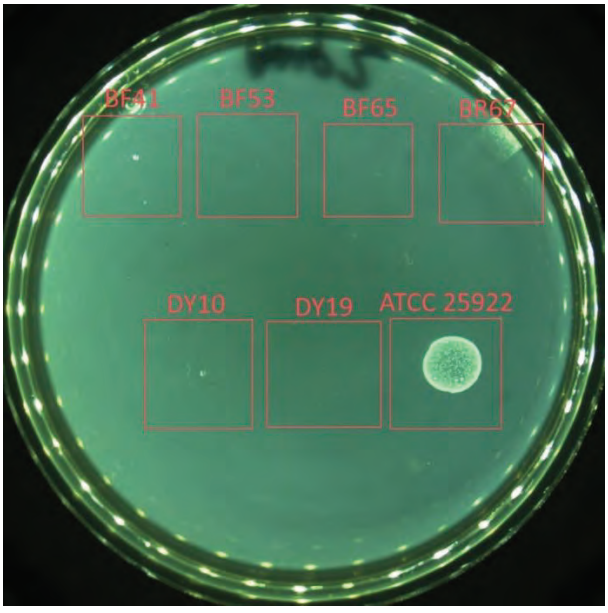
0 µg/mL Ampicillin



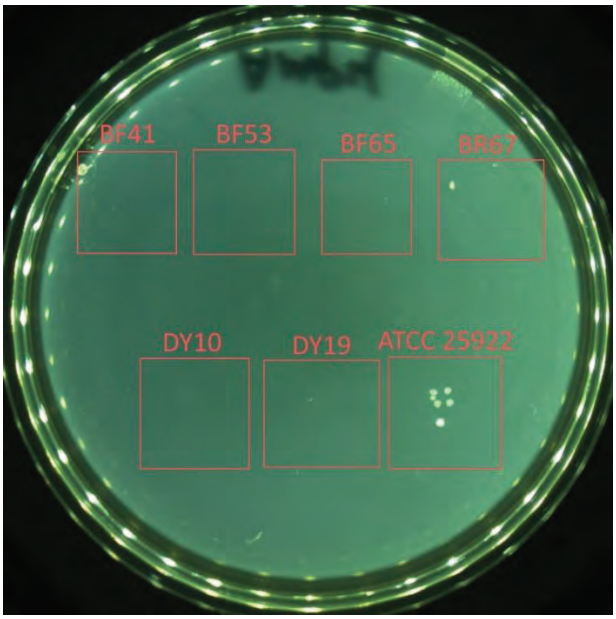
0.5 µg/mL Ampicillin



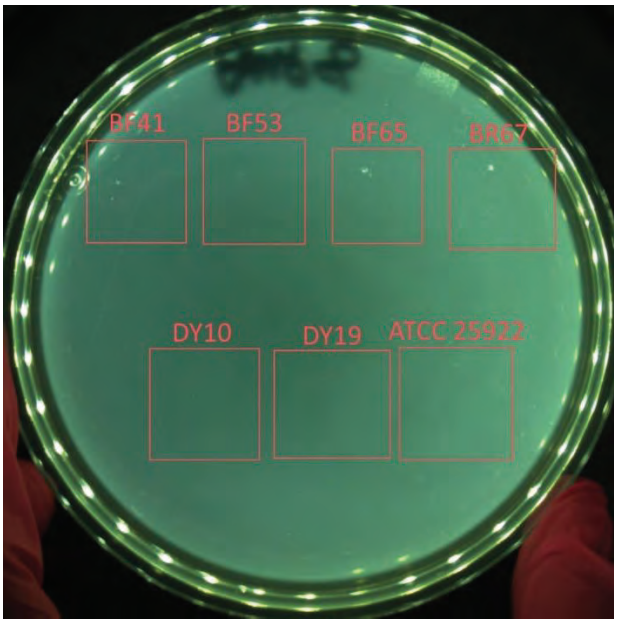
1 µg/mL Ampicillin



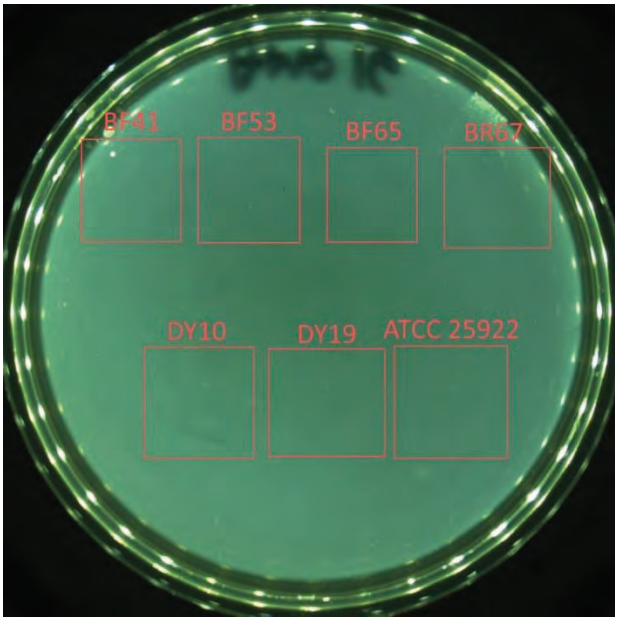
2 µg/mL Ampicillin



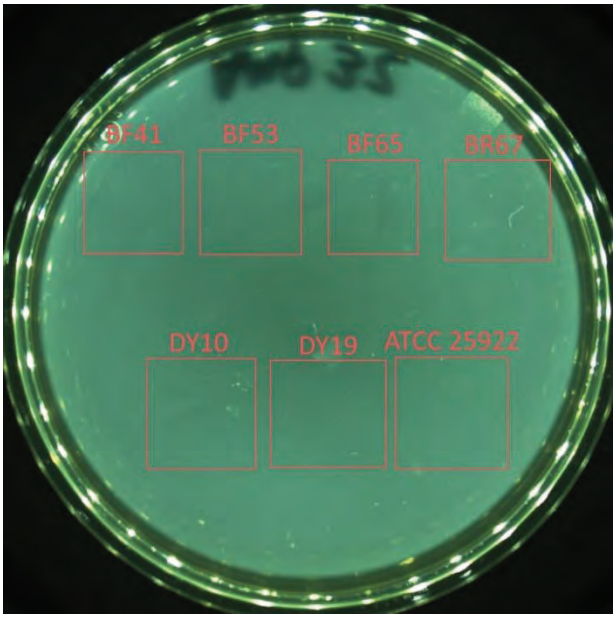
4 µg/mL Ampicillin



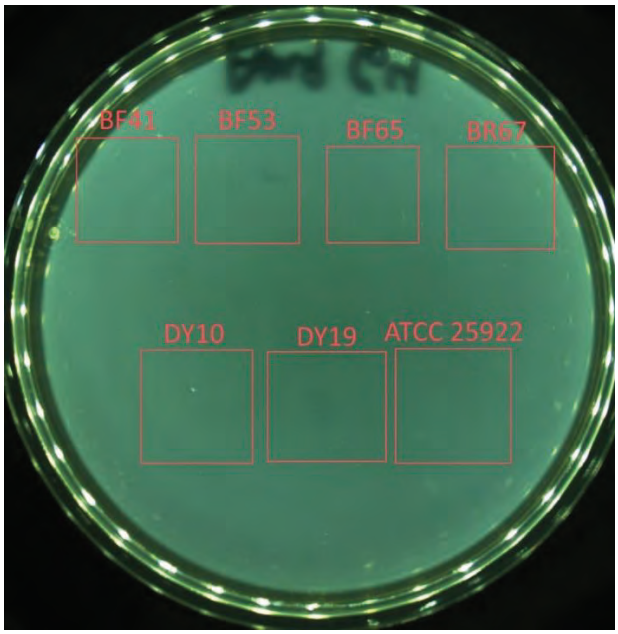
8 µg/mL Ampicillin



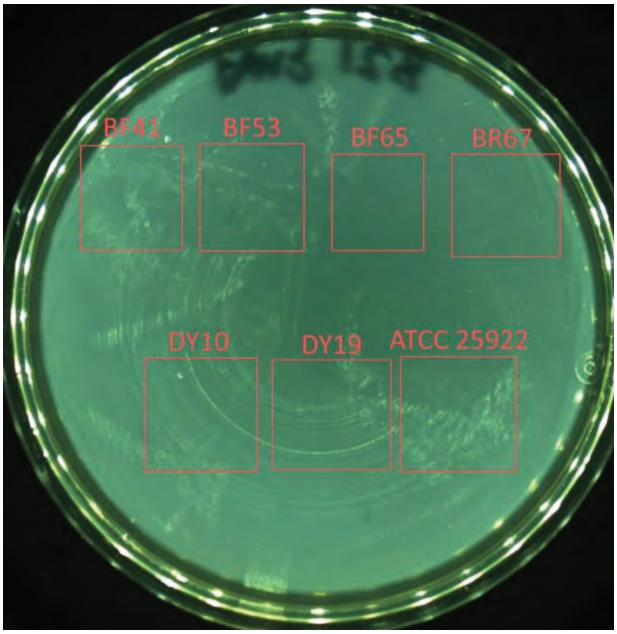
16 µg/mL Ampicillin



32 µg/mL Ampicillin



64 µg/ml Ampicillin



128 µg/ml Ampicillin

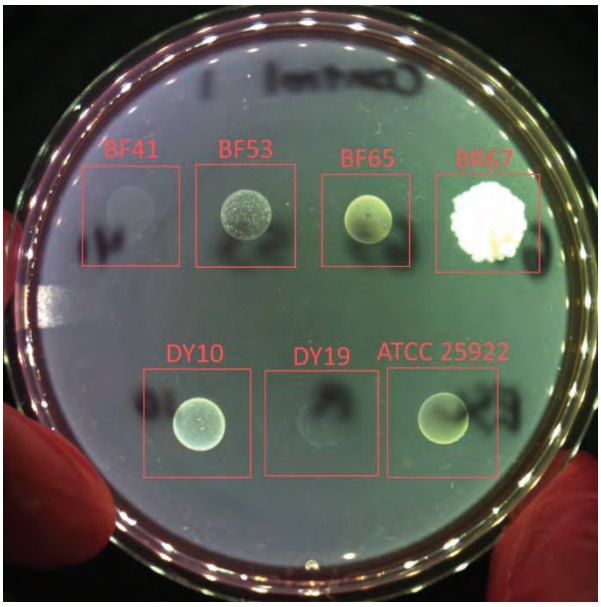
Table C-2. Agar Dilution Antibiotic Results and Susceptibility Photos: Chloramphenicol

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

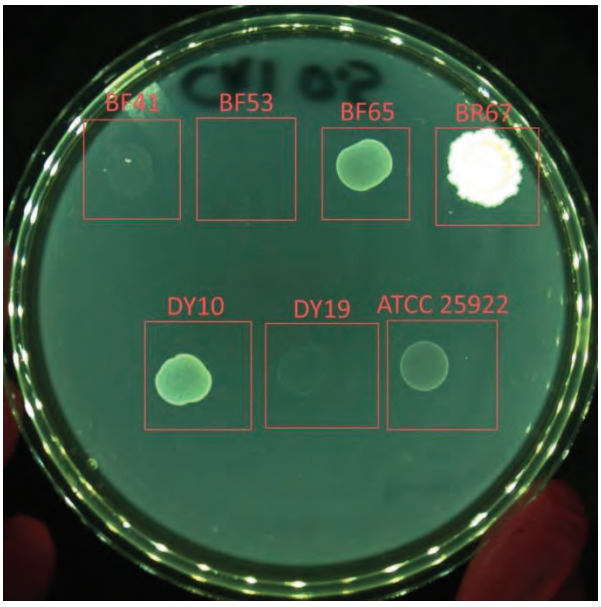
G = Growth

NG = No Growth

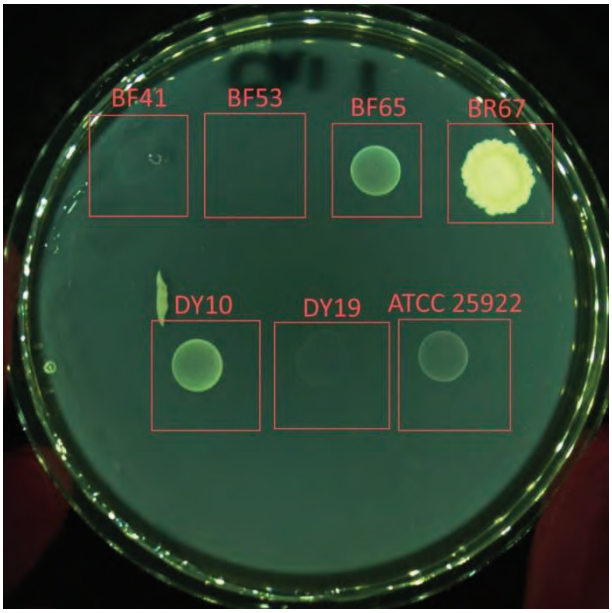
Agar Dilution Antibiotic Susceptibility Photos: Chloramphenicol



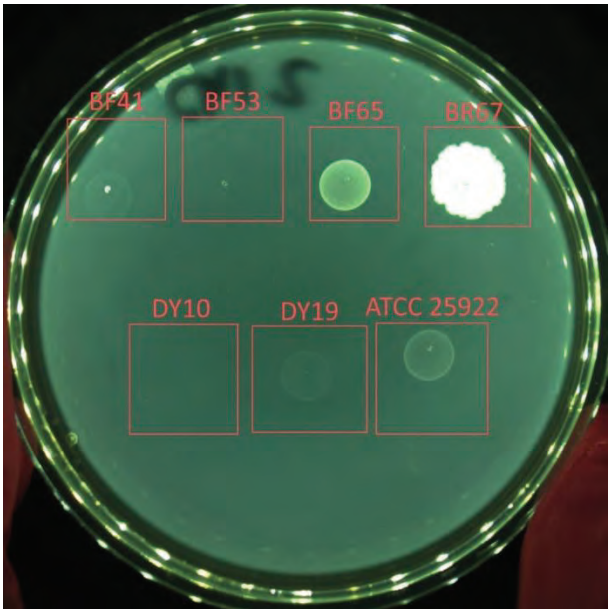
0 µg/mL Chloramphenicol



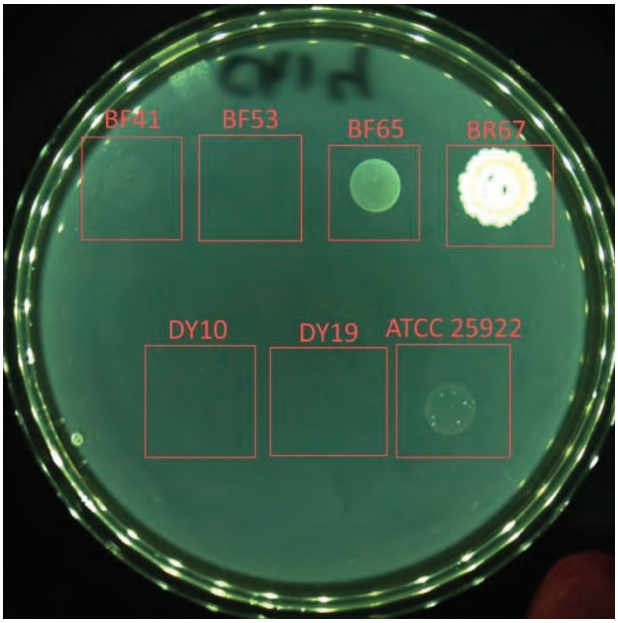
0.5 µg/mL Chloramphenicol



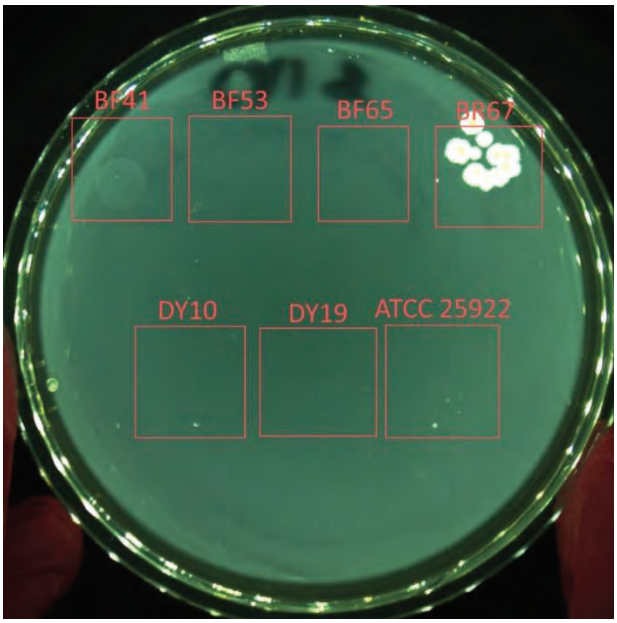
1 µg/mL Chloramphenicol



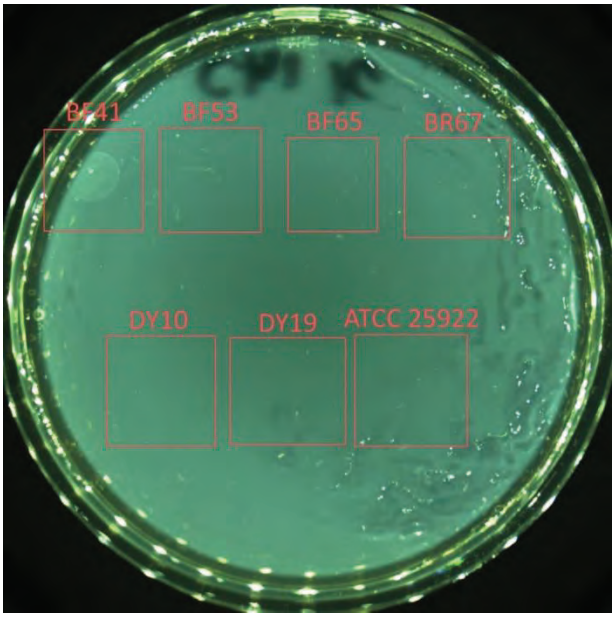
2 µg/mL Chloramphenicol



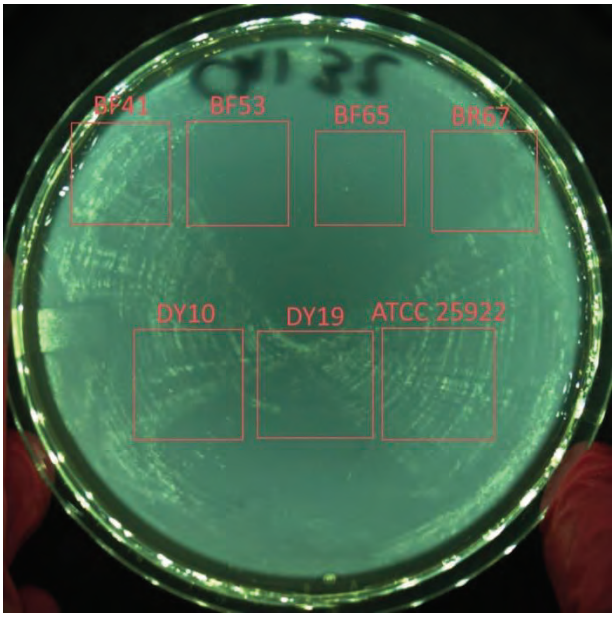
4 µg/mL Chloramphenicol



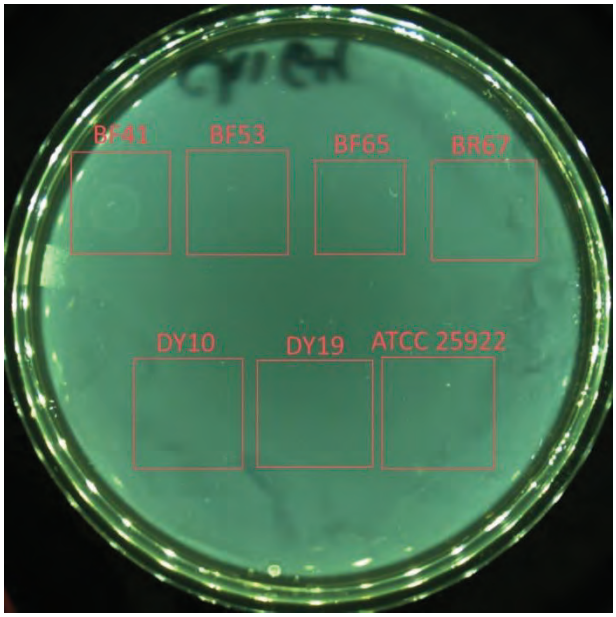
8 µg/mL Chloramphenicol



16 µg/mL Chloramphenicol



32 µg/mL Chloramphenicol



64 µg/ml Chloramphenicol

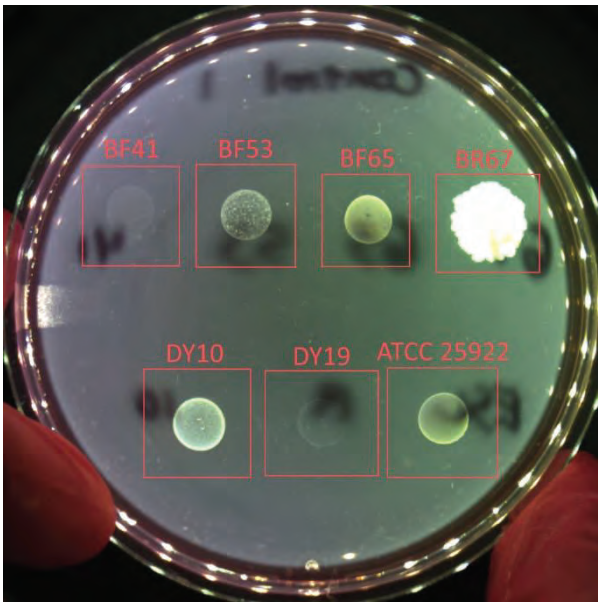
Table C-3. Agar Dilution Antibiotic Results and Susceptibility Photos: Clindamycin

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

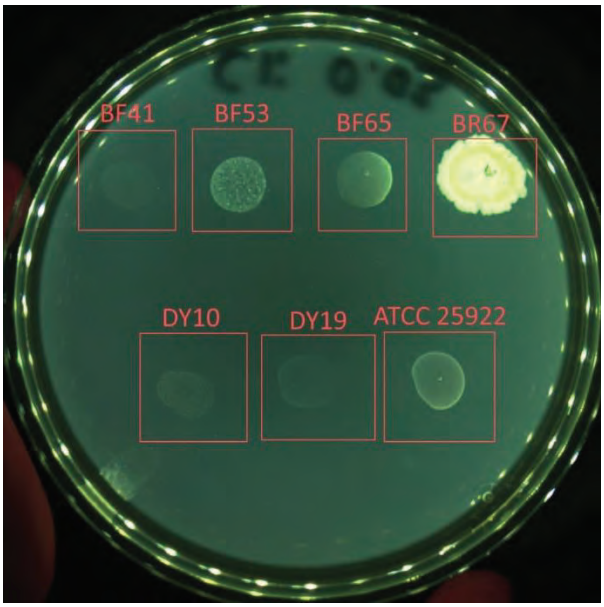
G = Growth

NG = No Growth

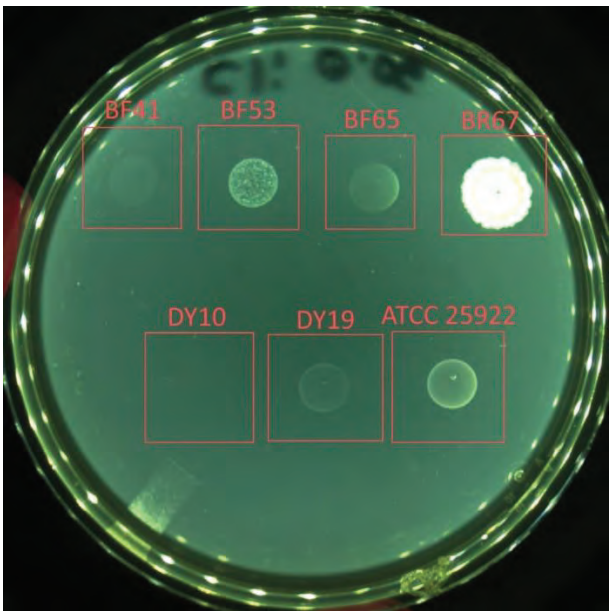
Agar Dilution Antibiotic Susceptibility Photos: Clindamycin



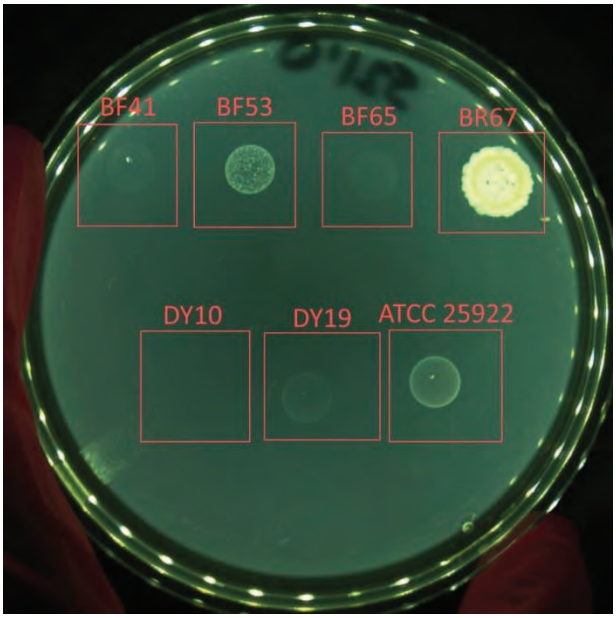
0 µg/mL Clindamycin



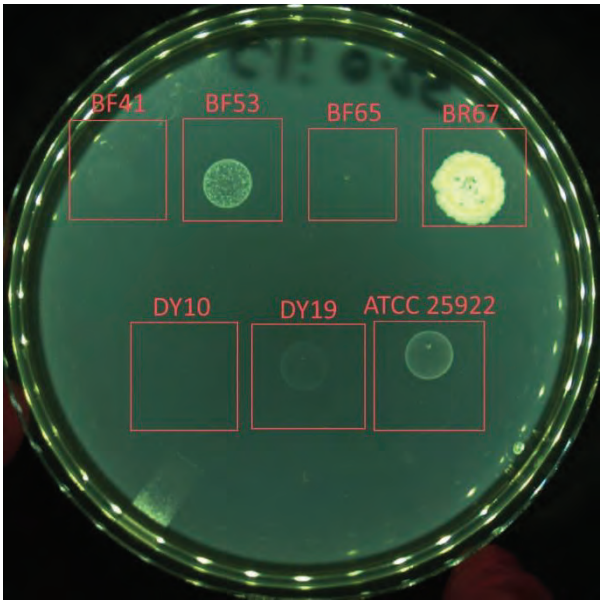
0.03125 µg/mL Clindamycin



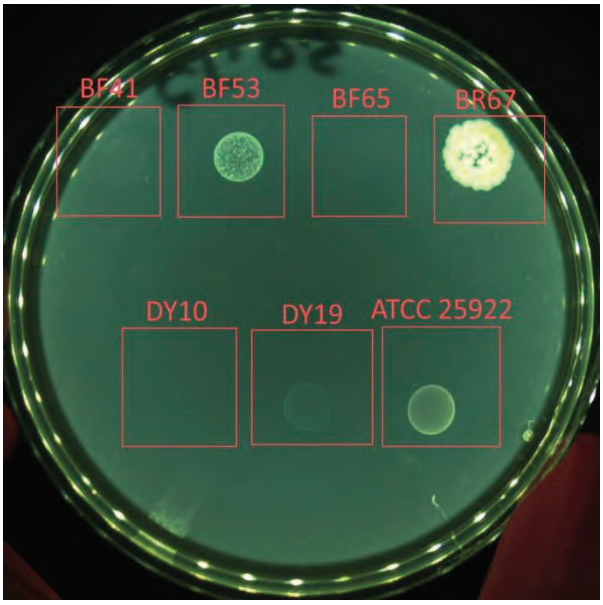
0.0625 µg/mL Clindamycin



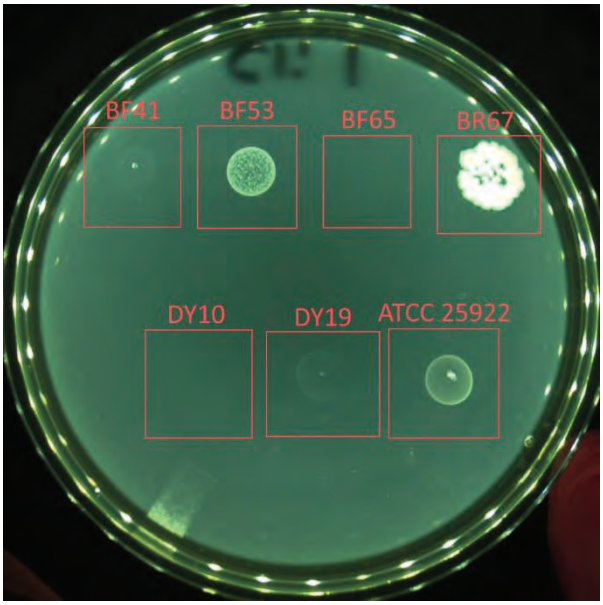
0.125 µg/mL Clindamycin



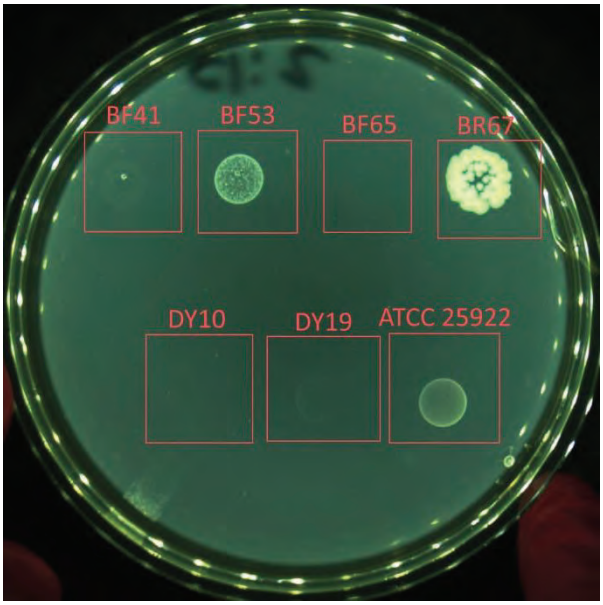
0.25 µg/mL Clindamycin



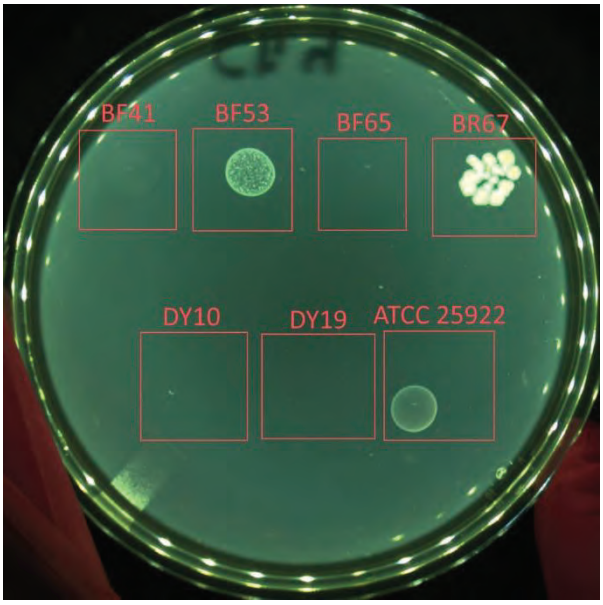
0.5 µg/mL Clindamycin



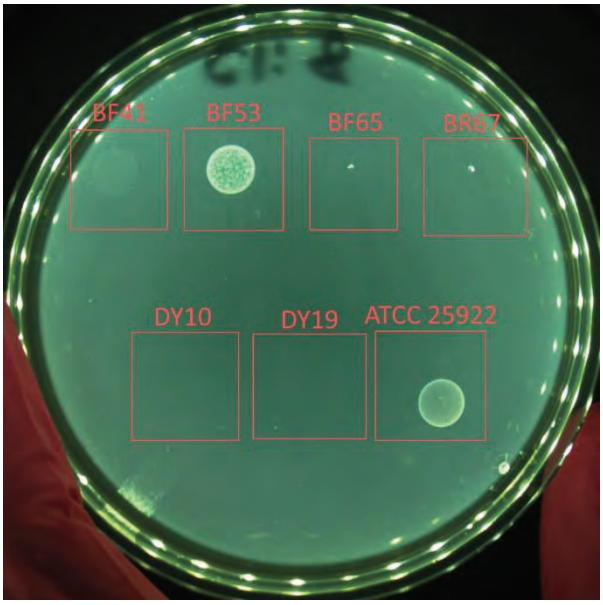
1 µg/mL Clindamycin



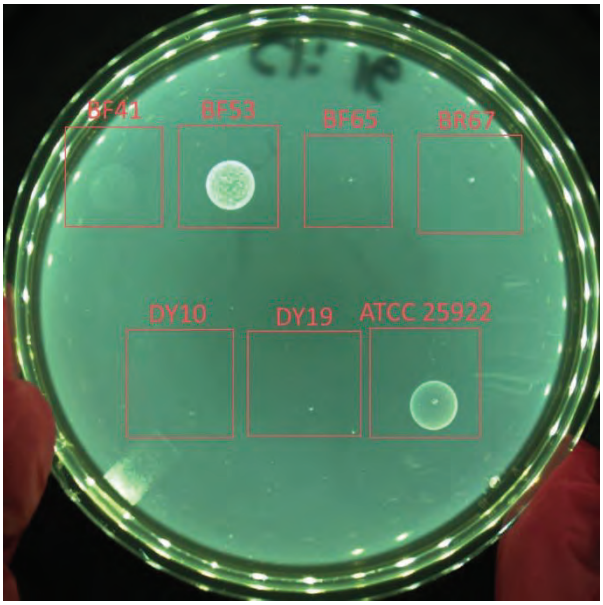
2 µg/mL Clindamycin



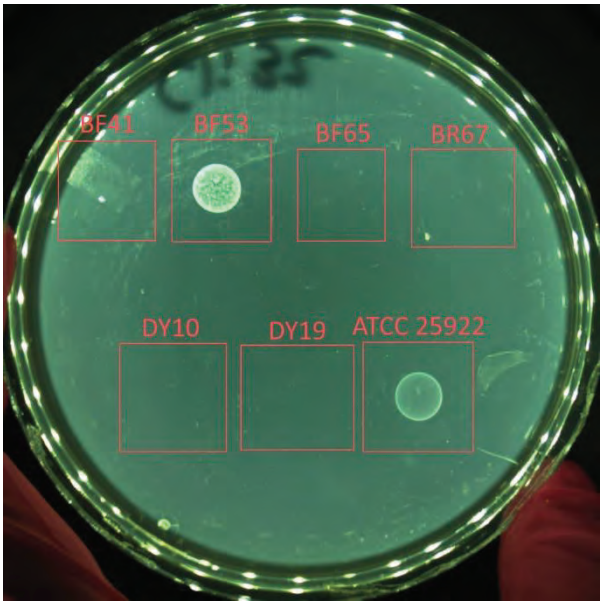
4 µg/mL Clindamycin



8 µg/mL Clindamycin



16 µg/mL Clindamycin



32 µg/mL Clindamycin

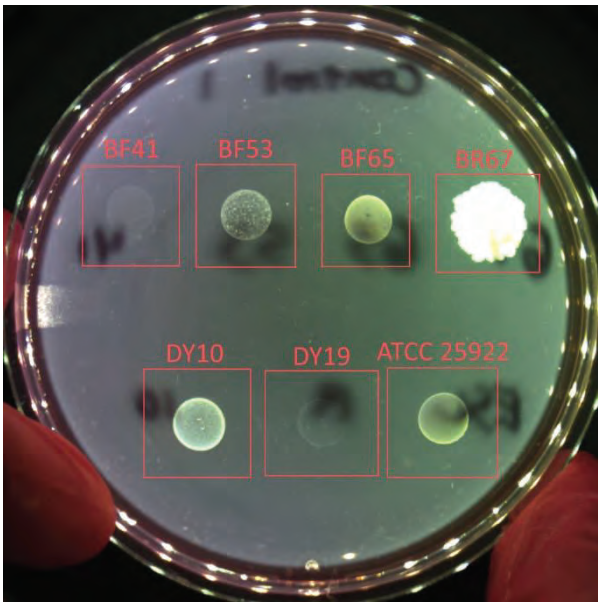
Table C-4. Agar Dilution Antibiotic Results and Susceptibility Photos: Erythromycin

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

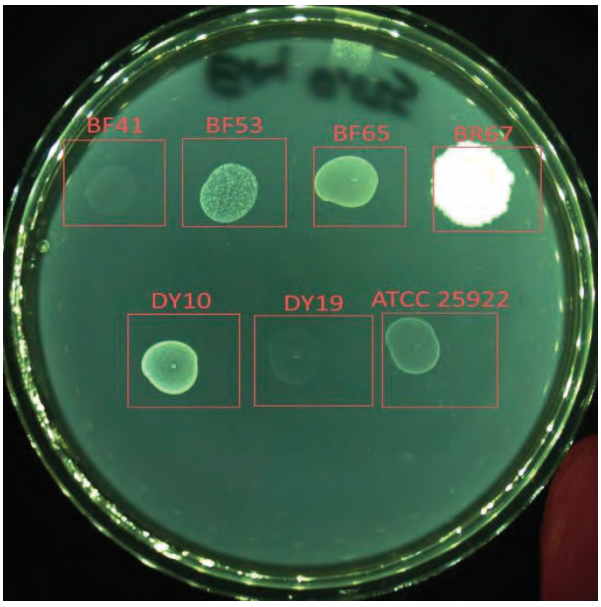
G = Growth

NG = No Growth

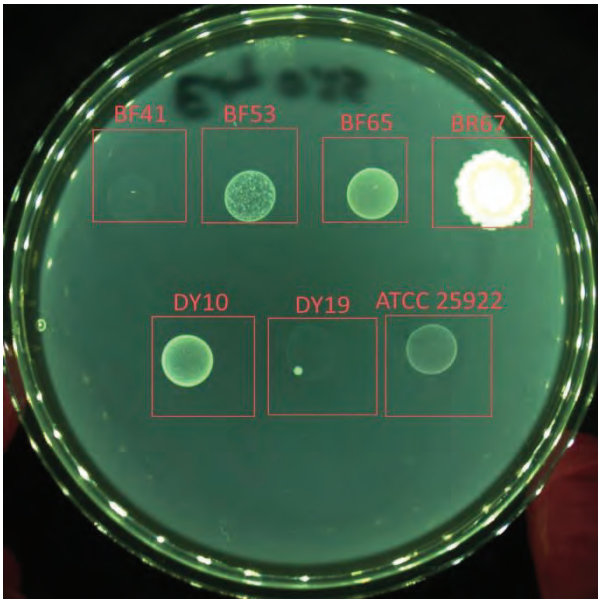
Agar Dilution Antibiotic Susceptibility Photos: Erythromycin



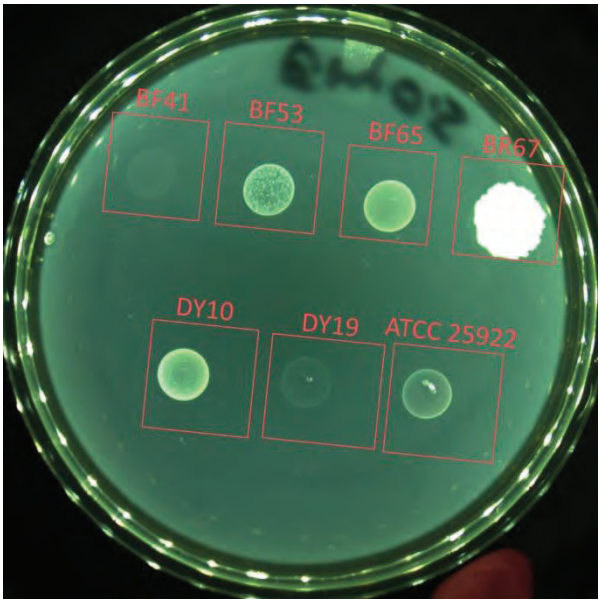
0 µg/mL Erythromycin



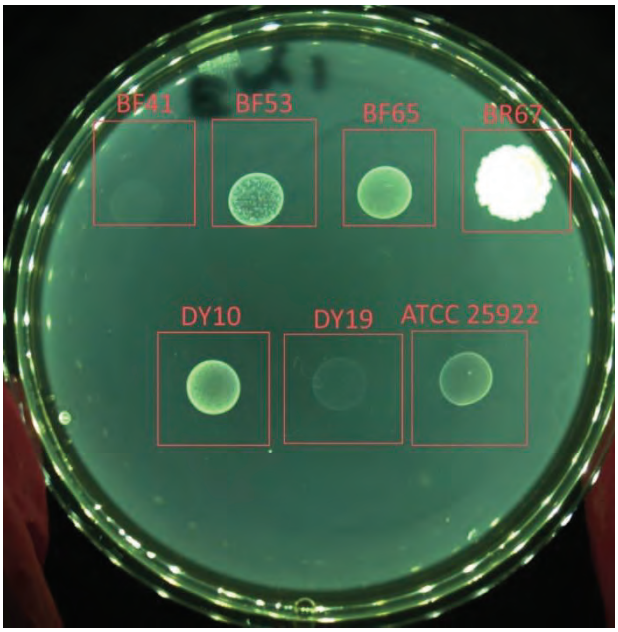
0.125 µg/mL Erythromycin



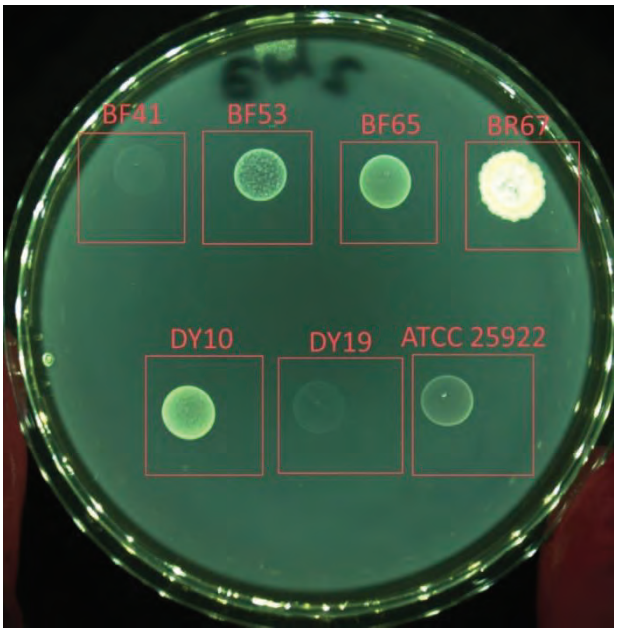
0.25 µg/mL Erythromycin



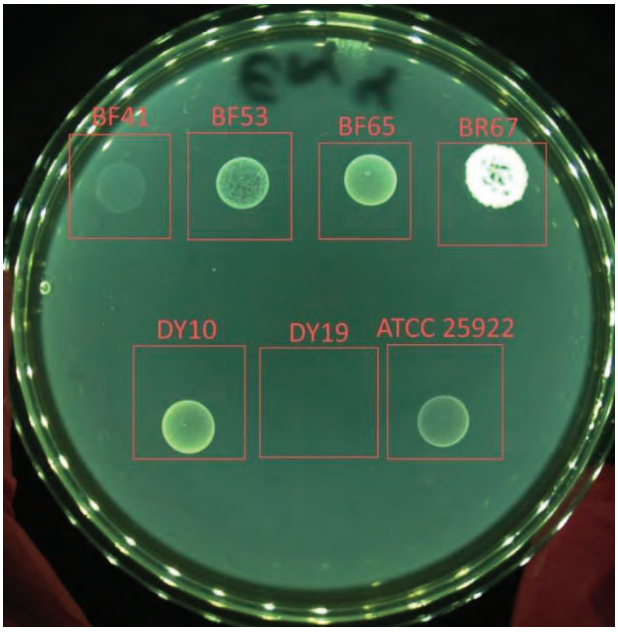
0.5 µg/mL Erythromycin



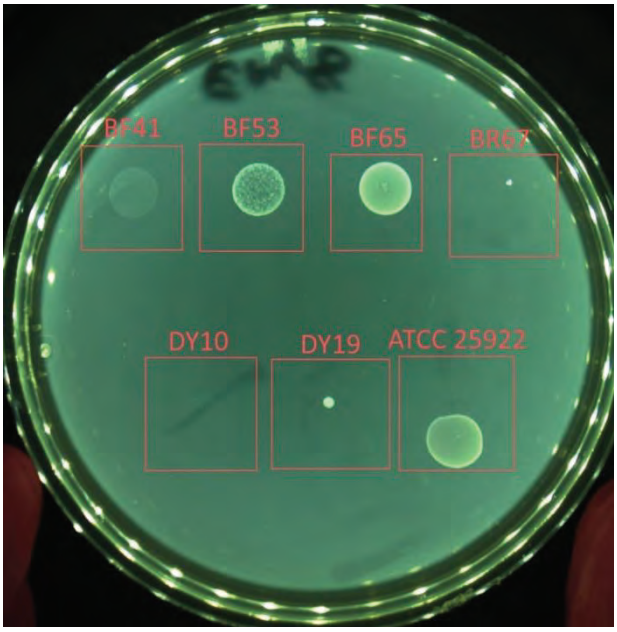
1 µg/mL Erythromycin



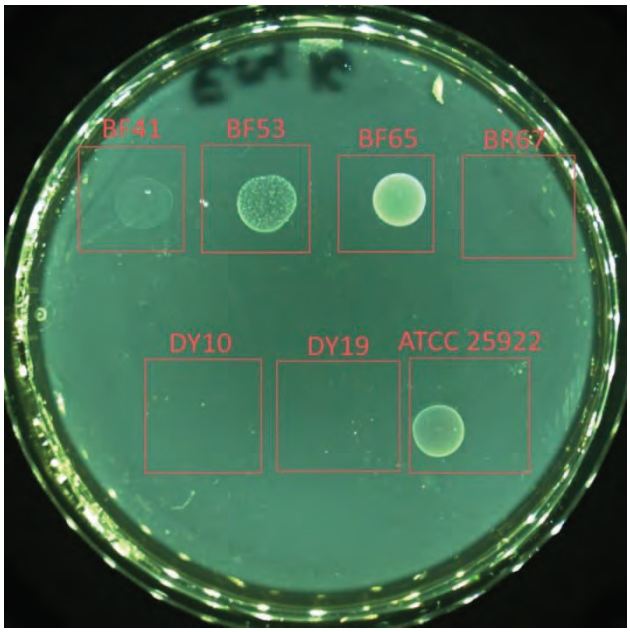
2 µg/mL Erythromycin



4 µg/mL Erythromycin



8 µg/mL Erythromycin



16 µg/ml Erythromycin

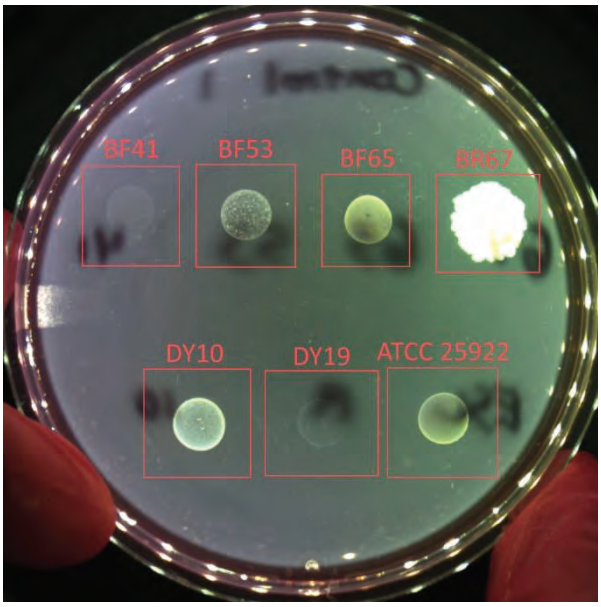
Table C-5. Agar Dilution Antibiotic Results and Susceptibility Photos: Gentamicin

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

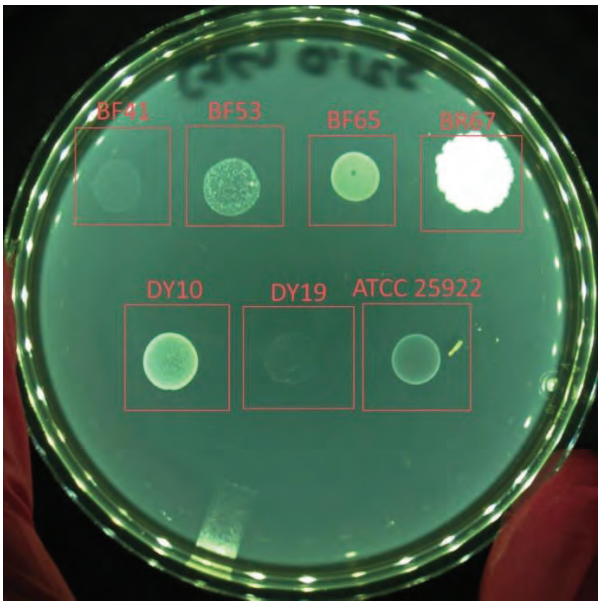
G = Growth

NG = No Growth

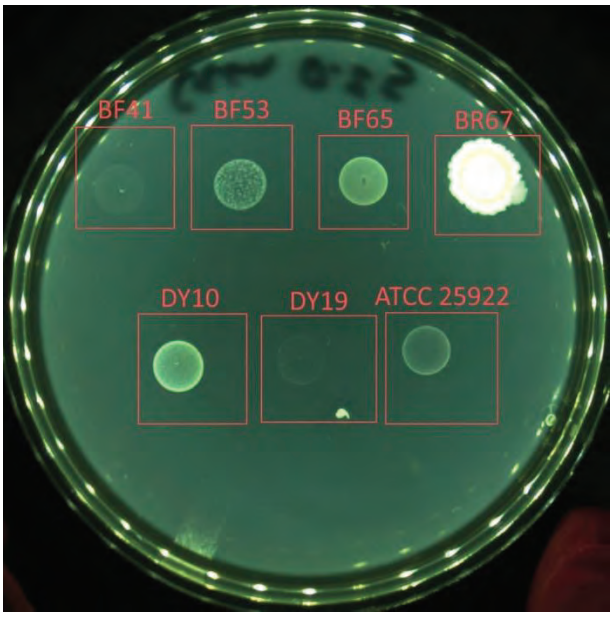
Agar Dilution Antibiotic Susceptibility Photos: Gentamicin



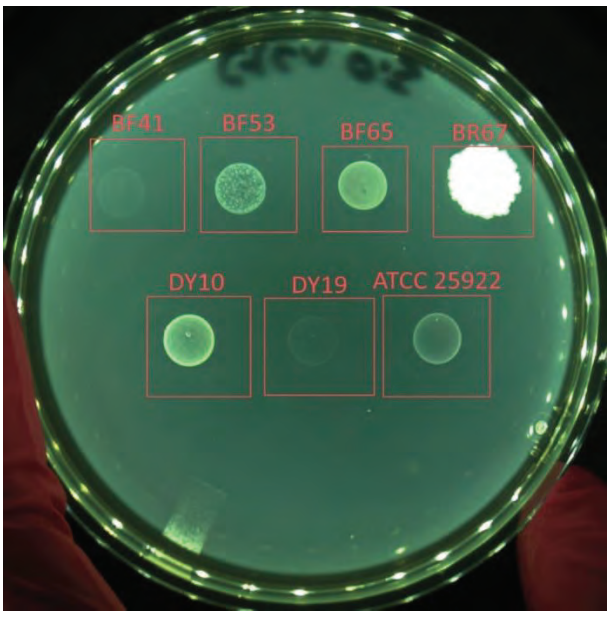
0 µg/mL Gentamicin



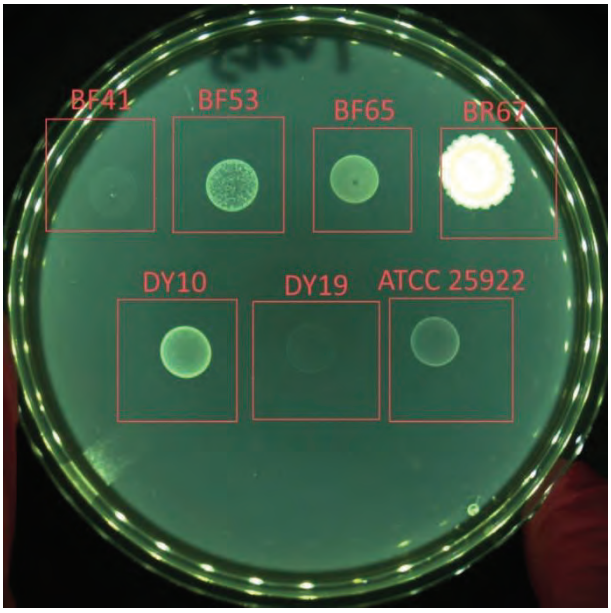
0.125 µg/mL Gentamicin



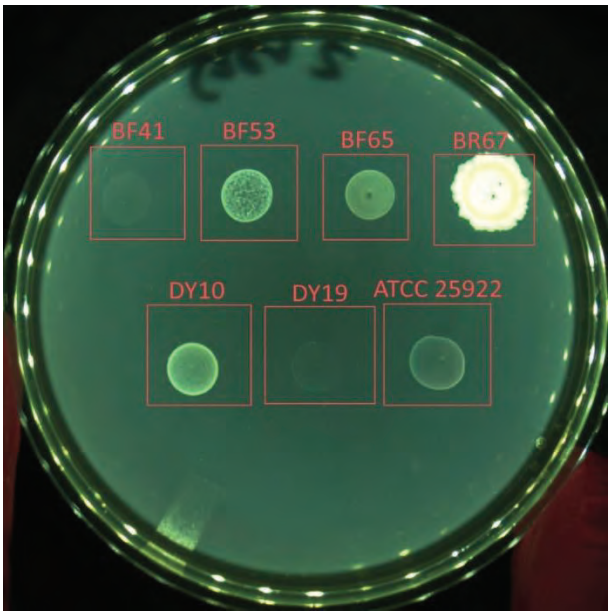
0.25 µg/mL Gentamicin



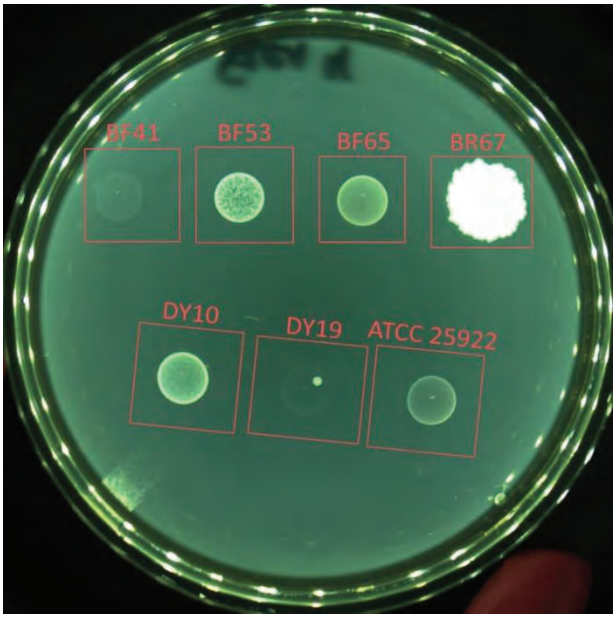
0.5 µg/mL Gentamicin



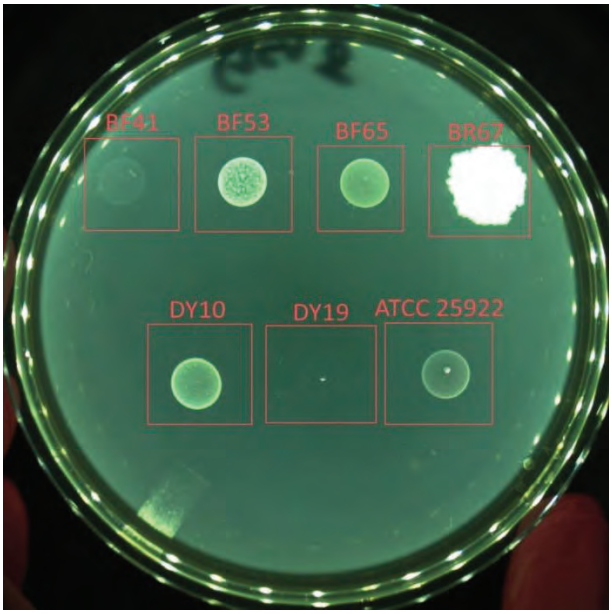
1 µg/mL Gentamicin



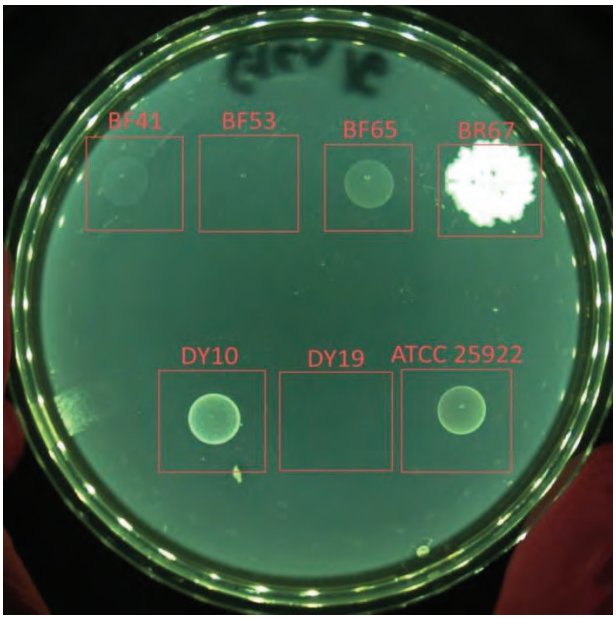
2 µg/mL Gentamicin



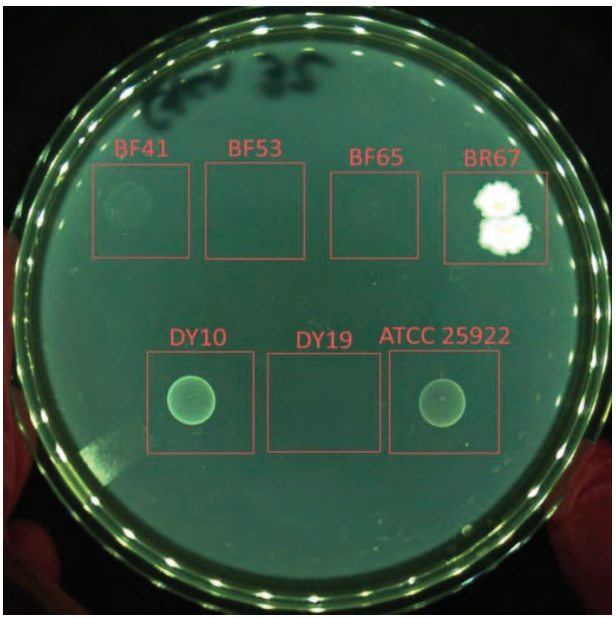
4 µg/mL Gentamicin



8 µg/mL Gentamicin



16 µg/mL Gentamicin



32 µg/mL Gentamicin

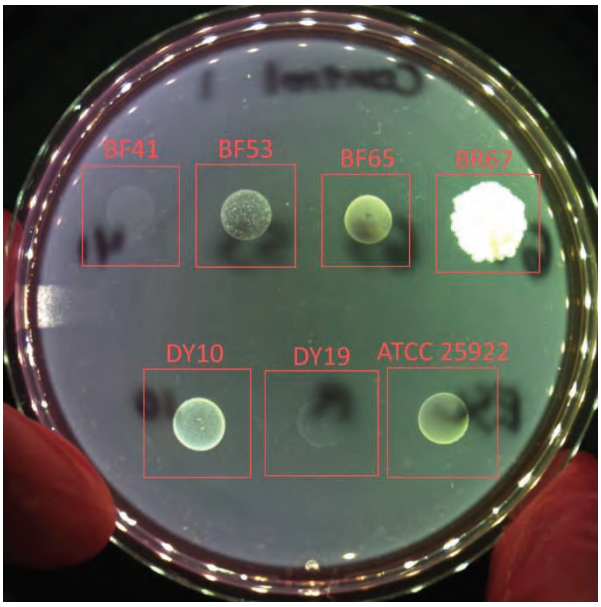
Table C-6. Agar Dilution Antibiotic Results and Susceptibility Photos: Kanamycin

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

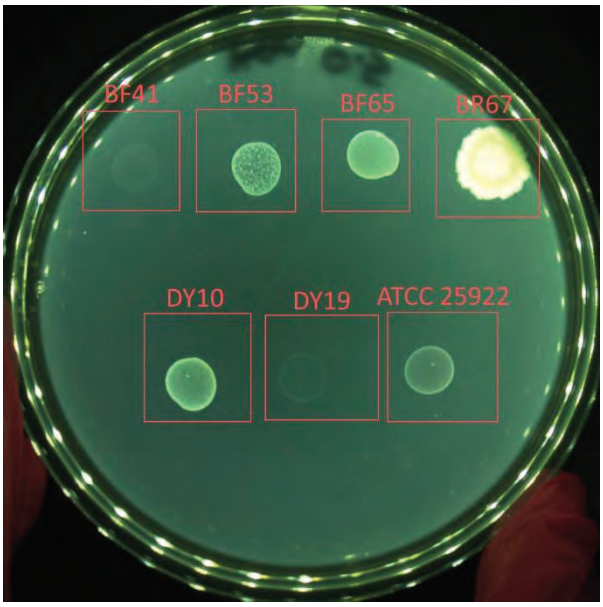
G = Growth

NG = No Growth

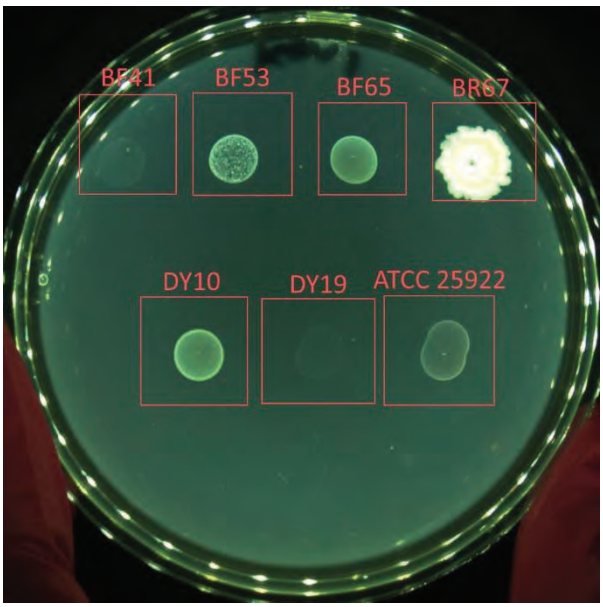
Agar Dilution Antibiotic Susceptibility Photos: Kanamycin



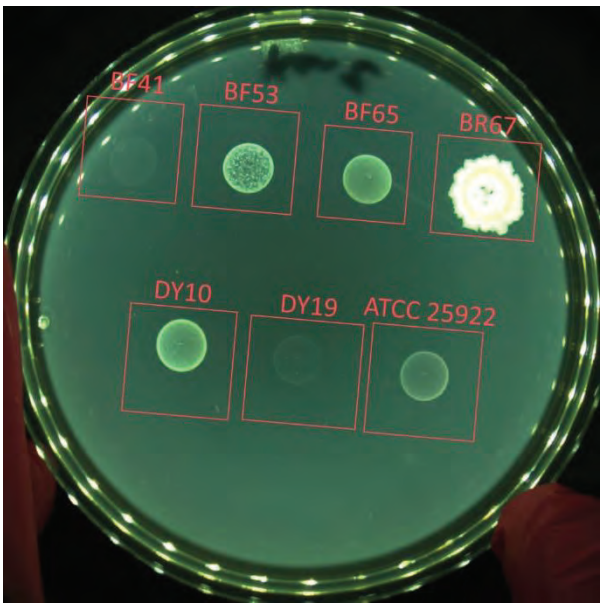
0 µg/mL Kanamycin



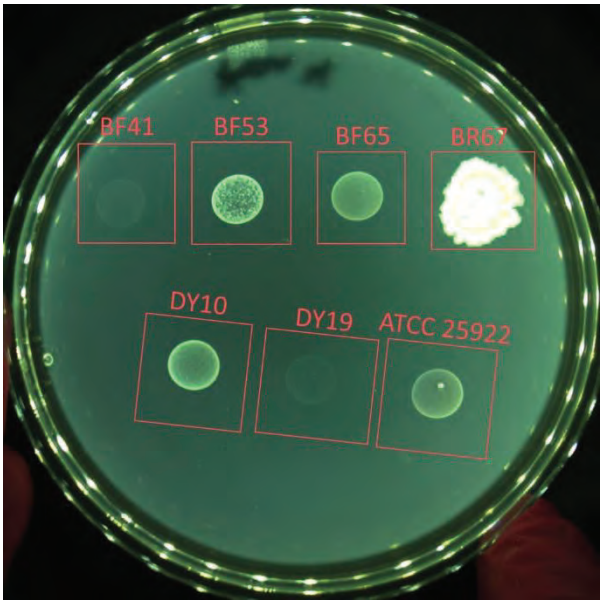
0.5 µg/mL Kanamycin



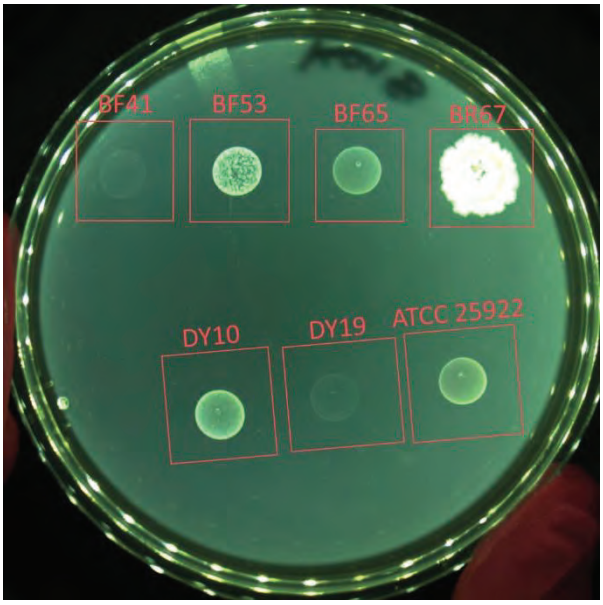
1 µg/mL Kanamycin



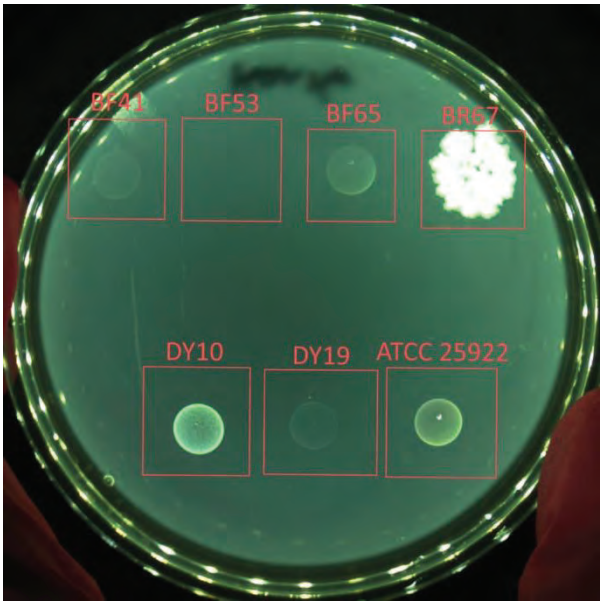
2 µg/mL Kanamycin



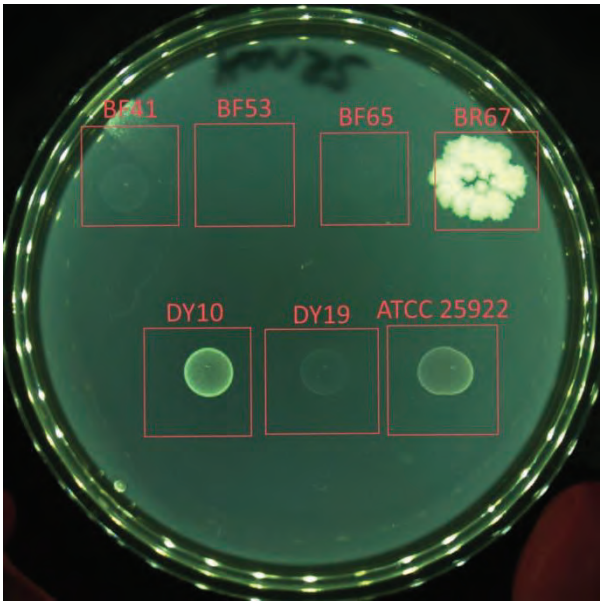
4 µg/mL Kanamycin



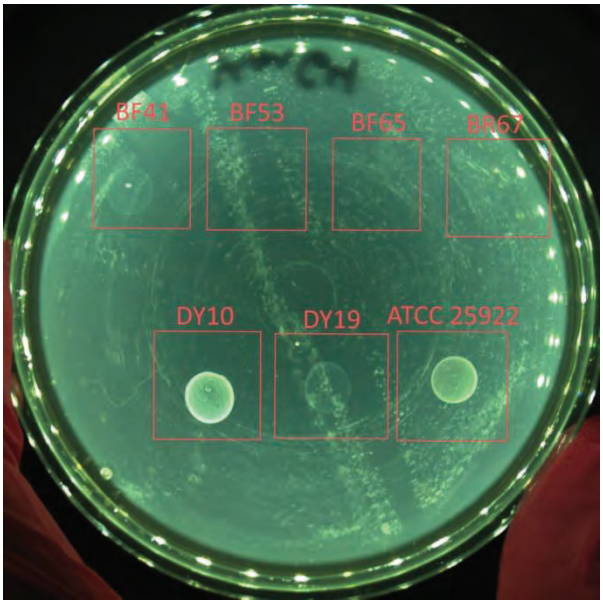
8 µg/mL Kanamycin



16 µg/mL Kanamycin



32 µg/mL Kanamycin



64 µg/mL Kanamycin

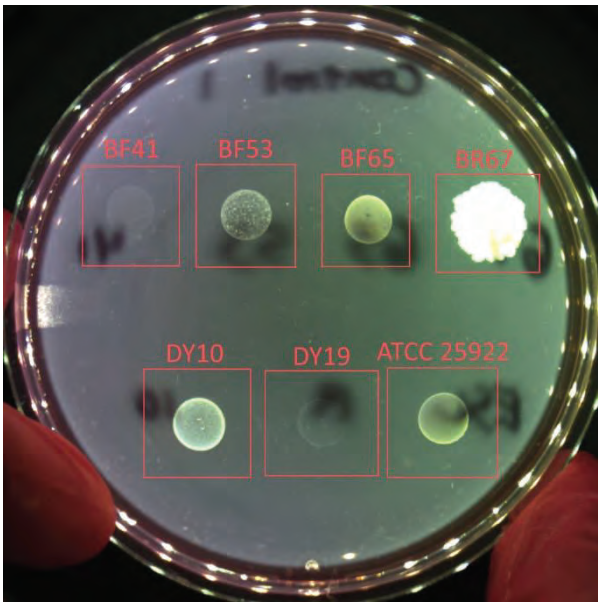
Table C-7. Agar Dilution Antibiotic Results and Susceptibility Photos: Streptomycin

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

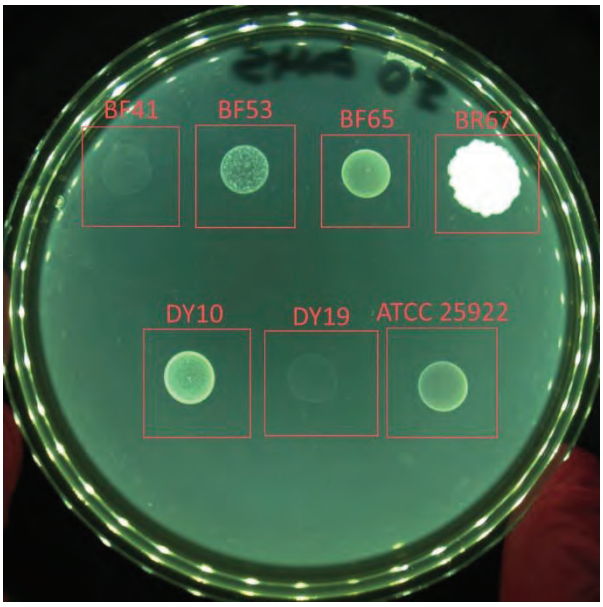
G = Growth

NG = No Growth

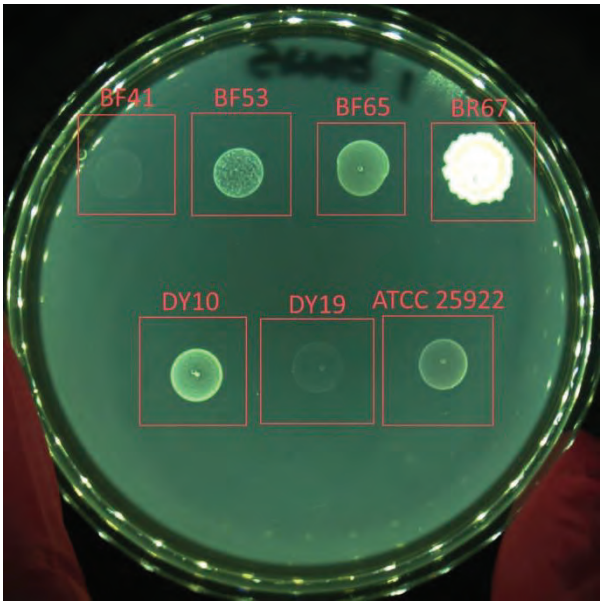
Agar Dilution Antibiotic Susceptibility Photos: Streptomycin



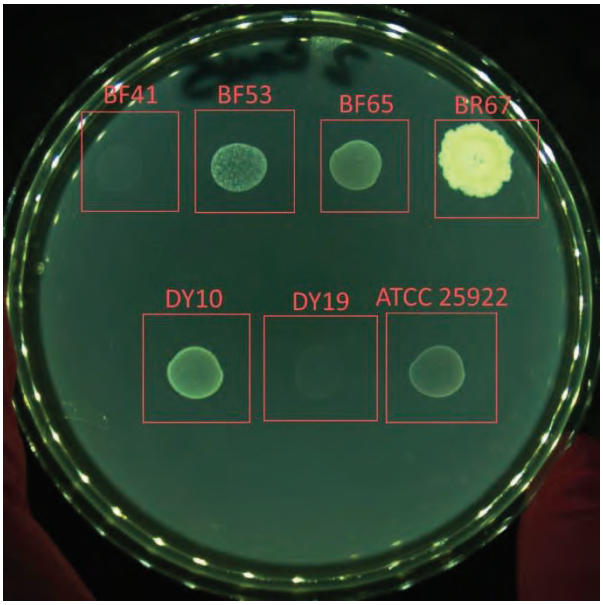
0 µg/mL Streptomycin



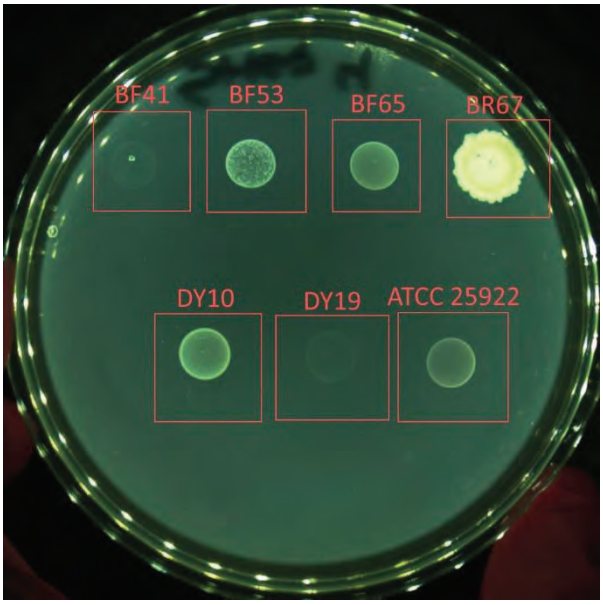
0.5 µg/mL Streptomycin



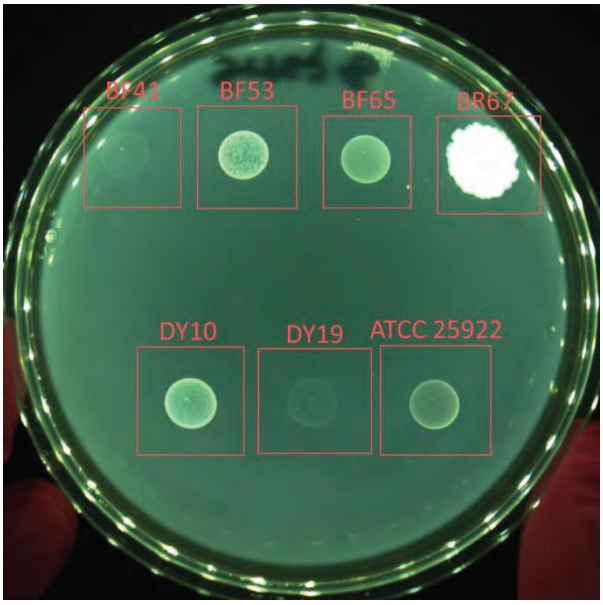
1 µg/mL Streptomycin



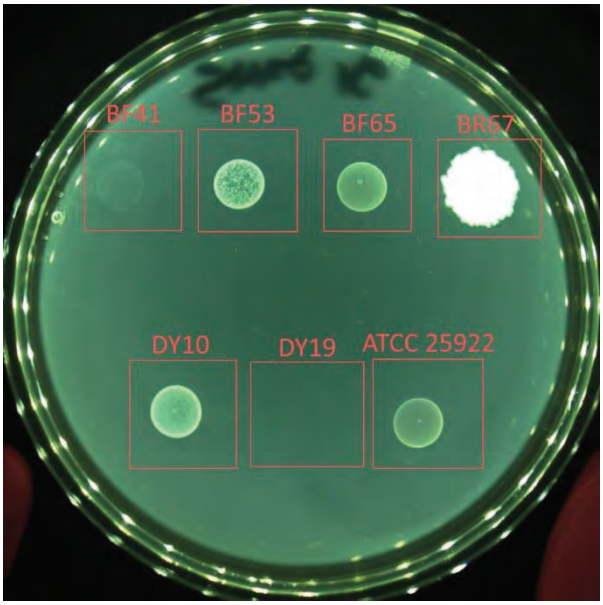
2 µg/mL Streptomycin



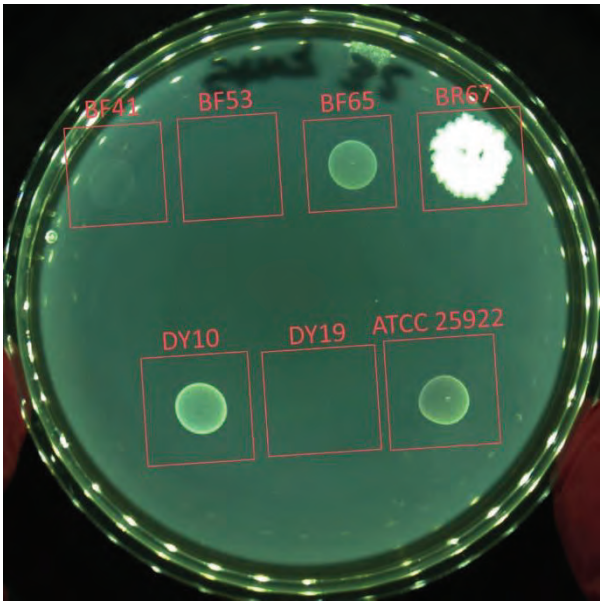
4 µg/mL Streptomycin



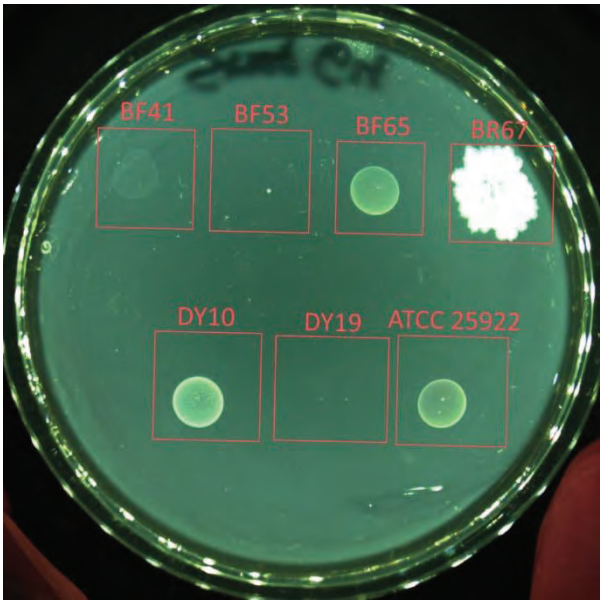
8 µg/mL Streptomycin



16 µg/mL Streptomycin



32 µg/mL Streptomycin



64 µg/mL Streptomycin

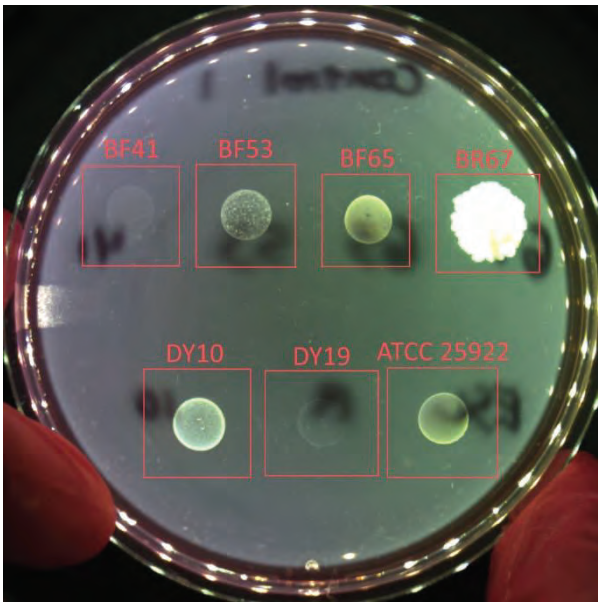
Table C-8. Agar Dilution Antibiotic Results and Susceptibility Photos: Tetracycline

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

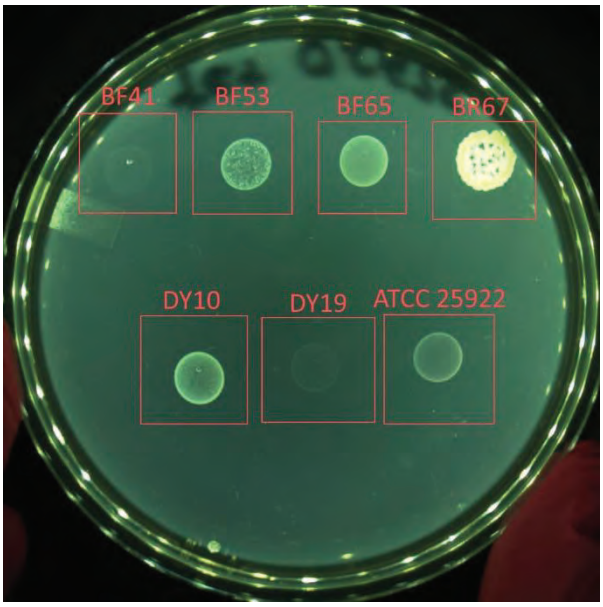
G = Growth

NG = No Growth

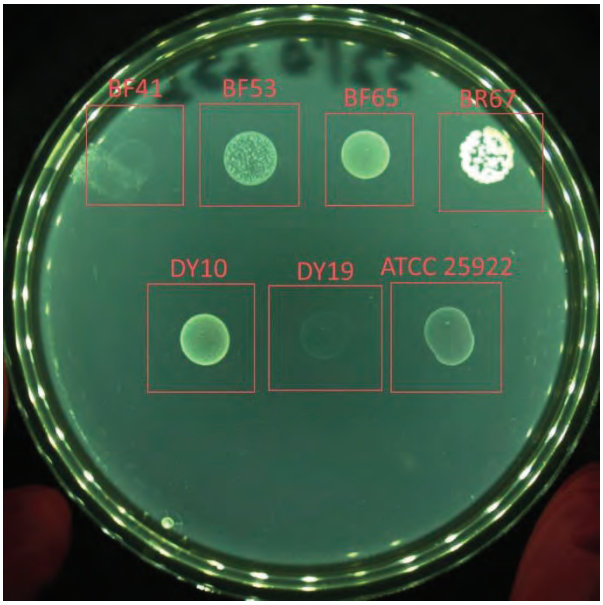
Agar Dilution Antibiotic Susceptibility Photos: Tetracycline



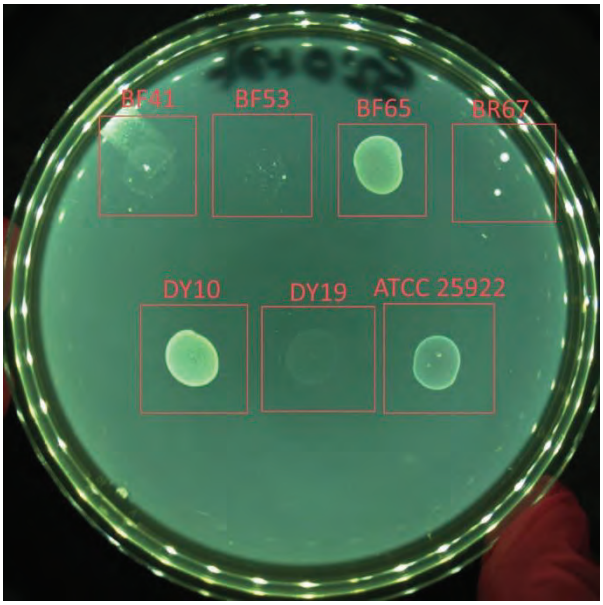
0 µg/mL Tetracycline



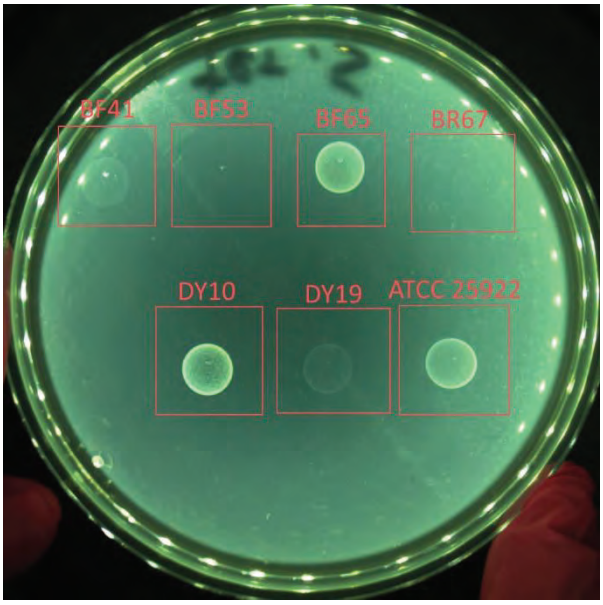
0.0625 µg/mL Tetracycline



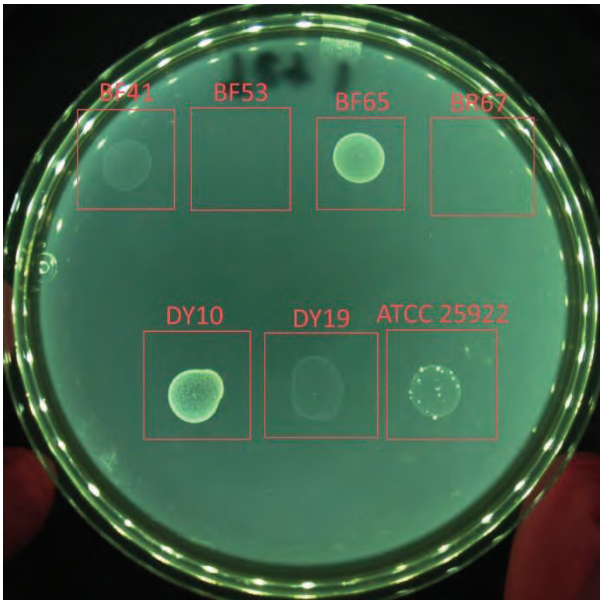
0.125 µg/mL Tetracycline



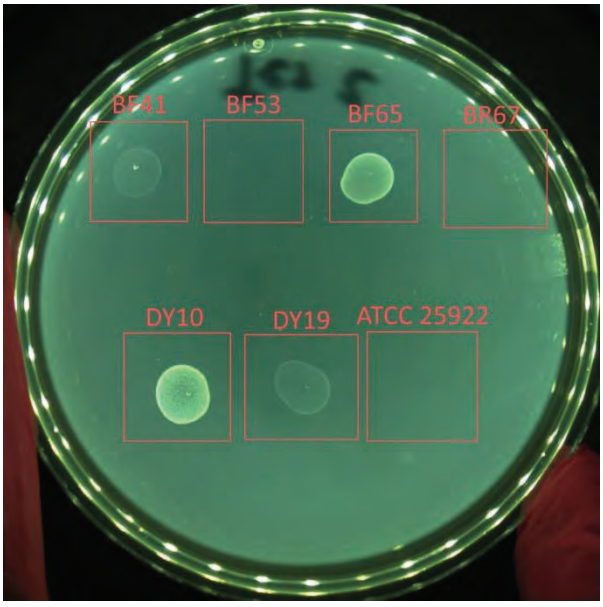
0.25 µg/mL Tetracycline



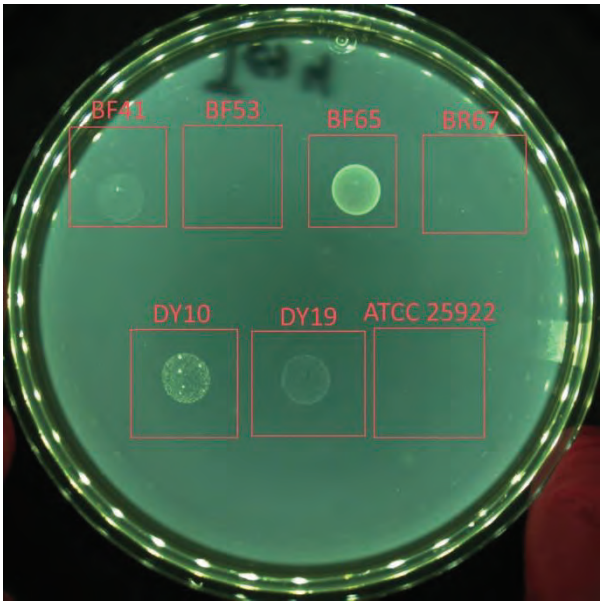
0.5 µg/mL Tetracycline



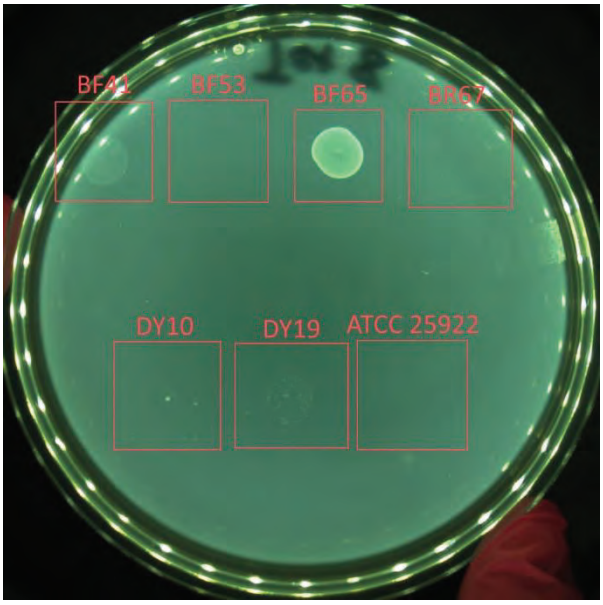
1 µg/mL Tetracycline



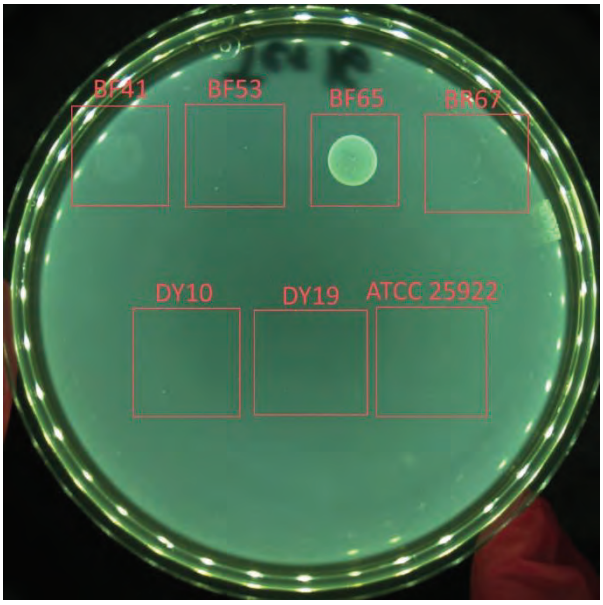
2 µg/mL Tetracycline



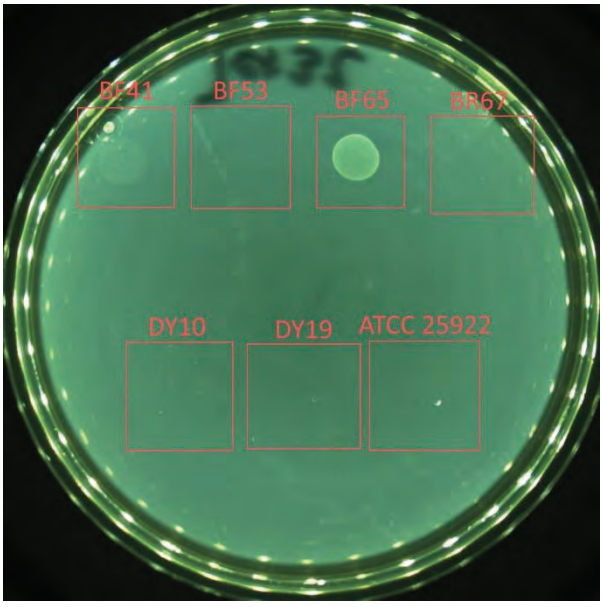
4 µg/mL Tetracycline



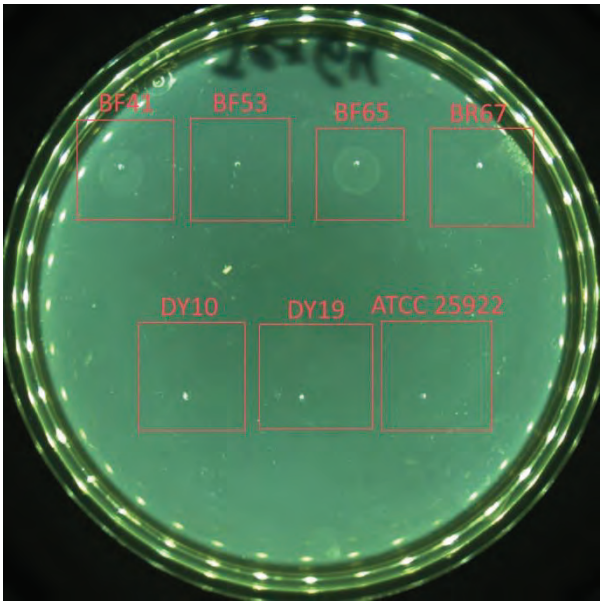
8 µg/mL Tetracycline



16 µg/mL Tetracycline



32 µg/mL Tetracycline



64 µg/mL Tetracycline

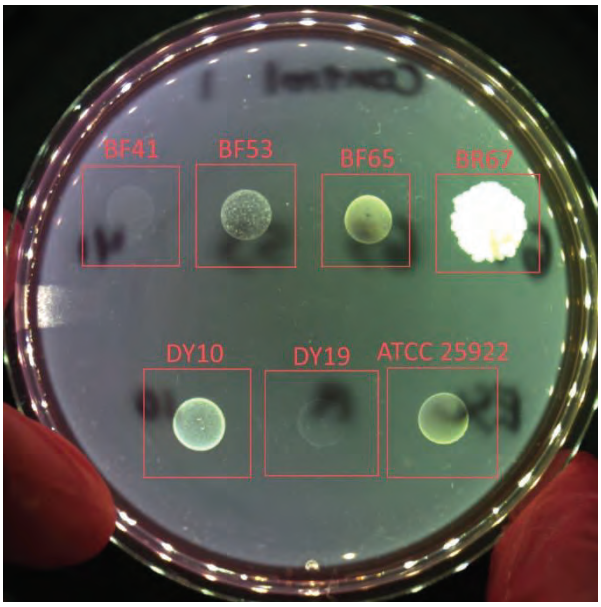
Table C-9. Agar Dilution Antibiotic Results and Susceptibility Photos: Vancomycin

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

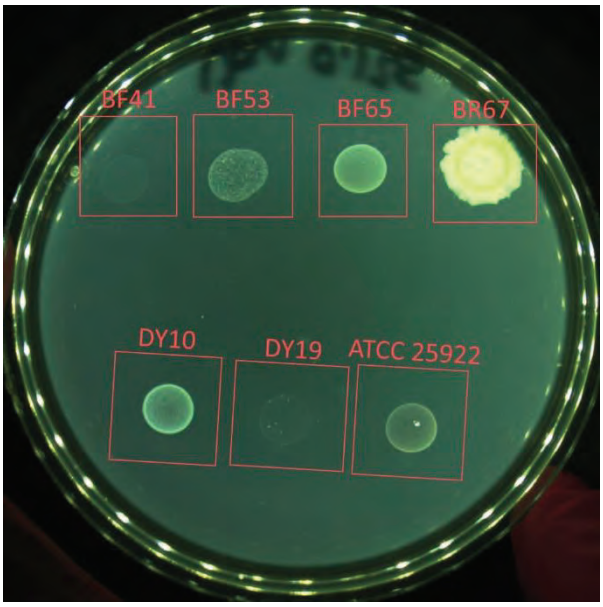
G = Growth

NG = No Growth

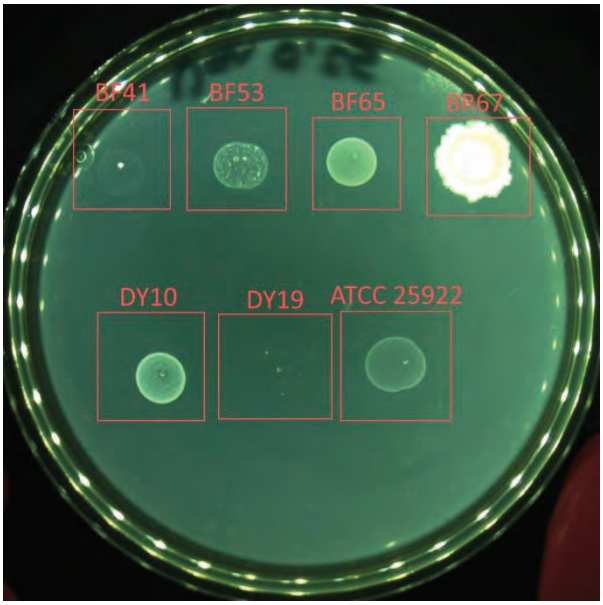
Agar Dilution Antibiotic Susceptibility Photos: Vancomycin



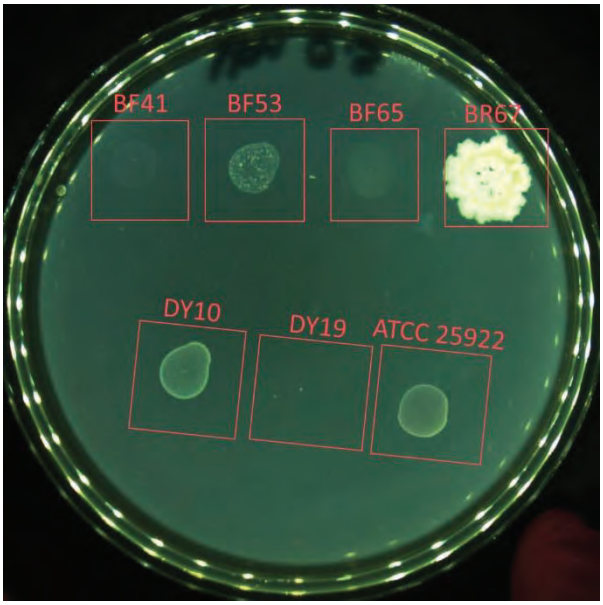
0 µg/mL Vancomycin



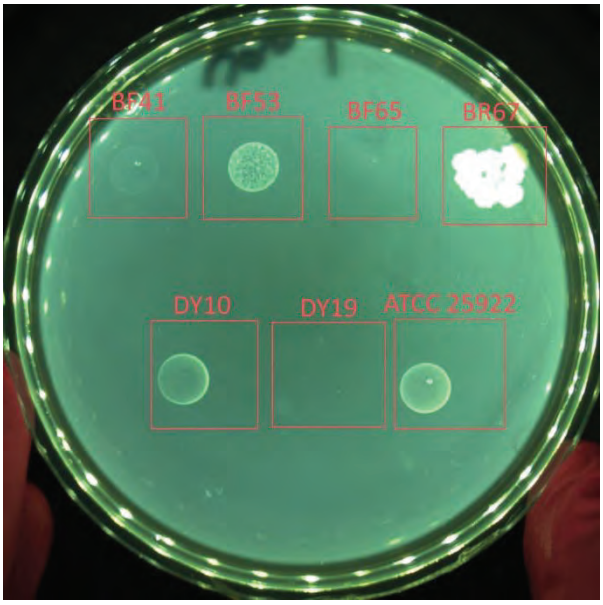
0.125 µg/mL Vancomycin



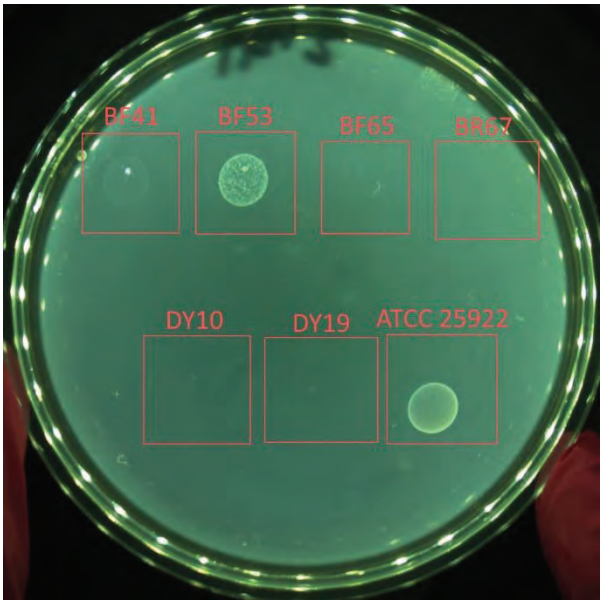
0.25 µg/mL Vancomycin



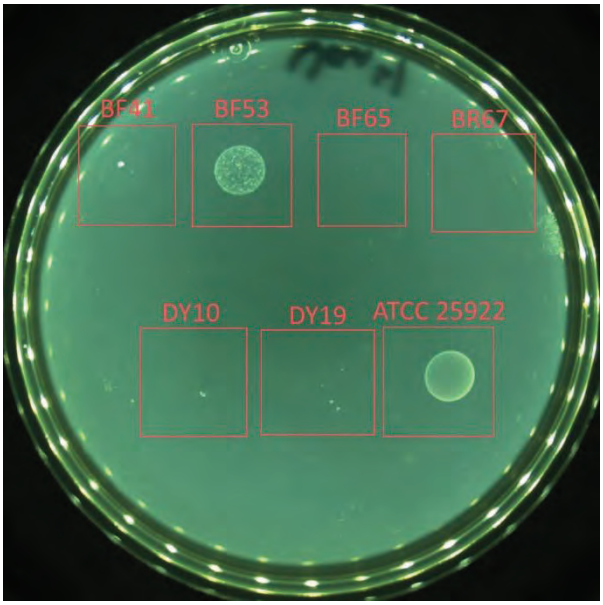
0.5 µg/mL Vancomycin



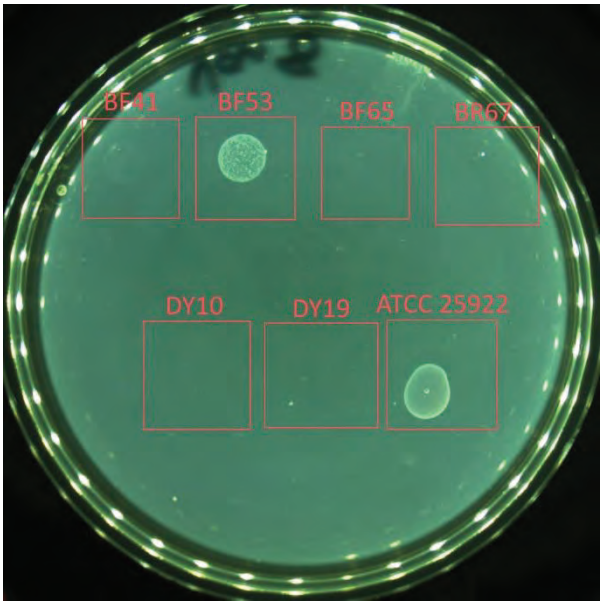
1 µg/mL Vancomycin



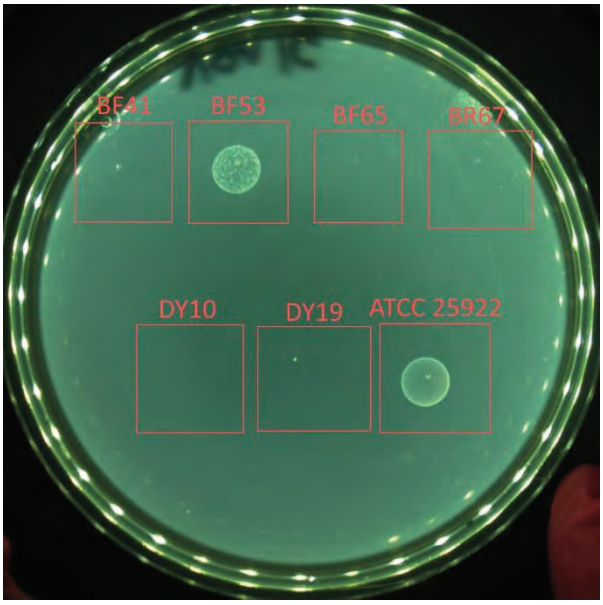
2 µg/mL Vancomycin



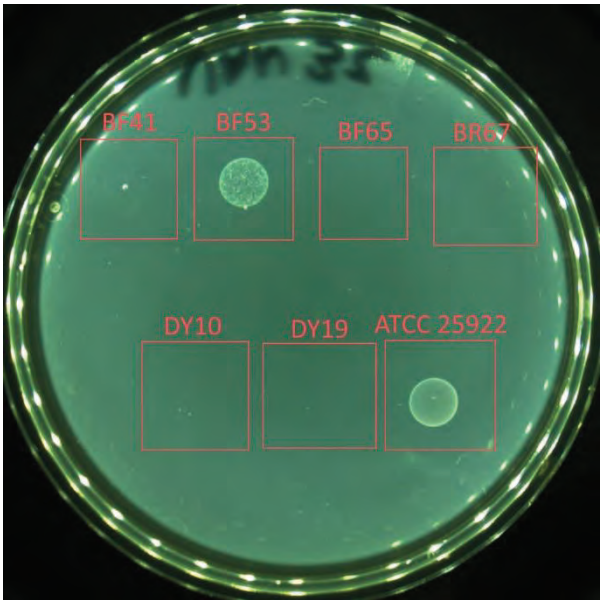
4 µg/mL Vancomycin



8 µg/mL Vancomycin



16 µg/mL Vancomycin



32 µg/mL Vancomycin

(b)
(4)

[redacted] (b) (4)

FINAL REPORT

TITLE: **Characterization of Ascus Biosciences *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19) Production Strain: Absence of Antimicrobial Activity**

INVESTIGATOR'S STUDY NUMBER: [redacted] (b) (4)

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

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

INVESTIGATOR: [redacted] (b) (4)

VERSION: FINAL

SIGNATURE: [redacted] (b) (4) 2/21/20
Date

[redacted] (b) (4)
Principal Investigator

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LIST OF TABLES AND APPENDICES

OBJECTIVES

To determine the antimicrobial properties of the *Butyrivibrio fibrisolvens* **ASCUSDY19** (Dairy-19) production strain supernatant.

STANDARDS OF COMPLIANCE

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

STUDY SITE

Antimicrobial property testing of the product was performed by [redacted]

MATERIALS

The sponsor-provided Dairy-19 supernatant (Lot number 20191105_V1) was prepared by centrifugation at 16,100 RCF (13,200RPM) for 20 minutes followed by sterile filtration with a 0.22µm membrane. The sample was received on November 20, 2019.

ANTIMICROBIAL PROPERTIES

A portion of the growth medium from a typical production batch of the *Butyrivibrio fibrisolvens* ASCUSDY19 (Dairy-19), or a scaled down version, was kept refrigerated (2-8°C) and shipped to [redacted] and used 13 days after receipt.

1.1. Preparation of Culture Plates

The following six organisms were tested against the supernatant:

Organism	ATCC number	[redacted]ode	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 Dairy-19 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 Dairy-19 Supernatant and Controls

Organism	ATCC number	(b) (4)	Zone Diameter for the indicated solution (mm)		
			Dairy-19 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 Dairy-19 supernatant exhibited no antibacterial activity against the 6 strains representative of Gram positive and Gram negative bacteria.

APPENDIX A. Protocol

[redacted]

STUDY PROTOCOL

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

TABLE OF CONTENTS

SIGNATURES

Sponsor Representative

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

Signature 11/14/19 Date

Investigator

(b) (4)

(b) (4)

Signature 11/14/19 Date

1. OBJECTIVES

Determination of the antimicrobial properties of various production strain supernatants.

2. STUDY TIMELINE

Anticipated study dates are:
Antimicrobial Properties: November 2019

3. STANDARDS OF COMPLIANCE

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

4. STUDY SITE

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

5. MATERIALS AND METHODS

5.1. Supernatant

It is anticipated that 5 supernatants will be provided by the Sponsor. The supernatants will be streaked onto trypticase soy agar containing (b) (4) (b) (4)

6. ABSENCE OF ANTIMICROBIAL PRODUCTION[1]

The presence of antimicrobial activity in the growth medium from the production strain supernatants will be tested. (b) (4) (b) (4)

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

6.1. Preparation of Culture Plates

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

Organism	ATCC number	(b) (4) ode	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)

6.2. Disk Preparation

(b) (4)

6.3. Incubation

(b) (4)

(b) (4)

6.4. Interpretation

(b) (4)

6.5. Quality Control

(b) (4)

7. RAW DATA, RECORDS, AND REPORTS

7.1. Data

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

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

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

7.2. Reporting of Results

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

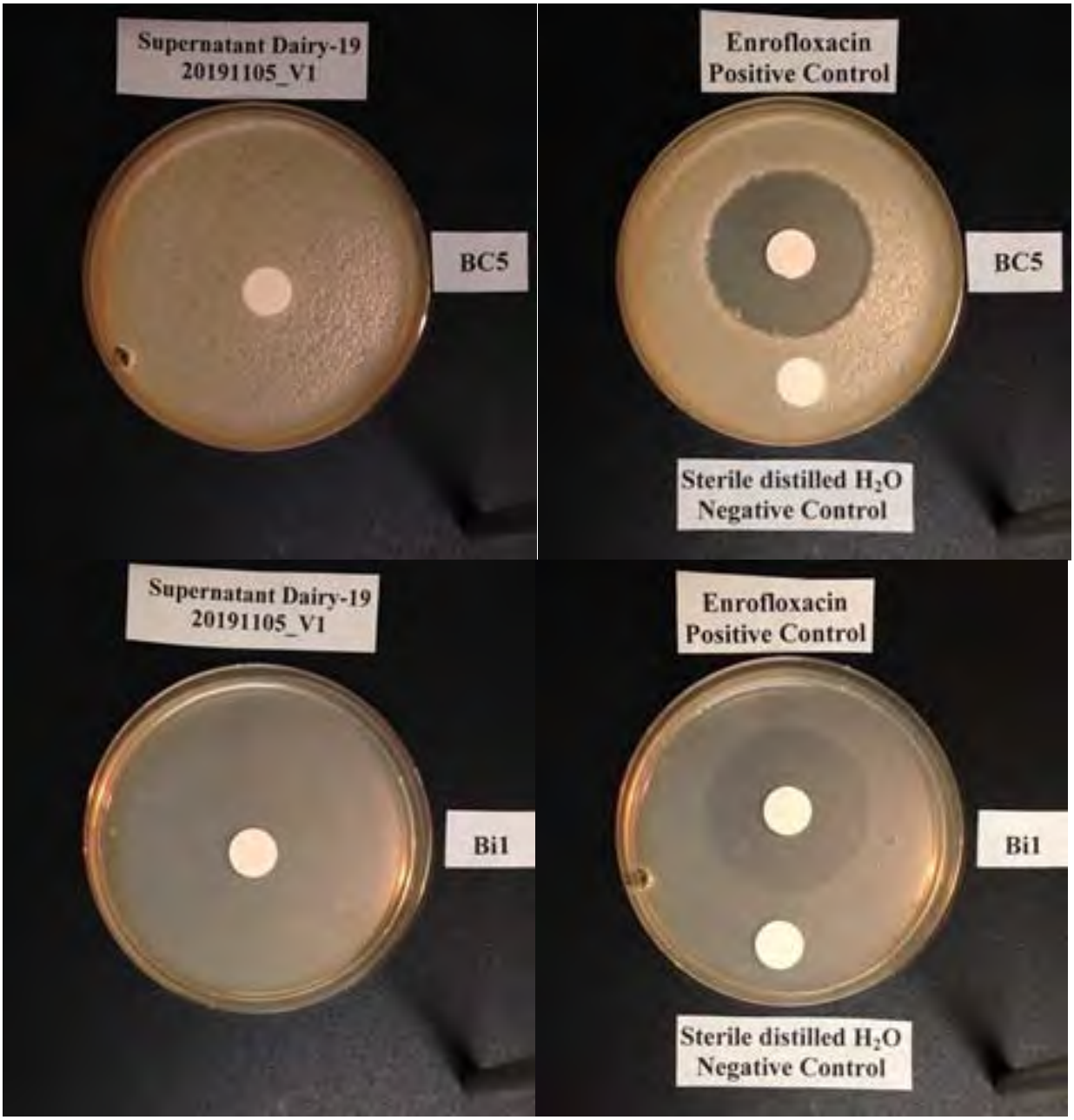
8. DISPOSITIONS

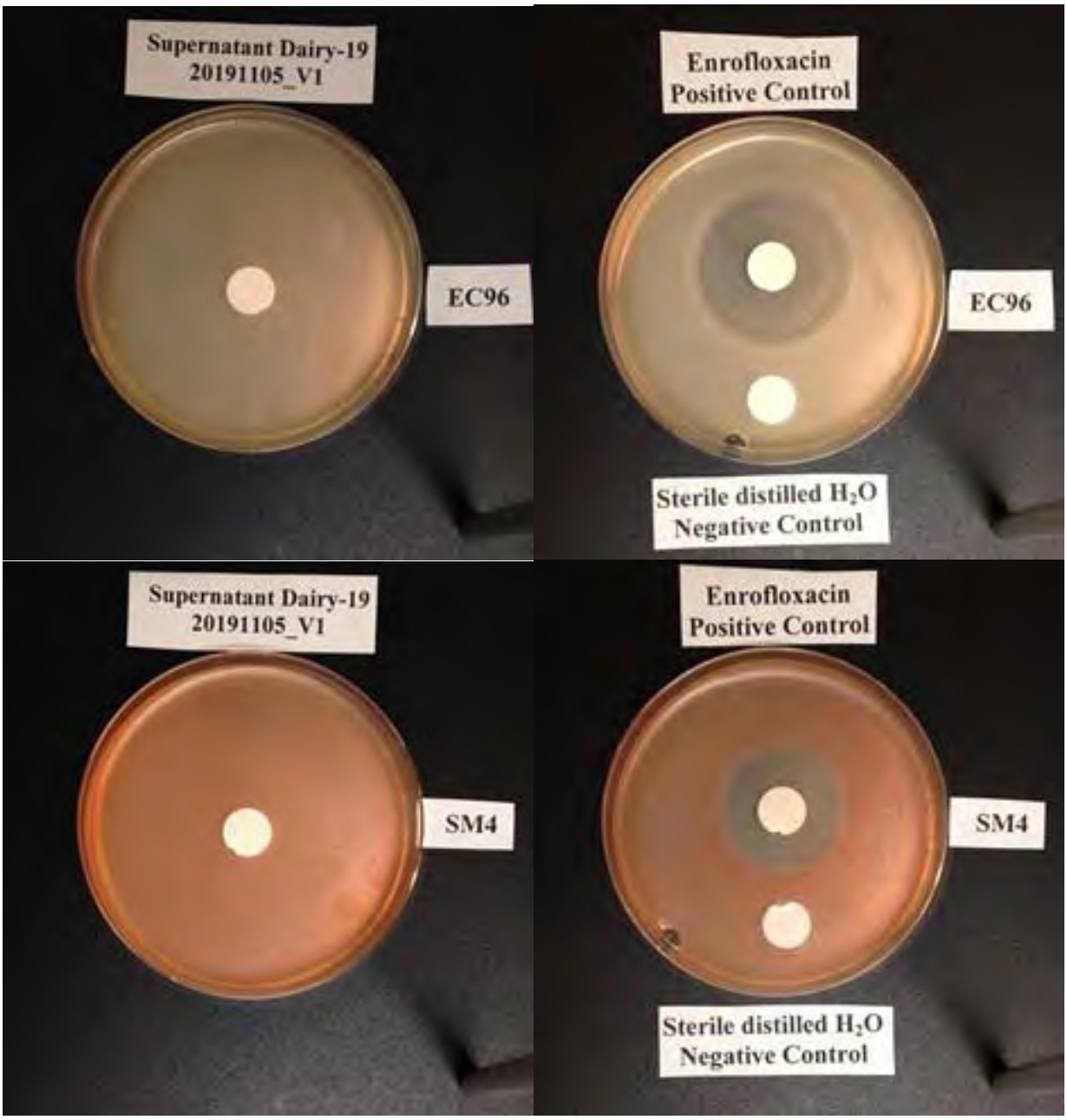
8.1. Supernatants
All surplus quantities of the provided supernatants will be discarded after autoclaving following report issue. No reserve samples will be maintained.

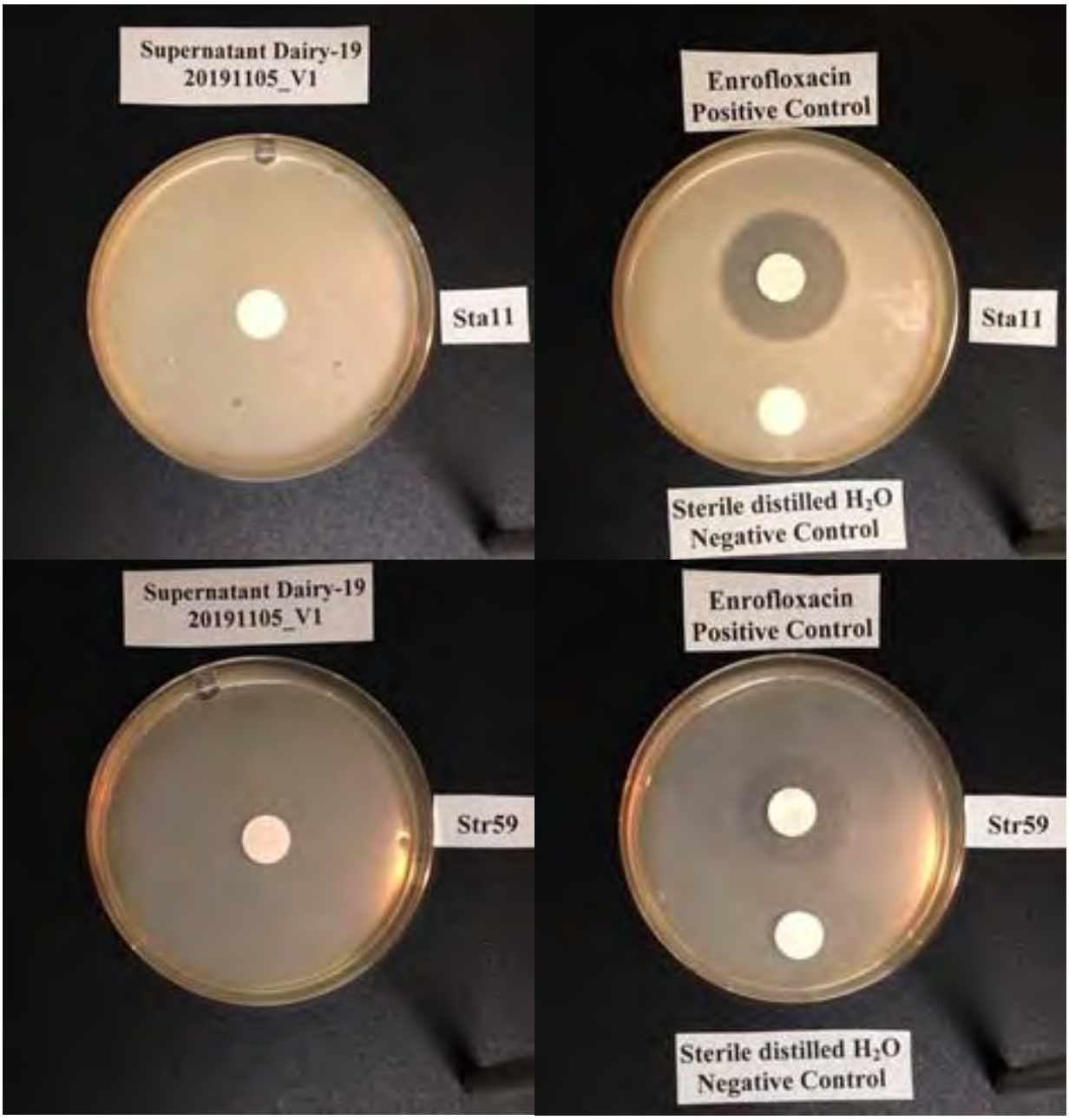
9. CHANGES TO PROTOCOL

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

APPENDIX B: Photos



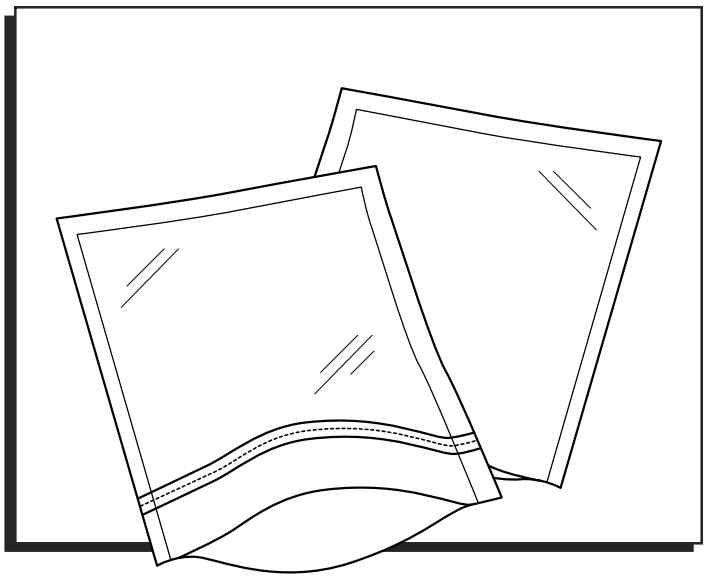

Supernatant Dairy-19
20191105_V1
EC96
Enrofloxacin
Positive Control
EC96
Sterile distilled H_2O
Negative Control
Supernatant Dairy-19
20191105_V1
SM4
Enrofloxacin
Positive Control
SM4
Sterile distilled H_2O
Negative Control


Supernatant Dairy-19
20191105_V1
Sta11
Enrofloxacin
Positive Control
Sta11
Sterile distilled H_2O
Negative Control
Supernatant Dairy-19
20191105_V1
Str59
Enrofloxacin
Positive Control
Str59
Sterile distilled H_2O
Negative Control

Para Español, vea página 2.
Pour le français, consulter la page 3.

ULINE S-18139
FOOD BAGS

1-800-295-5510
uline.com



PRODUCT SPECIFICATIONS

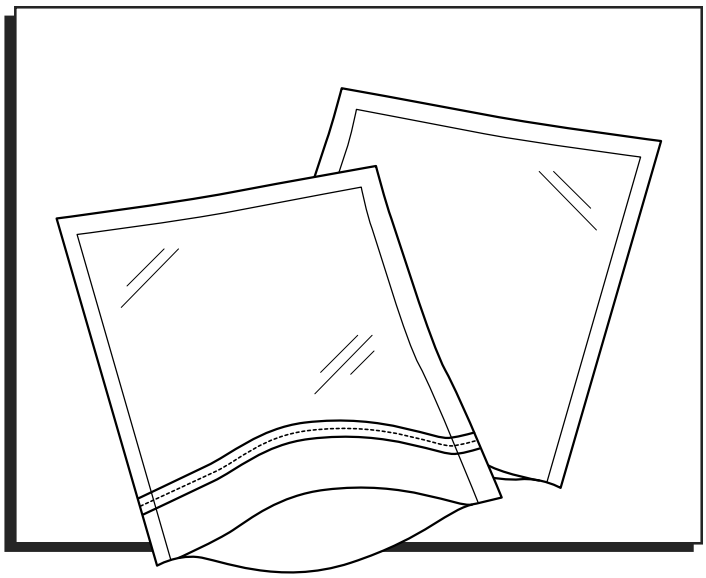
Uline Food Bags provide protection against moisture and corrosion for packaging of powders and food-related products. Uline Food Bags are FDA approved.

PHYSICAL FILM PROPERTIES	UNITS
Moisture Vapor Transmission Rate (ASTM F1249)	0.0003 gr./100 in^2 24 hrs.
O_2 Transmission Rate (73.4°F, 0% RH)(ASTM D 3985-05)	0.001/cc/m^2/24 hrs.
Bursting Strength (TAPPI T403)	69 psi.
Puncture (FED. 101)	> 18 psi.
Total Thickness	4.4 mil

Uline makes no warranty, expressed or implied, as to the suitability of these materials for any specific use. The values shown above were developed from random samples taken from production material. We believe them to be typical for the product. Actual values may vary somewhat from those depicted here. Customers should determine product suitability based upon their own internal criteria.

BOLSAS PARA ALIMENTOS

800-295-5510
uline.mx



ESPECIFICACIONES DEL PRODUCTO

Las bolsas para alimentos de Uline brindan protección ante la humedad y la corrosión para el empaque de productos en polvo y relacionados con la alimentación. Las bolsas para alimentos de Uline han sido aprobadas por la FDA.

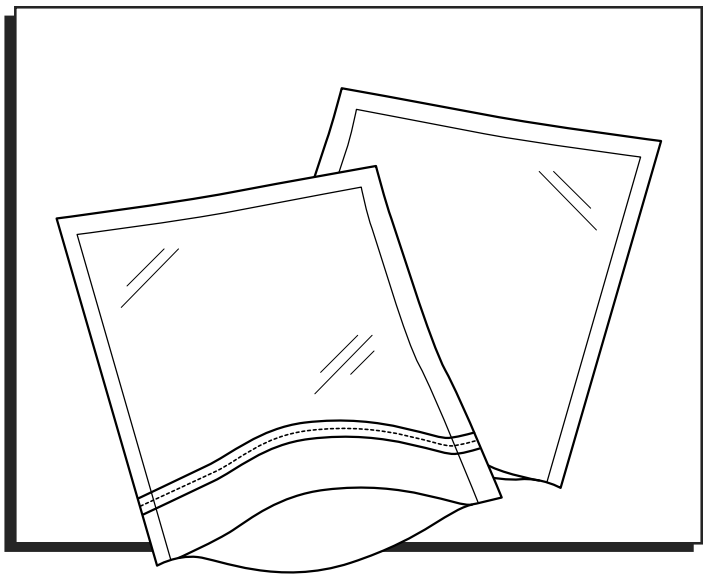
PROPIEDADES FÍSICAS DE LA PELÍCULA	UNIDADES
Tasa de Transmisión de Vapor de Agua (ASTM F1249)	0.0003 gr./100 pul.^{2} 24 h
Tasa de transmisión de O_2 [23°C (73.4°F), 0% RH](ASTM D 3985-05)	0.001/cc/m^2/24 hrs.
Resistencia al Reventamiento (TAPPI T403)	69 psi.
Perforaciones (FED. 101)	> 18 psi.
Grosor Total	4.4 mil

Uline no garantiza de forma alguna, ya sea implícita o explícita, la idoneidad de estos materiales para cualquier uso específico. Los valores arriba mostrados han sido desarrollados a partir de muestras aleatorias tomadas del material de producción. Creemos que son típicos del producto. Los valores reales podrían diferir con respecto a los mostrados aquí. Los clientes deberán determinar la idoneidad del producto de acuerdo con su propio criterio interno.

ULINE S-18139

SACS POUR ALIMENTS

1-800-295-5510
uline.ca



SPÉCIFICATIONS DU PRODUIT

Les sacs pour aliments Uline permettent d'emballer des produits alimentaires ou en poudre pour les protéger de l'humidité et de la corrosion. Les sacs pour aliments Uline sont approuvés par la FDA.

PROPRIÉTÉS PHYSIQUES DE LA PELLICULE	UNITÉS
Taux de transmission de vapeur humide (ASTM F1249)	0,0003 gr./100 po^2 24 h
Taux de transmission d'O_2 [23 °C (73.4 °F), 0 % RH](ASTM D 3985-05)	0,001/cc/m^2/24 h
Résistance à l'éclatement (TAPPI T403)	69 lb/po^2
Perforation (FED. 101)	> 18 lb/po^2
Épaisseur totale	4,4 mil

Uline n'offre aucune garantie, explicite ou implicite, quant à la convenance de ces matériaux pour tout usage précis. Les valeurs indiquées ci-dessus ont été élaborées à partir d'échantillons prélevés au hasard dans la production. Nous croyons qu'elles sont représentatives du produit. Les valeurs réelles peuvent varier quelque peu de celles qui sont énoncées ici. La convenance du produit à un usage précis reste à être déterminée par le client lui-même.

OXFORD

Journal of AOAC INTERNATIONAL, 103(2), 2020, 513–522

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Advance Access Publication Date: 12 March 2020
Article

FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the 3M™ Petrifilm™ Rapid *E. coli*/Coliform Count Plate for the Enumeration of *E. coli* and Coliforms: Collaborative Study, First Action: 2018.13

Patrick Bird,[1] Benjamin Bastin,[1] Nicole Klass,[1] Erin Crowley,[1] James Agin,[1] David Goins,[1] Hannah Bakken,[2] Cari Lingle,[2] and April Schumacher[2,3]

[1]Q Laboratories, Inc, 1400 Harrison Ave, Cincinnati, OH 45214, USA, [2]3M Food Safety Department, 3M Center, Bldg. 260-6B-01, St. Paul, MN 55144, USA

Collaborators: A. Calle, K. Suntharesan, V. Gohil, A. Donkers, R. Smith, D. Wood, S. Diederich, S. Kuchenberg, I. Satoshi, M. Brown, N. Alvarez, S. Corti, M. Hochreuter
Corresponding author's e-mail: ajschumacher@mmm.com

Abstract

Background: The 3M™ Petrifilm™ Rapid *E. coli*/Coliform Count Plate is a selective and differential sample-ready-culture medium designed for the rapid enumeration of *Escherichia coli* (*E. coli*) and coliforms in the food and beverage industries.
Objective: The 3M Petrifilm Rapid *E. coli*/Coliform Count Plate was compared to the U.S. Food and Drug Administration Bacteriological Analytical Manual (BAM) Chapter 4 Enumeration of *Escherichia coli* and the Coliform Bacteria, the International Organization of Standards (ISO) 4832:2006 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms—Colony-count technique, and ISO 16649-2:2017 Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli—Part 2 Colony-count technique at 44 degrees C using bromo-4-chloro-3- indolyl beta-D-glucuronide methods for the enumeration of *E. coli* and coliforms in dry dog kibble.
Method: The candidate method was evaluated using two diluents, Butterfield's phosphate buffered diluent and peptone salt solution, in a paired study design with each reference method in a multi-laboratory collaborative study following the current AOAC Validation Guidelines. Three target contamination levels and an uninoculated control level were evaluated.
Results: The candidate and reference methods were not statistically different at each contamination level. Reproducibility values obtained during the collaborative study were similar between the candidate and reference methods.
Conclusion: These results demonstrate that the candidate method is equivalent to the reference methods.
Highlight: 3M Petrifilm Rapid *E. coli*/Coliform Count Plate was recommended for Official First Action status for enumeration of *E. coli* and coliforms in a broad range of foods and environmental surfaces.

Coliform bacteria are a category of rod-shaped, non-spore forming Gram-negative bacteria. These organisms can be motile or non-motile and can ferment lactose with the production of acid and gas. While coliform bacteria are very common and normally harmless, coliform contamination in food or beverage products does pose a health risk. Since coliforms are commonly

Received: 20 December 2018; **Revised:** 25 September 2019; **Accepted:** 26 September 2019

found in soil and vegetation, when coliform contamination is found, it usually is caused by the environment. Coliform presence in food products raises the question of pathogen contamination occurring through a similar process. Many coliforms, including *Escherichia coli (E. coli)*, a subgroup of coliform, can be found in the human digestive tract. While some strains of *E. coli* are harmless, other strains can cause serious illness. Similar to coliforms, if *E. coli* contamination is detected it indicates that conditions exist in which pathogens may be present (1).

Test methods for coliforms and *E. coli* are designed to detect and enumerate typical phenotypes. For this reason, methods for coliforms and *E. coli* have some limitations. For example, The Compendium of Methods for the Microbiological Examination of Foods states that 92–99% of *E. coli* produce beta-glucuronidase, some pathogenic *E. coli* do not produce this enzyme and a few strains of non-*E. coli* organisms can produce beta-glucuronidase (2). This limitation should be understood for any beta-glucuronidase-based method, such as: 4-methylumbelliferyl-β-glucuronide (MUG) most probable number, violet red bile (VRB) agar with MUG (FDA-BAM), and other alternative methods including 3M Petrifilm Plate methods (2).

Traditional screening and confirmation of *E. coli* or coliform bacteria can require 3 to 7 days and can be very labor intensive for laboratories. The 3M Petrifilm Rapid *E. coli*/Coliform Count Plate allows for the simple, rapid enumeration and differentiation of coliform and *E. coli* in food and environmental samples. Test portions are diluted in an appropriate diluent and a sample aliquot is plated onto the plate. The plates can be incubated at multiple temperatures (for dairy: 30 ± 1 °C or 32 ± 1 °C for *E.* coli and coliforms, and 42 ± 1 °C for *E. coli;* for all other foods; 35 ± 1 °C or 37 ± 1 °C for *E.* coli and coliforms, and 42 ± 1 °C for *E. coli*) with enumeration occurring in as little as 18 h.

The 3M Petrifilm Rapid *E. coli*/Coliform Count Plate was validated according to AOAC Validation Guidelines (3) following the AOAC® *Official Methods of Analysis*℠ process. The objective of these studies was to demonstrate that the candidate method accurately enumerated *E. coli* and coliforms in a broad range of foods and select environmental surfaces as claimed by the manufacturer and that no difference in repeatability was observed between the candidate method and the reference methods. For the pre-collaborative studies, 33 matrices were evaluated. The following matrices were evaluated with Butterfield's Phosphate Buffered Diluent (BPBD) only: pasteurized whole milk, butter, nonfat dry milk, raw ground pork, lamb chop, raw ground chicken, chicken carcass rinsate, shell eggs, liquid egg whites, powdered egg whites, fresh raw bean sprouts, frozen cranberries, infant formula with probiotics, infant formula without probiotics, infant rice cereal without probiotics, dry dog kibble, dry cat food, all-purpose flour, and chocolate chip cookie dough. The following matrices were evaluated with BPBD and peptone salt solution (PSS): raw ground beef (73% lean), raw frozen chicken wings, raw milk, whole liquid egg, tuna sushi, smoked salmon, bunched raw spinach, pasteurized carrot juice, ready-made sandwiches (bread, deli meat, and cheese), raw vegetable salad with mayonnaise-based dressing, chicken feed, soybean meal, stainless steel environmental sponges, and sealed concrete environmental sponges. Additional pre-collaborative parameters (inclusivity and exclusivity testing) satisfied the requirements for *Official Methods of Analysis* approval.

The purpose of this collaborative study was to compare the 3M Petrifilm Rapid *E. coli*/Coliform Count Plate to the U.S. Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) Chapter 4: *Enumeration of Escherichia coli and the Coliform Bacteria* (4), the International Organization of Standards (ISO) 4382:2006 *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coliforms—Colony-count technique* (5), and ISO 16649-2:2001 *Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of β-glucuronidase-positive Escherichia coli—Part 2: Colony count technique at 44 °C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide* (6) reference methods for the enumeration of coliform and *E. coli* in dry pet food.

Collaborative Study

Study Design

One matrix, dry dog kibble, was evaluated in this study. The matrix was obtained from a local retailer and screened for the presence of naturally occurring *E. coli* and coliforms by the FDA BAM and ISO reference methods. No natural contamination was observed; four separate levels of contamination were targeted for the evaluation: uninoculated [0 colony-forming unit (CFU)/g], low (10–100 CFU/g), medium (100–1000 CFU/g), and high (1000–10 000 CFU/g). To obtain the required contamination levels, bulk lots of the matrix were artificially contaminated with a lyophilized culture of *E. coli* [Q Laboratories (QL) isolate 11007-8 (origin – beef hide)] and *Klebsiella pneumonia* [QL isolate 11007-7 (origin – raw hamburger)] at each target contamination level. Two replicate samples from each of the four contamination levels were analyzed by both the candidate and reference methods in a paired study design.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, test portion preparation and documentation of results was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of the Inocula and Test Portions

The isolates used in this evaluation were lyophilized prior to inoculation. The cultures were first propagated onto tryptic soy agar with 5% sheep blood (SBA) from a QL frozen stock culture stored at -70°C. To prepare the culture for lyophilization, a single, well isolated colony from SBA was transferred into brain heart infusion broth and incubated at 37 ± 2 °C for 18–24 h. The cultures were diluted in a sterile cryoprotectant, reconstituted in 10% non-fat dry milk, and freeze dried for 48–72 h. A bulk lot of the test matrix was inoculated with each culture at a high level. An aliquot of the high-level inoculated matrix was further mixed with uninoculated matrix to produce the medium and low level inoculum by mixing by hand and shaking in sterile containers. After inoculation, the matrix was held for a minimum of 2 weeks at ambient temperature (20–25 °C). The inoculated test product was packaged into separate 10 g (ISO) and 50 g (BAM) samples in sterile Whirl-Pak® bags and shipped to the collaborators. All data was received from each temperature probe, showing all shipments remained under similar conditions throughout shipment.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3-digit number affixed to the sample container. Nine participants from 8 separate locations participated. Test portions were shipped in leak-proof insulated containers via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by International Air Transport Association. Test portions were shipped at ambient temperatures (20–25°C). Upon receipt, samples were held at ambient temperature until analysis was initiated. In addition to each of the test portions, collaborators

also received a test portion for the matrix labeled as Aerobic Plate Count (APC), to determine total background count in the matrix using the FDA BAM Chapter 3 *Aerobic Plate Count* reference method (7). The APC background screen samples were prepared from the bulk lot of test matrix, prior to inoculation. Additionally, a temperature probe was included in the shipment. Participants were instructed to submit the data from the temperature probe upon receipt of the shipment.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol provided to them in the collaborator instructions (Version 2, August, 2018). Each collaborator received 16 test portions (2 high, 2 medium, 2 low, and 2 uninoculated for paired analysis with the 3M Petrifilm Rapid *E. coli*/Coliform Count Plate and ISO methods, and 2 high, 2 medium, 2 low, and 2 uninoculated for analysis with the 3M™ Petrifilm™ Plate and BAM method).

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and BAM

A 50 g test portion was diluted with 450 mL of BPBD, allowed to sit for 20 min to soften the dry dog kibble, and homogenized with a paddle blender for 2 min ± 10 s. Ten-fold serial dilutions of each sample were prepared in BPBD and a 1.0 mL aliquot of each dilution was plated onto a single 3M Petrifilm Rapid *E. coli*/Coliform Count Plate for each dilution. The plate was incubated at 35 ± 1 °C for 18–24 h. After incubation, plates were enumerated for total coliforms and *E. coli*. The total coliform count is indicated by red colonies with gas production and blue colonies with and without gas production (*E. coli*). Colonies can go through further identification based on user requirements. Plates containing greater than 100 CFU were recorded as too numerous to count (TNTC). Final results were determined by multiplying the counts by the dilution factor for that plate.

Each test portion analyzed by the candidate method was also analyzed using the FDA BAM Chapter 4 reference method in a paired study design. A 1.0 mL aliquot from each sample dilution was plated onto a Petri dish. To each plate, 10 mL of VRB agar was added and allowed to solidify. To avoid spreading colonies and surface growth, an overlay of 5 mL of VRB agar with MUG was added. All plates were inverted, incubated for 18–24 h at 35 ± 1 °C and enumerated. Purple-red colonies that were 0.5 mm or larger in diameter and surrounded by a zone of precipitated bile acids were enumerated as coliform colonies. *E. coli* colonies were determined by observing bluish fluorescence when viewed under a longwave ultraviolet light. Counts of 25–250 CFU/plate were considered countable, while counts outside that range were considered estimates. For both the reference method test portions and the 3M test portions, coliform colonies were confirmed by transferring typical colonies to tubes containing brilliant green lactose bile (BGLB) broth and incubating at 35 ± 1 °C. Tubes were examined at 24 and 48 h for gas production. The reference method test portions and the 3M test portions' *E. coli* colonies were confirmed by transferring presumptive colonies to EC-MUG broth and incubating at 35 ± 1 °C for 48 ± 2 h. Tubes were then examined for fluorescence. In addition, for each positive sample, a single colony was confirmed using API® 20E, AOAC *Official Methods of Analysis* **978.24** (8).

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and ISO

A 10 g test portion was diluted with 90 mL of PSS, allowed to sit for 20 min to soften the dry dog kibble, and homogenized with a paddle blender for 2 min ± 10 s. Ten-fold serial dilutions of each sample were prepared in PSS and a 1.0 mL aliquot of each dilution was plated onto duplicate 3M Petrifilm Rapid *E. coli*/Coliform Count Plates for each dilution. One plate was incubated at 37 ± 1 °C for 18–24 h and the other plate was incubated at 42 ± 1 °C for 18–24 h. After incubation, plates were enumerated for total coliform (37 °C) and *E. coli* (37 °C and 42 °C). The total coliform count is indicated by red colonies with or without gas production and blue colonies with or without gas production (*E. coli*). Plates containing greater than 100 colonies were recorded as TNTC. Final results were determined by multiplying the counts by the dilution factor for that plate.

Each test portion analyzed by the candidate method was also analyzed with the ISO 4382 and ISO 16649-2 reference methods in a paired study design. For ISO 4832, serial dilutions for each sample were plated in sterile Petri dishes followed by the addition of VRB agar with lactose (VRBL). To avoid spreading colonies and surface growth, an overlay of 5 mL of VRBL was added. Agar plates were incubated for 24 ± 2 h at 37 ± 1 °C. Typical colonies in the countable range (10–150) were enumerated using a standard colony counter. If atypical colonies were present, further confirmation was conducted by transferring colonies to BGLB broth. For ISO 16649-2, serial dilutions for each sample were plated in singular using tryptone bile X-glucuronide medium. Agar plates were incubated for 18–24 h at 44 ± 1 °C. Typical colonies in the countable range (10–150) were enumerated using a standard colony counter.

Statistical Analysis

Each collaborating laboratory recorded the CFU/g results for the reference methods and the candidate method on the electronic spreadsheet provided. The data sheets were submitted to the study director at the end of the study for analysis. A logarithmic transformation (CFU/g + 0.1f, where f is the reported CFU/g corresponding to the smallest reportable result). A Youden plot was prepared to identify discrepancies between test replicates (Figures 1–5). Outliers were identified using the Cochran and Grubbs' tests. The differences of means, including 95% upper and lower confidence limits, were determined for each contamination level (9). If the difference of means between the two methods was < 0.5 Log_{10}, it was considered that no statistical difference existed between the two methods (10). The repeatability (s_r) and reproducibility (s_R) of the methods were also determined (11).

AOAC Official Method 2018.13

Enumeration of *Escherichia coli* and Coliform in a Broad Range of Foods and Select Environmental Surfaces

3M Petrifilm Rapid *E. coli*/Coliform Count Plate

First Action 2018

[Applicable to the enumeration of *E. coli* and coliform from pasteurized whole milk, butter, non-fat dry milk, raw ground pork, raw lamb chop, raw ground chicken, chicken carcass rinsate, shell eggs, liquid egg whites, powdered egg whites, fresh raw bean sprouts, frozen cranberries, infant formula with probiotics, infant formula without probiotics, infant rice cereal without probiotics, dry dog kibble, dry cat food, all-purpose flour, chocolate chip cookie dough, raw ground beef (73% lean), raw frozen chicken wings, raw milk, whole liquid egg, tuna sushi, smoked salmon, bunched raw spinach, pasteurized carrot juice, ready-made sandwiches (bread, deli meat and cheese), raw vegetable salad with mayonnaise-based dressing, chicken

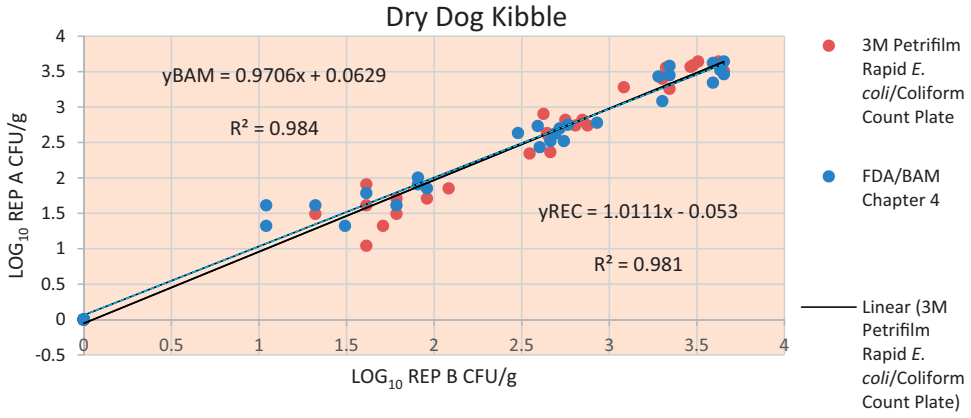

Figure 1. Youden's Plot for 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (35 °C) and FDA BAM for Coliforms.

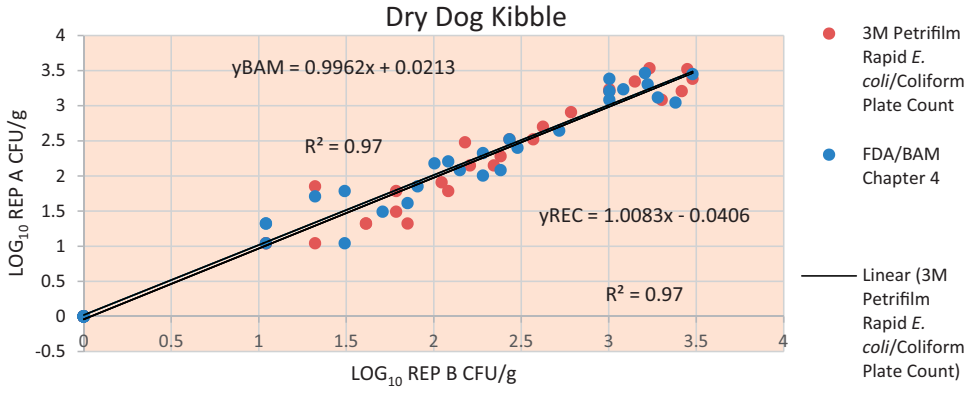

Figure 2. Youden's Plot for 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (35 °C) and FDA BAM for *E. coli*.

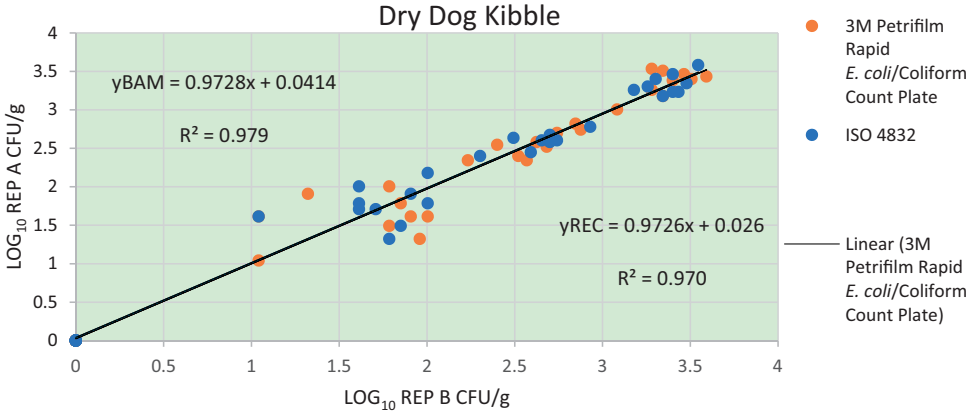

Figure 3. Youden's Plot for 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (37 °C) and ISO 4832 for Coliforms.

feed, soybean meal, stainless steel environmental sponges, and sealed concrete environmental sponges]

See Table 2018.13A and B for a summary of results of the collaborative study.

See Tables 2018.13C–G for detailed results of the collaborative study.

A. Principle

The 3M Petrifilm Rapid *E. coli*/Coliform Count Plate is a self-contained, sample-ready-culture-medium system which contains a cold-water-soluble gelling agent and two different indicators; 5-bromo-4-chloro-3-indolyl-D-glucuronide that indicates glucuronidase activity and tetrazolium that facilitates colony enumeration. The 3M Petrifilm Rapid *E. coli*/Coliform Count Plate is intended for the enumeration of both *E. coli* and coliforms in various food and beverage products and from environmental surfaces. The 3 M Petrifilm Rapid *E.coli*/Coliform Count Plates can be incubated for 18–24 h at 30 ± 1 °C or 32 ± 1 °C for *E. coli* and coliforms, and 42 ± 1 °C for *E. coli* for dairy products; for all other foods; 35 ± 1 °C or 37 ± 1 °C for *E. coli* and coliforms, and 42 ± 1 °C for *E. coli*. The typical colony morphology for *E. coli* is blue to

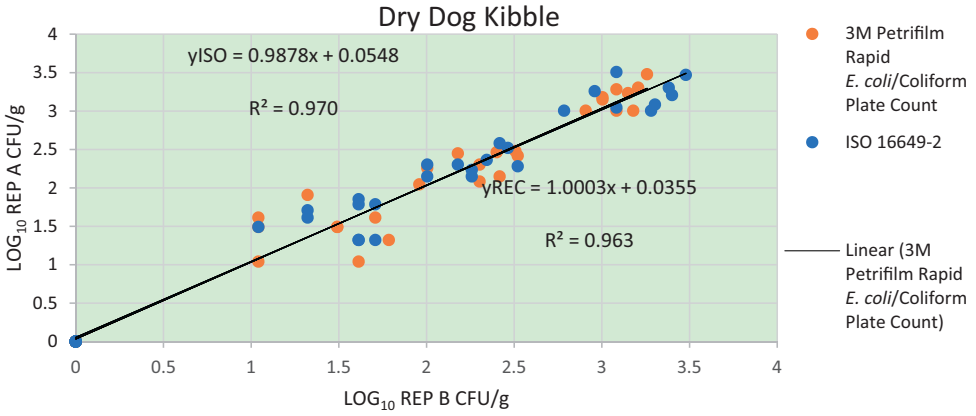

Figure 4. Youden's Plot for 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (37 °C) and ISO 16649-2 for *E. coli*.

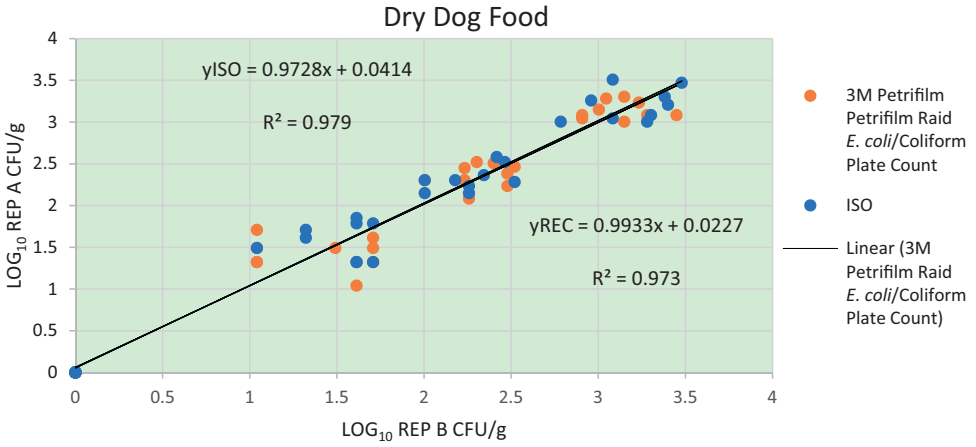

Figure 5. Youden's Plot for 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (42 °C) and ISO 16649-2 for *E. coli*.

Table 2018.13A. Interlaboratory study results of 3M Petrifilm Rapid *E. coli*/Coliform Count Plate vs. FDA BAM Chapter 4

3M Petrifilm Rapid *E. coli*/Coliform Count Plate-Coliform (35°C)						FDA BAM Chapter 4 Coliform						
Matrix	Lot	N[a]	Mean Log_{10} CFU/g	s_r[b]	s_R[c]	Lot	N[a]	Mean Log_{10} CFU/g	s_r	s_R	Difference of Means[d]	Difference of Means[e] 95% LCL, UCL
Dry Pet Kibble	Uninoculated	9	0.000	0.00	0.00	Uninoculated	9	0.000	0.00	0.00	0.00	0.00, 0.00
	Low	9	1.645	0.22	0.25	Low	9	1.617	0.18	0.31	−0.30	−0.20, 0.14
	Medium	9	2.687	0.12	0.16	Medium	9	2.660	0.10	0.12	−0.30	−0.09, 0.03
	High	9	3.458	0.10	0.17	High	9	3.474	0.12	0.17	0.02	−0.06, 0.10
3M Petrifilm Rapid *E. coli*/Coliform Count Plate—*E. coli* (35°C)						FDA BAM Chapter 4 *E. coli*					Difference of Means[d]	Difference of Means[e] 95% LCL, UCL
Dry Pet Kibble	Uninoculated	9	0.000	0.00	0.00	Uninoculated	9	0.000	0.00	0.00	0.00	0.00, 0.00
	Low	9	1.422	0.22	0.31	Low	9	1.448	0.20	0.32	0.02	−0.17, 0.22
	Medium	9	2.298	0.12	0.26	Medium	9	2.292	0.11	0.22	−0.01	−0.12, 0.11
	High	9	3.236	0.14	0.22	High	9	3.220	0.15	0.17	−0.02	−0.14, 0.10

[a] Number of collaborators that reported complete results.
[b] s_r = Repeatability.
[c] s_R = Reproducibility.
[d] A Difference of means <0.5 indicates no statistically significant difference between methods.
[e] 95% Lower and Upper Confidence Limits.

Table 2018.13B. Interlaboratory Study Results of 3M Petrifilm Rapid *E. coli*/Coliform Count Plate vs. ISO 4832 and 16649-2

3M Petrifilm Rapid *E. coli*/Coliform Count Plate—Coliform (37°C)						ISO 4832						
Matrix	Lot	N[a]	Mean Log_{10} CFU/g	s_r[b]	s_R[c]	Lot	N[a]	Mean Log_{10} CFU/g	s_r	s_R	Difference of Means[d]	Difference of Means[e] 95% LCL, UCL
Dry Pet Kibble	Uninoculated	9	0.000	0.00	0.00	Uninoculated	9	0.000	0.00	0.00	0.00	0.00, 0.00
	Low	9	1.616	0.28	0.34	Low	9	1.692	0.22	0.24	0.08	−0.09, 0.25
	Medium	9	2.583	0.09	0.19	Medium	9	2.557	0.09	0.23	−0.03	−0.12, 0.07
	High	9	3.359	0.09	0.16	High	9	3.352	0.09	0.12	−0.01	−0.10, 0.08
3M Petrifilm Rapid *E. coli*/Coliform Count Plate—*E. coli* (37°C)						ISO 16649-2						
Dry Pet Kibble	Uninoculated	9	0.000	0.00	0.00	Uninoculated	9	0.000	0.00	0.00	0.00	0.00, 0.00
	Low	9	1.419	0.28	0.28	Low	9	1.542	0.22	0.22	0.12	−0.04, 0.29
	Medium	9	2.291	0.12	0.19	Medium	9	2.296	0.11	0.17	0.01	−0.11, 0.12
	High	9	3.140	0.11	0.14	High	9	3.202	0.16	0.21	0.04	−0.07, 0.16
3M Petrifilm Rapid *E. coli*/Coliform Count Plate—*E. coli* (42°C)						ISO 16649-2					Difference of Means[d]	Difference of Means[e] 95% LCL, UCL
Dry Pet Kibble	Uninoculated	9	0.000	0.00	0.00	Uninoculated	9	0.000	0.00	0.00	0.00	0.00, 0.00
	Low	9	1.400	0.27	0.27	Low	9	1.542	0.22	0.22	0.14	0.02. 0.27
	Medium	9	2.342	0.13	0.15	Medium	9	2.296	0.11	0.17	−0.05	−0.15. 0.06
	High	9	3.133	0.14	0.14	High	9	3.202	0.16	0.21	0.05	−0.09, 0.19

[a] Number of collaborators that reported complete results.
[b] s_r = Repeatability.
[c] s_R = Reproducibility.
[d] A Difference of means <0.5 indicates no statistically significant difference between methods.
[e] 95% Lower and Upper Confidence Limits.

Table 2018.13C. Log_{10} Coliform Counts for Dry Dog Kibble with 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (35°C) and FDA BAM Chapter 4

Uninoculated					Low				Medium				High			
	3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g	
Collaborator	A[a]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	0.000	0.000	0.000	0.000	1.613	1.613	1.322	1.041	2.364	2.664	2.520	2.741	3.400	3.303	3.344	3.592
2	0.000	0.000	0.000	0.000	1.708	1.959	1.785	1.613	2.741	2.807	2.733	2.592	3.281	3.083	3.083	3.303
3	0.000	0.000	0.000	0.000	1.851	2.083	2.004	1.908	2.344	2.545	2.433	2.603	3.644	3.507	3.581	3.344
4	0.000	0.000	0.000	0.000	1.491	1.322	1.613	1.785	2.592	2.654	2.634	2.479	3.558	3.324	3.464	3.654
5	0.000	0.000	0.000	0.000	1.041	1.613	1.613	1.322	2.820	2.846	2.749	2.764	3.569	3.464	3.433	3.281
6	0.000	0.000	0.000	0.000	1.708	1.785	1.851	1.959	2.741	2.876	2.779	2.930	3.258	3.344	3.449	3.344
7	0.000	0.000	0.000	0.000	1.908	1.613	1.908	1.908	2.904	2.624	2.624	2.691	3.644	3.624	3.644	3.654
8	0.000	0.000	0.000	0.000	1.322	1.708	1.322	1.491	2.634	2.644	2.520	2.664	3.592	3.479	3.520	3.634
9	0.000	0.000	0.000	0.000	1.491	1.785	1.613	1.041	2.820	2.749	2.700	2.717	3.507	3.654	3.624	3.592

[a] A and B = Indicated duplicate test portions.

blue-green colonies with or without gas production, regardless of size or color intensity. Other coliform colonies will appear as red colonies with entrapped gas (within approximately one colony diameter) for enumeration when comparing to FDA BAM, or red colonies with and without gas production when comparing to ISO 4832:2006. Plates containing more than 100 CFU for the total coliform count or more than 100 CFU for the *E. coli* count can either be estimated or recorded as TNTC.

B. Apparatus and Reagents

(a) *3M Petrifilm Rapid E. coli/Coliform Count Plates*.—Available from 3M Food Safety - CAT No. 6436/6437

(b) *3M™ Petrifilm Flat Spreader*.—CAT No. 6425

(c) *Sterile Diluent*.—PSS and BPBD.

(d) *Pipettes*.—capable of pipetting 1000 μL or a serological pipette.

(e) *Sterile pipette tips*.—capable of 1000 μL.

(f) *Laboratory paddle-blender*.—Seward 400 or equivalent.

(g) *Filter Stomacher bags*.–Seward or equivalent.

(h) *Incubators*.—Capable of maintaining 30 ± 1°C, 32 ± 1°C, 35 ± 1°C, 37 ± 1°C, or 42 ± 1°C.

(i) *Refrigerator*.—capable of maintaining 2–8°C, for storing plates.

(j) *Freezer*.—capable of maintaining –20–0°C for storing plates.

(k) *Standard Colony Counter or Illuminated Magnifier*.

(l) *Top-loading balance*.—capable of weighing 1–2000 g.

Table 2018.13D. Log_{10} *E. coli* Counts for Dry Dog Kibble with 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (35°C) and FDA BAM Chapter 4

Uninoculated					Low				Medium				High			
	3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		BAM Chapter 4 Log_{10} CFU/g	
Collaborator	A[a]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	0.000	0.000	0.000	0.000	1.322	1.613	1.041	1.041	1.785	2.083	2.083	2.382	3.233	3.004	3.045	3.382
2	0.000	0.000	0.000	0.000	1.322	1.851	1.708	1.322	2.149	2.344	2.400	2.479	2.908	2.785	3.083	3.004
3	0.000	0.000	0.000	0.000	1.785	1.785	1.851	1.908	1.908	2.045	2.083	2.149	3.382	3.479	3.464	3.207
4	0.000	0.000	0.000	0.000	1.041	1.041	1.491	1.708	2.281	2.382	2.179	2.004	3.533	3.233	3.449	3.479
5	0.000	0.000	0.000	0.000	1.041	1.322	1.322	1.041	2.700	2.624	2.520	2.433	3.207	3.004	3.303	3.223
6	0.000	0.000	0.000	0.000	1.491	1.785	1.785	1.491	2.520	2.433	2.644	2.717	3.083	3.303	3.382	3.004
7	0.000	0.000	0.000	0.000	1.851	1.322	1.613	1.851	2.479	2.179	2.207	2.083	3.520	3.449	3.117	3.281
8	0.000	0.000	0.000	0.000	1.041	1.041	1.041	1.491	2.149	2.207	2.004	2.281	3.344	3.149	3.207	3.004
9	0.000	0.000	0.000	0.000	1.322	1.613	1.322	1.041	2.520	2.569	2.324	2.281	3.207	3.417	3.233	3.083

[a] A and B = Indicated duplicate test portions.

Table 2018.13E. Log_{10} Coliform Counts for Dry Dog Kibble with 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (37°C) and ISO 4832

Uninoculated					Low				Medium				High			
	3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 4832 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 4832 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 4832 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 4832 Log_{10} CFU/g	
Collaborator	A[a]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	0.000	0.000	0.000	0.000	2.004	1.785	1.708	1.613	2.344	2.233	2.179	2.004	3.004	3.083	3.179	3.344
2	0.000	0.000	0.000	0.000	1.908	1.322	1.613	1.041	2.545	2.400	2.603	2.654	3.179	3.344	3.303	3.258
3	0.000	0.000	0.000	0.000	1.613	1.041	1.708	1.708	2.520	2.682	2.449	2.592	3.382	3.400	3.581	3.545
4	0.000	0.000	0.000	0.000	1.041	1.041	1.322	1.785	2.344	2.569	2.603	2.741	3.258	3.281	3.464	3.400
5	0.000	0.000	0.000	0.000	1.785	1.851	1.785	1.613	2.400	2.520	2.673	2.700	3.433	3.592	3.233	3.400
6	0.000	0.000	0.000	0.000	1.613	1.908	1.908	1.908	2.581	2.624	2.400	2.303	3.400	3.507	3.258	3.179
7	0.000	0.000	0.000	0.000	1.322	1.959	1.785	2.004	2.741	2.876	2.779	2.930	3.507	3.344	3.233	3.433
8	0.000	0.000	0.000	0.000	1.491	1.785	2.004	1.613	2.820	2.846	2.581	2.700	3.464	3.464	3.344	3.479
9	0.000	0.000	0.000	0.000	1.613	2.004	1.491	1.851	2.700	2.741	2.634	2.493	3.533	3.281	3.400	3.303

[a] A and B = Indicated duplicate test portions.

Table 2018.13F. Log_{10} *E. coli* Counts for Dry Dog Kibble with 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (37°C) and ISO 16649-2

Uninoculated					Low				Medium				High			
	3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g	
Collaborator	A[a]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	0.000	0.000	0.000	0.000	1.613	1.708	1.613	1.322	2.083	2.303	2.149	2.004	3.004	2.908	3.083	3.303
2	0.000	0.000	0.000	0.000	1.908	1.322	1.491	1.041	2.258	2.004	2.364	2.344	3.004	3.179	3.258	2.959
3	0.000	0.000	0.000	0.000	1.491	1.041	1.708	1.322	2.045	1.959	2.303	2.179	3.281	3.083	3.470	3.479
4	0.000	0.000	0.000	0.000	1.041	1.041	1.322	1.613	2.149	2.417	2.581	2.417	3.004	3.083	3.303	3.382
5	0.000	0.000	0.000	0.000	1.613	1.041	1.785	1.613	2.449	2.179	2.520	2.464	3.179	3.004	3.507	3.083
6	0.000	0.000	0.000	0.000	1.491	1.491	1.785	1.708	2.303	2.303	2.149	2.258	3.149	3.004	3.004	2.785
7	0.000	0.000	0.000	0.000	1.322	1.785	1.322	1.708	2.417	2.520	2.281	2.520	3.479	3.258	3.207	3.400
8	0.000	0.000	0.000	0.000	1.041	1.613	1.851	1.613	2.479	2.507	2.233	2.258	3.233	3.149	3.045	3.083
9	0.000	0.000	0.000	0.000	1.491	1.491	1.322	1.613	2.464	2.400	2.303	2.004	3.303	3.207	3.004	3.281

[a] A and B = Indicated duplicate test portions.

Table 2018.13G. Log_{10} *E. coli* Counts for Dry Dog Kibble with 3M Petrifilm Rapid *E. coli*/Coliform Count Plate (42°C) and ISO 16649-2

Uninoculated					Low				Medium				High			
	3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g		3M Petrifilm Rapid *E. coli*/ Coliform Count Plate Log_{10} CFU/g		ISO 16649-2 Log_{10} CFU/g	
Collaborator	A[a]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	0.000	0.000	0.000	0.000	1.322	1.041	1.613	1.322	2.303	2.233	2.149	2.004	3.045	2.908	3.083	3.303
2	0.000	0.000	0.000	0.000	1.708	1.041	1.491	1.041	2.52	2.303	2.364	2.344	3.004	3.149	3.258	2.959
3	0.000	0.000	0.000	0.000	1.322	1.041	1.708	1.322	2.083	2.258	2.303	2.179	3.083	2.908	3.470	3.479
4	0.000	0.000	0.000	0.000	1.491	1.041	1.322	1.613	2.233	2.479	2.581	2.417	3.083	3.281	3.303	3.382
5	0.000	0.000	0.000	0.000	1.491	1.491	1.785	1.613	2.449	2.233	2.520	2.464	3.233	3.233	3.507	3.083
6	0.000	0.000	0.000	0.000	1.491	1.708	1.785	1.708	2.303	2.004	2.149	2.258	3.303	3.149	3.004	2.785
7	0.000	0.000	0.000	0.000	1.041	1.613	1.322	1.708	2.382	2.479	2.281	2.520	3.281	3.045	3.207	3.400
8	0.000	0.000	0.000	0.000	1.322	1.708	1.851	1.613	2.464	2.520	2.233	2.258	3.083	3.449	3.045	3.083
9	0.000	0.000	0.000	0.000	1.613	1.708	1.322	1.613	2.507	2.400	2.303	2.004	3.149	3.004	3.004	3.281

[a] A and B = Indicated duplicate test portions.

C. General Instructions

(a) Read the instruction manual carefully before use.
(b) Storage conditions: Store the plates at –20 to 8°C. Allow the plates to warm to ambient temperature (20–25°C/<60% relative humidity) prior to use. After opening the pouch, unused plates should be placed back in the pouch, sealed and stored at ambient temperature for no longer than 4 weeks. If the temperature of the site is > 25°C with a relative humidity greater than 50%, it is recommended to place the plates in a sealed container and store in a freezer for no more than 4 weeks.
(c) Plates containing more than 100 CFU for the total coliform count or more than 100 CFU for the *E. coli* count can either be estimated or recorded as TNTC. Note, the enumeration for *E. coli* or total coliforms may occur on separate dilutions.

Safety Precautions:

Do not use this plate for the specific detection of *E. coli* O:157 because most *E. coli* O:157 strains are atypical, glucuronidase negative, and will not be detected as *E. coli* but only as coliforms. After use, 3M Petrifilm Rapid *E. coli*/Coliform Count Plates may contain microorganisms that may be a potential biohazard. Follow current industry standards and local regulations for disposal of biohazardous waste.

To reduce the risks associated with release of contaminated product:

(a) Follow all product storage instructions contained in the instructions for use.
(b) Do not use beyond the use by date.
(c) Do not use 3M Petrifilm Rapid *E. coli*/Coliform Count Plates that show discoloration.
(d) Do not use diluents containing citrate, bisulfate or thiosulfate; they can inhibit growth.

To reduce the risks associated with bacterial infection and workplace contamination:

(a) Perform testing in a properly equipped laboratory under the control of a skilled microbiologist.
(b) The user must train their personnel in current proper testing techniques.

To reduce the risk associated with misinterpretation of results:

(a) 3M has not documented 3M Petrifilm Rapid *E. coli*/Coliform Count Plates for use in industries other than food and beverage. 3M has not documented 3M Petrifilm Rapid *E. coli*/Coliform Count Plates for testing water, pharmaceuticals, or cosmetics.
(b) Do not use 3M Petrifilm Rapid *E. coli*/Coliform Count Plates in the diagnosis of conditions in humans or animals.
(c) 3M Petrifilm Rapid *E. coli*/Coliform Count Plates do not differentiate any one *E. coli* or coliform strain from another.
(d) A few strains of bacteria can produce β-glucuronidase such as *Shigella, Salmonella, Enterobacter, Citrobacter,* and *Klebsiella* and will produce blue to blue-green colonies on the 3M Petrifilm Rapid *E. coli*/Coliform Count Plate.
(e) Foods with high sugar content may increase the potential for gas production from non-coliform *Enterobacteriaceae*.

D. Sample Preparation

(a) Use appropriate sterile diluents (BPBD or PSS). Do not use diluents containing citrate, bisulfate, or thiosulfate with the 3M Petrifilm Rapid *E. coli*/Coliform Count Plates, as they can inhibit growth.
(b) For food samples, prepare test portion (10, 11, or 50 g) or equivalent ratio of sample to diluent to create a 1:10 dilution as appropriate to the sample being tested.
(c) For environmental surface samples, add 25 mL of the appropriate diluent to each sponge sample.
(d) Blend or homogenize sample as appropriate.
(e) For optimal growth and recovery of microorganisms in acidic products (< pH 5), adjust the pH of the sample suspension to greater than pH 5. For acidic products, adjust with pH 1N NaOH.
(f) Remove all required plates and allow to come to ambient temperature (20–25°C).
(g) Retract the top film to fully expose the culture medium and dispense 1.0 mL of sample onto the center of the plate.
(h) Reapply the cover by rolling down the film. Place the 3M Petrifilm Flat Spreader on the center of the plate and press gently to allow sample to spread evenly over the medium

causing gel to form. Let the plate sit undisturbed for at least 1 min.

(i) Incubate the plates.
   (1) For dairy products: 30 ± 1°C or 32 ± 1°C for coliforms and *E. coli* or 42 ± 1°C for *E. coli* for 18–24 h.
   (2) For all other foods: 35 ± 1°C or 37 ± 1°C for coliforms and *E. coli* or 42 ± 1°C for *E. coli* for 18–24 h.

(j) Enumerate all blue to blue-green colonies with or without gas regardless of size or intensity of color as *E. coli*.

(k) Interpretation of non-*E. coli* coliform colonies varies by reference method.
   (1) The U.S. FDA/BAM Chapter 4 defines coliforms as Gram negative rods, which produce acid and gas from lactose during metabolic fermentation. Enumerate red colonies which are closely associated with entrapped gas within one colony diameter of the colony. Colonies not associated with gas (a distance greater than one colony diameter between colony and gas bubble) are not counted as coliforms. The total coliform count consists of both the red colonies with gas and blue colonies with and without gas, estimates can be made on plates containing > 100 colonies.
   (2) ISO defines coliforms by their ability to grow in method-specific, selective media. ISO 4832 enumerates typical coliform colonies on VRBL with confirmation of atypical colonies. Enumerate red colonies with and without gas production. The total coliform count is indicated by red colonies with or without gas production and blue colonies with and without gas production, estimates can be made on plates containing > 100 colonies.

(l) Multiply the count by the dilution factor to get final coliform and *E. coli* count.

Results of the Collaborative Study

In this collaborative study, the 3M Petrifilm Rapid *E. coli*/Coliform Count Plate was compared to the FDA BAM Chapter 4, ISO 4832, and 16649-2 for the enumeration of *E. coli* and coliforms. A total of 9 participants across 8 laboratories throughout the United States, Canada, Mexico, and Switzerland participated in the evaluation. All participants who received samples performed analysis and submitted results.

The candidate method results along with the reference method results reported by each laboratory were converted to logarithmic values for statistical analysis and were plotted using a Youden's plot. The Log_{10} individual laboratory results are presented in Tables 2018.13C–G. Figures 1–5 present the Youden plots of each matrix. The transformed data were analyzed for outliers by the Cochran and Grubb's tests. No outliers were identified. The difference of means (including 95% confidence intervals), repeatability (s_r), and reproducibility (s_R) were determined for each contamination level. The results of the interlaboratory data analyses are presented in Tables 2018.13A and B. In addition to the test portions, each participant that performed testing submitted results for an APC test to determine the total microbial load of the test matrix. The average APC result obtained by the collaborators was 1.8×10^5 CFU/g (low to high range of 3.4×10^4 CFU/g to 5.2×10^5 CFU/g). Table 1 presents the results of the APC for each collaborator.

Table 1. Results of aerobic plate count for collaborating laboratories

Lab	Dry Dog Kibble (CFU/g)[a]
1	1.2×10^5
2	3.4×10^4
3	6.6×10^4
4	3.7×10^5
5	5.2×10^5
6	1.1×10^5
7	6.6×10^4
8	8.9×10^4
9	2.2×10^5

[a] Samples analyzed by FDA BAM Chapter 3.

Dry Dog Kibble

Four contamination levels were evaluated (uninoculated, low, medium, and high) by both the candidate and reference method. Nine collaborators submitted data, and all data sets were included in the statistical analysis.

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and BAM Coliform

Difference of means values (0.00, -0.28, 0.28, and 0.17) for the uninoculated, low, medium, and high contamination levels, indicate the candidate and reference method are not statistically different. Repeatability (0.00, 0.22, 0.12, and 0.10) and reproducibility (0.00, 0.25, 0.16, and 0.17) values for each contamination level indicate that the method performed similarly within sample replicates and between laboratories throughout the range of contamination levels.

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and BAM *E. coli*

Difference of means values (0.00, -0.02, -0.01, and -0.02) for the uninoculated, low, medium, and high contamination levels, indicate the candidate and reference method are not statistically different. Repeatability (0.00, 0.22, 0.12, and 0.14) and reproducibility (0.00, 0.31, 0.26, and 0.22) values for each contamination level indicate that the method performed similarly within sample replicates and between laboratories throughout the range of contamination levels.

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and ISO Coliform 37 °C

Difference of means values (0.00, 0.08, -0.03, -0.01) for the uninoculated, low, medium, and high contamination levels, indicate the candidate and reference method are not statistically different. Repeatability (0.00, 0.28, 0.09, 0.09) and reproducibility (0.00, 0.34, 0.19, 0.16) values for each contamination level indicate that the method performed similarly within sample replicates and between laboratories throughout the range of contamination levels.

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and ISO *E. coli* 37 °C

Difference of means values (0.00, 0.12, 0.01, and 0.04) for the uninoculated, low, medium, and high contamination levels, indicate the candidate and reference method are not statistically different. Repeatability (0.00, 0.28, 0.12, and 0.11) and reproducibility (0.00, 0.28, 0.19, and 0.14) values for each contamination level indicate that the method performed similarly within sample replicates and between laboratories throughout the range of contamination levels.

3M Petrifilm Rapid *E. coli*/Coliform Count Plate and ISO *E. coli* 42 °C

Difference of means values (0.00, 0.14, -0.05, and 0.05) for the uninoculated, low, medium, and high contamination levels, indicate the candidate and reference method are not statistically different. Repeatability (0.00, 0.27, 0.13, and 0.14) and reproducibility (0.00, 0.27, 0.15, and 0.14) values for each contamination level indicate that the method performed similarly within sample replicates and between laboratories throughout the range of contamination levels.

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regard to the performance of the candidate method. No statistically significant difference was observed between the candidate method and the reference methods, when compared using a difference of means of < 0.5 and a confidence interval between -0.5 and 0.5. Based on the data presented, the reproducibility values obtained for all contamination levels were generally similar between the candidate and reference methods, indicating that both the between-laboratory variations and within-laboratory variations were consistent between the candidate and reference method. These values indicate that for reproducibility, no meaningful statistical differences (absolute value of <0.50 Log_{10}) were observed in the data between the candidate and reference methods when test portions were analyzed by different analysts at each laboratory or within each sample set at a given laboratory.

Recommendations

It is recommended that the 3M Petrifilm Rapid *E. coli*/Coliform Count Plate be adopted as Official First Action status for the enumeration of *E. coli* and coliforms in a broad range of foods and environmental surface established from testing pasteurized whole milk, butter, non-fat dry milk, raw ground pork, lamb chop, raw ground chicken, chicken carcass rinsate, shell eggs, liquid egg whites, powdered egg whites, sprouts, cranberries, infant formula with probiotics, infant formula without probiotics, infant rice cereal without probiotics, dry dog kibble, dry cat food, flour, chocolate chip cookie dough, raw ground beef (73% lean), raw frozen chicken wings, raw milk, whole liquid egg, tuna sushi, smoked salmon, bunched spinach, pasteurized carrot juice, ready-made sandwiches, raw vegetable salad with dressing, chicken feed, soybean meal, stainless steel environmental sponges, and sealed concrete environmental sponges.

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Approving Body and Method Feedback

This method was approved by the AOAC Expert Review Panel for Microbiology Methods for Food and Environmental Surfaces as First Action. The Expert Review Panel invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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BAM: Clostridium botulinum

January 2001

Bacteriological Analytical Manual
Chapter 17
Clostridium botulinum

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Clostridium botulinum is an anaerobic, rod-shaped sporeforming bacterium that produces a protein with characteristic neurotoxicity. Under certain conditions, these organisms may grow in foods producing toxin(s). Botulism, a severe form of food poisoning results when the toxin-containing foods are ingested. Although this food illness is rare, its mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 (2) involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks, the foods implicated contained both types A and B toxins. Due to a limited number of reports, type C and D toxins have been questioned as the causative agent of human botulism. It is suspected that these toxins are not readily absorbed in the human intestine. However, all types except F and G, which have not been as studied thoroughly, are important causes of animal botulism.

Antigenic types of *C. botulinum* are identified by the complete neutralization of their toxins using the homologous antitoxin. Cross-neutralization of a specific toxin by heterologous antitoxins does not occur or is minimal. There are seven recognized antigenic types: A through G. Cultures of five of these types apparently produce only one type of toxin but all are given type designations corresponding to their toxin production. Types C and D cross-react with antitoxins to each other because they each produce more than one toxin and have at least one common toxin component. Type C produces predominantly C_1 toxin with lesser amounts of D and C_2, or only C_2, and type D produces predominantly type D toxin along with smaller amounts of C_1 and C_2. Mixed toxin production by a single strain of *C. botulinum* may be more common than previously realized. There is a slight reciprocal cross-neutralization with types E and F, and recently a strain of *C. botulinum* was shown to produce a mixture of predominantly type A toxin, with a small amount of type F.

Aside from toxin type, *C. botulinum* can be differentiated into general groups on the basis of cultural, biochemical, and physiological characteristics. Cultures producing types C and D toxins are not proteolytic on coagulated egg white or meat and have a common metabolic pattern which sets them apart from the others. All cultures that produce type A toxin and some that produce B and F toxins are proteolytic. All type E strains and the remaining B and F strains are nonproteolytic, with carbohydrate metabolic patterns differing from the C and D nonproteolytic groups. Strains that produce type G toxin have not been studied in sufficient detail for effective and satisfactory characterization.

C. botulinum is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to

contaminated fish or other seafoods. Types A and B are most commonly encountered in foods associated with soil contamination. In the United States, home-canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of foodborne illness caused by these types.

Measures to prevent botulism include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all botulinal spores in the food. Heat processing is the most common method of destruction. Properly processed canned foods will not contain viable *C. botulinum*. Home-canned foods are more often a source of botulism than are commercially canned foods, which probably reflects the commercial canners' great awareness and better control of the required heat treatment.

A food may contain viable *C. botulinum* and still not be capable of causing botulism. If the organisms do not grow, no toxin is produced. Although many foods satisfy the nutritional requirements for the growth of *C. botulinum*, not all of them provide the necessary anaerobic conditions. Both nutritional and anaerobic requirements are supplied by many canned foods and by various meat and fish products. Growth in otherwise suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity, a high concentration of NaCl, an inhibitory concentration of $NaNO_2$ or other preservative, or two or more of these conditions in combination. Refrigeration will not prevent growth and toxin formation by nonproteolytic strains unless the temperature is precisely controlled and kept below 3°C. Foods processed to prevent spoilage but not usually refrigerated are the most common vehicles of botulism.

Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for nonproteolytic strains it is 26-28°C. Nonproteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the nonproteolytics do not manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form. These and other differences can be important in epidemiological and laboratory considerations of botulism outbreaks. Clinical diagnosis of botulism is most effectively confirmed by identifying botulinal toxin in the blood, feces, or vomitus of the patient. Specimens must be collected before botulinal antitoxin is administered to the patient. Identifying the causative food is most important in preventing additional cases of botulism. See Examination of Canned Foods, Chapter 21.

Botulism in infants 6 weeks to 1 year of age was first recognized as a distinct clinical entity in 1976. This form of botulism results from growth and toxin production by *C. botulinum* within the intestinal tract of infants rather than from ingestion of a food with preformed toxin. It is usually caused by *C. botulinum* types A or B, but a few cases have been caused by other types. Infant botulism has been diagnosed in most U.S. states and in every populated continent except Africa (1).

Constipation almost always occurs in infant botulism and usually precedes characteristic signs of neuromuscular paralysis by a few days or weeks. Illnesses have a broad range of severity. Some infants show only mild weakness, lethargy, and reduced feeding and do not require hospitalization. Many have shown more severe symptoms such as weakened suck, swallowing, and cry; generalized muscle weakness; and diminished gag reflex with a pooling of oral secretions. Generalized muscle weakness and loss of head control in some infants reaches such a degree of severity that the patient appears "floppy." In some hospitalized cases, respiratory arrest has occurred, but most were successfully

resuscitated, and with intense supportive care have ultimately recovered. As a result, the case-fatality rate (2%) for this form of botulism is low. Recovery usually requires at least several weeks of hospitalization (1).

Honey, a known source of *C. botulinum* spores, has been implicated in some cases of infant botulism. In studies of honey, up to 13% of the test samples contained low numbers of *C. botulinum* spores (3). For this reason, the FDA, the Centers for Disease Control and Prevention (CDC), and the American Academy of Pediatrics recommend not feeding honey to infants under one year old.

The mouse bioassay is a functional assay that detects biologically active toxin. The assay requires a three part approach: toxin screening, toxin titer, and finally toxin neutralization using monovalent antitoxins. The process requires two days of analysis at each step.

Recently, rapid, alternative, in-vitro procedures have been developed for the detection of types A, B, E, and F botulinal toxin producing organisms and their toxins. The toxins generated in culture media can be detected using ELISA techniques such as the DIG-ELISA and the amp-ELISA. Biologically active and non-active toxins are detected since the assay detects the toxin antigen. The ELISA assays require one day of analysis. The toxin genes of viable organisms can be detected using the polymerase chain reaction technique and require one days of analysis after overnight incubation of botulinal spores or vegetative cells. In-vitro assays that are positive are confirmed using the mouse bioassay.

I. Mouse Bioassay for *Clostridium botulinum* Toxin

   A. Equipment and Materials

      1. Refrigerator
      2. Clean dry towels

3. Bunsen burner
4. Sterile can opener (bacteriological or puncture type)
5. Sterile mortar and pestle
6. Sterile forceps
7. Sterile cotton-plugged pipets
8. Mechanical pipetting device (NEVER pipet by mouth)
9. Sterile culture tubes (at least a few should be screw-cap tubes)
10. Anaerobic jars (GasPak or Case-nitrogen replacement)
11. Transfer loops
12. Incubators, 35 and 28°C
13. Sterile, reserve sample jars
14. Culture tube racks
15. Microscope slides
16. Microscope, phase-contrast or bright-field
17. Sterile petri dishes, 100 mm
18. Centrifuge tubes
19. Centrifuge, refrigerated, high-speed
20. Trypsin (1:250; Difco Laboratories, Detroit, MI)
21. Syringes, 1 and or 3 ml, sterile, with 25 gauge, 5/8 inch needles for injecting mice
22. Mice, 16-24 g (for routine work, up to 34 g)
23. Mouse cages, feed, water bottles, etc.
24. Millipore filters: 0.45 µm pore size

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

1. Alcoholic solution of iodine (4% iodine in 70% ethanol) (R18 (/food/laboratory-methods/bam-r18-disinfectants))
2. Chopped liver broth (M38 (/food/laboratory-methods/bam-media-m38-chopped-liver-broth)) or cooked meat medium (M42 (/food/laboratory-methods/bam-media-m42-cooked-meat-medium))
3. Trypticase-peptone-glucose-yeast extract (TPGY) (M151 (/food/laboratory-methods/bam-media-m151-trypticase-peptone-glucose-yeast-extract-broth-tpgy)) broth or with trypsin (TPGYT) (M151a (/food/laboratory-methods/bam-media-m151a-trypticase-peptone-glucose-yeast-extract-broth-trypsin-tpgyt))
4. Liver-veal-egg yolk agar (M84 (/food/laboratory-methods/bam-media-m84-liver-veal-egg-yolk-agar)) or anaerobic egg yolk agar (M12 (/food/laboratory-methods/bam-media-m12-anaerobic-egg-yolk-agar))
5. Sterile, gel-phosphate buffer, pH 6.2 (R29 (/food/laboratory-methods/bam-r29-gel-phosphate-buffer))
6. Absolute ethanol
7. Gram stain reagents (R32 (/food/laboratory-methods/bam-r32-gram-stain)), crystal violet (R16 (/food/laboratory-methods/bam-r16-crystal-violet-stain-bacteria)), or methylene blue (R45 (/food/laboratory-methods/bam-r45-methylene-blue-stain-loefflers)) solutions

8. Sterile physiological saline solution (R63 (/food/laboratory-methods/bam-r63-physiological-saline-solution-085-sterile))
9. Monovalent antitoxin preparations, types A-F (obtain from CDC)
10. Trypsin solution (prepared from Difco 1:250)
11. 1 N Sodium hydroxide solution (R73 (/food/laboratory-methods/bam-r73-1-n-sodium-hydroxide-solution))
12. 1 N Hydrochloric acid solution (R36 (/food/laboratory-methods/bam-r36-1-n-hydrochloric-acid))

C. Sample preparation

Preliminary examination. Refrigerate samples until testing, except unopened canned foods, which need not be refrigerated unless badly swollen and in danger of bursting. Before testing, record product designation, manufacturer's name or home canner, source of sample, type of container and size, labeling, manufacturer's batch, lot or production code, and condition of container. Clean and mark container with laboratory identification codes.

Solid and liquid foods. Aseptically transfer foods with little or no free liquid to sterile mortar. Add equal amount of gel-phosphate buffer solution and grind with sterile pestle before inoculation. Alternatively, inoculate small pieces of product directly into enrichment broth with sterile forceps. Inoculate liquid foods directly into enrichment broth with sterile pipets. Reserve sample; after culturing, aseptically remove reserve portion to sterile sample jar for tests which may be needed later. Refrigerate reserve sample.

Opening of canned foods (see Chapter 21).

Examine product for appearance and odor. Note any evidence of decomposition. DO NOT TASTE the product under any circumstances. Record the findings.

D. Detection of viable *C. botulinum*

1. Enrichment. Remove dissolved oxygen from enrichment media by steaming 10-15 min and cooling quickly without agitation before inoculation.

   Inoculate 2 tubes of cooked meat medium with 1-2 g solid or 1-2 ml liquid food per 15 ml enrichment broth. Incubate at 35°C.

   Inoculate 2 tubes of TPGY broth as above. Incubate at 28°C. Use TPGYT as alternative only when organism involved is strongly suspected of being a nonproteolytic strain of types B, E, or F.

   Introduce inoculum slowly beneath surface of broth to bottom of tube. After 5 days of incubation, examine enrichment cultures. Check for turbidity, gas production, and digestion of meat particles. Note the odor.

   Examine cultures microscopically by wet mount under high-power phase contrast, or a smear stained by Gram reagent, crystal violet, or methylene blue under bright-field illumination. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells. A typical clostridial cell resembles a tennis racket. At this time test each enrichment culture for toxin, and if present, determine toxin type according to procedure in F, below. Usually, a 5-day incubation is the period of active growth giving the highest concentration

of botulinal toxin. If enrichment culture shows no growth at 5 days, incubate an additional 10 days to detect possible delayed germination of injured spores before discarding sample as sterile. For pure culture isolation save enrichment culture at peak sporulation and keep under refrigeration.

2. Isolation of pure cultures. *C. botulinum* is more readily isolated from the mixed flora of an enrichment culture or original specimen if sporulation has been good.

   Pre-treatment of specimens for streaking. Add equal volume of filter-sterilized absolute alcohol to 1 or 2 ml of enrichment culture in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. To isolate from sample, take 1 or 2 ml of retained portion, and add an equal volume of filter-sterilized absolute alcohol in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. Alternatively, heat 1 or 2 ml of enrichment culture or sample to destroy vegetative cells (80°C for 10-15 min). DO NOT use heat treatment for nonproteolytic types of *C. botulinum*.

   Plating of treated cultures. With inoculating loop, streak 1 or 2 loopfuls of ethanol or heat-treated cultures to either liver- veal-egg yolk agar or anaerobic egg yolk agar (or both) to obtain isolated colonies. If necessary, dilute culture to obtain well-separated colonies. Dry agar plates well before use to prevent spreading of colonies. Incubate streaked plates at 35°C for about 48 h under

anaerobic conditions. A Case anaerobic jar or the GasPak system is adequate to obtain anaerobiosis; however, other systems may be used.

E. Selection of typical *C. botulinum* colonies

Selection. Select about 10 well-separated typical colonies, which may be raised or flat, smooth or rough. Colonies commonly show some spreading and have an irregular edge. On egg yolk medium, they usually exhibit surface iridescence when examined by oblique light. This luster zone, often referred to as a pearly layer, usually extends beyond and follows the irregular contour of the colony. Besides the pearly zone, colonies of *C. botulinum* types C, D, and E are ordinarily surrounded by a wide zone (2-4 mm) of yellow precipitate. Colonies of types A and B generally show a smaller zone of precipitation. Considerable difficulty may be experienced in picking toxic colonies since certain other members of the genus *Clostridium* produce colonies with similar morphological characteristics but do not produce toxins.

Inoculation. Use sterile transfer loop to inoculate each selected colony into tube of sterile broth. Inoculate *C. botulinum* type E into TPGY broth. Inoculate other toxin types of *C. botulinum* into chopped liver broth or cooked meat medium. Incubate as described in D-1, above, for 5 days. Test for toxin production as described in F, below. To determine toxin type, see F-3, below.

Isolation of pure culture. Restreak toxic culture in duplicate on egg yolk agar medium. Incubate one plate anaerobically at 35°C. Incubate second plate aerobically at 35°C. If colonies typical of *C. botulinum* are found only on anaerobic plate (no growth on aerobic plate), the culture may

be pure. Failure to isolate *C. botulinum* from at least one of the selected colonies means that its population in relation to the mixed flora is probably low. Repeated serial transfer through additional enrichment steps may increase the numbers sufficiently to permit isolation. Store pure culture in sporulated state either under refrigeration, on glass beads, or lyophilized.

F. Detection and identification of botulinal toxin

1. Preparation of food sample. Culture one portion of sample for detection of viable *C. botulinum*; remove another portion for toxicity testing, and store remainder in refrigerator. Centrifuge samples containing suspended solids under refrigeration and use supernatant fluid for toxin assay. Extract solid foods with equal volume of gel-phosphate buffer, pH 6.2, by macerating food and buffer with pre-chilled mortar and pestle. Centrifuge macerated sample under refrigeration and use supernatant fluid for toxin assay. Rinse empty containers suspected of having held toxic foods with a few milliliters of gel-phosphate buffer. Use as little buffer as possible to avoid diluting toxin beyond detection. To avoid or minimize nonspecific death of mice, filter supernatant fluid through a millipore filter before injecting mice. For non-proteolytic samples or cultures, trypsinize after filtration.

2. Determination of toxicity in food samples or cultures

   Trypsin treatment. Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Therefore, treat a portion of food supernatant fluid, liquid food, or TPGY culture with trypsin before testing

for toxin. Do not treat TPGYT culture with trypsin since this medium already contains trypsin and further treatment may degrade any fully activated toxin that is present. Adjust portion of supernatant fluid, if necessary, to pH 6.2 with 1 N NaOH or HCl. Add 0.2 ml aqueous trypsin solution to 1.8 ml of each supernatant fluid to be tested for toxicity. (To prepare trypsin solution, place 0.5 g of Difco 1:250 trypsin in clean culture tube and add 10 ml distilled water, shake, and warm to dissolve. Analysts who are allergic to trypsin should weigh it in a hood or wear a face mask.) Incubate trypsin- treated preparation at 35-37°C for 1 h with occasional gentle agitation.

Toxicity testing. Conduct parallel tests with trypsin-treated materials and untreated duplicates. Dilute a portion of untreated sample fluid or culture to 1:5, 1:10, and 1:100 in gel-phosphate buffer. Make the same dilutions of each trypsinized sample fluid or culture. Inject each of separate pairs of mice intraperitoneally (i.p.) with 0.5 ml untreated undiluted fluid and 0.5 ml of each dilution of untreated test sample, using a 1 or 3 ml syringe with 5/8 inch, 25 gauge needle. Repeat this procedure with trypsin-treated duplicate samples. Heat 1.5 ml of untreated supernatant fluid or culture for 10 min at 100°C. Cool heated sample and inject each of a pair of mice with 0.5 ml undiluted fluid. These mice should not die, because botulinal toxin, if present, will be inactivated by heating.

Observe all mice periodically for 48 h for symptoms of botulism. Record symptoms and deaths. Typical botulism signs in mice begin usually in the first 24 h with ruffling of fur, followed in sequence by labored

breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure. Death of mice without clinical symptoms of botulism is not sufficient evidence that injected material contained botulinal toxin. On occasion, death occurs from other chemicals present in injected fluid, or from trauma.

If after 48 h of observation, all mice except those receiving the heated preparation have died, repeat the toxicity test, using higher dilutions of supernatant fluids or cultures. It is necessary to have dilutions that kill and dilutions that do not kill in order to establish an endpoint or the minimum lethal dose (MLD) as an estimate of the amount of toxin present. The MLD is contained in the highest dilution killing both mice (or all mice inoculated). From these data, the number of MLD/ml can be calculated.

3. Typing of toxin. Rehydrate antitoxins with sterile physiological saline. Do not use glycerin water. Dilute monovalent antitoxins to types A, B, E, and F in physiological saline to contain 1 international unit (IU) per 0.5 ml. Prepare enough of these antitoxin solutions to inject 0.5 ml of antitoxin into each of 2 mice for each dilution of toxic preparation to be tested. Use the toxic preparation that gave the higher MLD, either untreated or trypsinized. Prepare dilutions of the toxic sample to cover at least 10, 100, and 1000 MLD below the previously determined endpoint of toxicity if possible (see 2, above). The untreated toxic preparation can be the same as that used for testing toxicity. If a trypsinized

preparation was the most lethal, it will be necessary to prepare a freshly trypsinized fluid. The continued action of trypsin may destroy the toxin.

Inject the mice with the monovalent antitoxins, as described above, 30 min to 1 h before challenging them with i.p. injection of the toxic preparations. Inject pairs of mice (protected by specific monovalent antitoxin injection) i.p. with each dilution of the toxic preparation. Also inject a pair of unprotected mice (no injection of antitoxin) with each toxic dilution as a control. The use of 4 monovalent antitoxins (types A, B, E, and F) for the unknown toxic sample prepared at 3 dilutions requires a total of 30 mice — 6 mice for each antitoxin (24 mice) plus 2 unprotected mice for each of the 3 dilutions (6 mice) as controls. Observe mice for 48 h for symptoms of botulism and record deaths. If test results indicate that toxin was not neutralized, repeat test, using monovalent antitoxins to types C and D, plus polyvalent antitoxin pool of types A through F.

II. Mouse Screening Procedure for *Clostridium botulinum* Type E Spores in Smoked Fish

A. Equipment and Materials

1. 12 mice (16-24 g, or up to 34 g) per subsample (24 or more required for positives)
2. Types A, B, E antisera
3. Saline, sterile, 0.85% NaCl (R63 (/food/laboratory-methods/bam-r63-physiological-saline-solution-085-sterile))

4. Trypsin (Difco); 1:250, 5% solution
5. Syringes, 1 and 3 ml, 25 gauge, 5/8 inch needle
6. Incubator 28°C
7. TPGY medium (M151 (/food/laboratory-methods/bam-media-m151-trypticase-peptone-glucose-yeast-extract-broth-tpgy))
8. Water bath, 37°C
9. Gel-phosphate diluent (R29 (/food/laboratory-methods/bam-r29-gel-phosphate-buffer))
10. Centrifuge, refrigerated
11. Plastic bags, strong and water-tight

B. Procedure

Incubation. Place each smoked fish subsample (which may consist of 1 or more fish, depending on size, and may be either vacuum-packed or bulk-smoked fish) in a strong water-tight plastic bag. Add freshly steamed and cooled TPGY broth to subsample. NOTE: Add enough TPGY broth to completely cover fish. Squeeze bag to expel as much air as possible and seal it with hot-iron bag sealer or other air-tight closure device. Incubate at 28°C for 5 days. Precautions should be taken during incubation period since bag may swell and split from gas formation.

Cultures. At end of incubation period, centrifuge 20 ml of TPGY culture from each subsample at 7500 × *g* rpm for 20 min. Use refrigerated centrifuge. Determine pH of TPGY. If above 6.5, adjust to 6.0-6.2 with HCl. Refrigerate for overnight storage.

Trypsinization. To 3.6 ml of culture, adjusted to pH 6.0-6.2, add 0.4 ml of 5% solution of trypsin. Incubate at 35-37°C for 1 h. Remove culture and let cool to room temperature before injecting mice. Trypsinized extract cannot be stored overnight.

Toxicity screening. Dilute trypsinized and nontrypsinized broth cultures to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (NOTE: Do not store trypsinized material overnight.) Inject mice i.p. with 0.5 ml of each dilution. Inject 2 mice per dilution, i.e., trypsinized and nontrypsinized (total 12 mice per subsample). Observe mice for botulism symptoms and record condition of mice at frequent intervals for 48 h. If no deaths occur, no further tests are indicated. Deaths are presumptive evidence of toxin and should be confirmed.

Confirmation with protected mice. Dilute new portion of nontrypsinized or trypsinized culture (whichever showed the highest titer) to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.) Inject 6 mice i.p. with 0.5 ml of 1:5 saline dilution of type E antiserum. These will be compared to 6 mice without this protection (controls). After 30 min, inject 0.5 ml of each dilution into 2 mice protected with antiserum and into 2 mice not so protected. Record their condition at intervals up to 48 h. If unprotected mice die and protected mice live, the presence of type E toxin is indicated. If all protected mice die, repeat confirmation with higher dilutions of toxic culture in type E-protected mice and with mice protected against *C. botulinum* types A and/or B antiserum. If all antiserum-protected mice die, send toxic culture media on dry ice to Division of Microbiological Studies (HFS-516), FDA, *5100 Paint Branch Pkwy, College Park, MD 20740*, for further tests. Isolate and identify cultures from samples containing toxin of type E, if possible.

Obtain *C. botulinum* antisera from Centers for Disease Control and Prevention, Atlanta, GA 30333, USA. Reconstitute lyophilized antisera with sterile saline. Dilute sera 1:5 with sterile saline for mouse injection.
If you have questions about the method, contact *Shashi Sharma*, FDA. Telephone *(240)-402-1570*.

General Hints Regarding *C. botulinum* Toxin Analysis

1. The first 24 hours are the most important time regarding symptoms and death of mice: 98-99% of animals die within 24 hours. Typical symptoms of botulism and death may occur within 4 to 6 hours.
2. If deaths occur after 24 hours, be very suspicious, unless typical botulism symptoms are clearly evident.
3. If deaths occur in mice injected with the 1:2 or 1:5 dilution but not with any higher dilution, be very suspicious. Deaths may have been from nonspecific causes.
4. Mice can be marked on tails with dye to represent various dilutions. Dye does not come off easily.
5. Mice injected with botulinal toxin may become hyperactive before symptoms occur.
6. Food and water may be given to the mice right away; it will not interfere with the test.
7. Rehydrated antitoxin may be kept up to 6 months under refrigeration, and may be frozen indefinitely.
8. TPGY medium is relatively stable and can be kept 2-3 weeks under refrigeration.
9. With cooked meat medium, vortex tubes completely; toxin may adhere to meat particles.

10. Trypsin is not filtered. Use 0.5 g in 10 ml of distilled water. It can be kept up to 1 week under refrigeration.

Interpretation of Data (NOTE: Laboratory tests are designed to identify botulinal toxin and/or organisms in foods)

1. Toxin in a food means that the product, if consumed without thorough heating, could cause botulism.
2. Viable *C. botulinum* but no toxin in foods is not proof that the food in question caused botulism.
3. The presence of toxin in food is required for an outbreak of botulism to occur.
4. Ingested organisms may be found in the alimentary tract, but are considered to be unable to multiply and produce toxin in vivo, except in infants.
5. Presence of botulinal toxin and/or organisms in low-acid (i.e., above pH 4.6) canned foods means that the items were underprocessed or were contaminated through post-processing leakage.
    - Swollen cans are more likely than flat cans to contain botulinal toxin since the organism produces gas during growth.
    - Presence of toxin in a flat can may imply that the seams were loose enough to allow gas to escape.
    - Botulinal toxin in canned foods is usually of a type A or a proteolytic type B strain, since spores of the proteolytics can be among the more heat-resistant.
    - Spores of nonproteolytics, types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.
6. The protection of mice from botulism and death with one of the

monovalent botulinal antitoxins confirms the presence of botulinal toxin and determines the serological type of toxin in a sample.

7. The following reasons may explain why deaths occur in mice that are protected by one of the monovalent antitoxins:
    - There may be too much toxin in the sample.
    - More than one kind of toxin may be present.
    - Deaths may be due to some other cause.

    Retesting at higher dilutions of toxic fluids is required, and mixtures of antitoxins must be used in place of monovalent antiserum. Some other toxic material, which is not heat-labile, could be responsible if both heated and unheated fluids cause death. The heat-stable toxic substance could possibly mask botulinal toxin.

Safety Precautions for the *Clostridium botulinum* Laboratory

1. Place biohazard signs on doors to restrict entrance and keep the number of people in the laboratory to a minimum.
2. All workers in the laboratory should wear laboratory coats and safety glasses.
3. Use 1% hypochlorite solution to wipe laboratory table tops before and after work.
4. NEVER PIPETTE ANYTHING BY MOUTH. USE MECHANICAL PIPETTORS.
5. Use a biohazard hood for transfer of toxic material, if possible.
6. Centrifuge toxic materials in a hermetically closed centrifuge with safety cups.
7. Personally take all toxic material to the autoclave and see that it is sterilized immediately.

8. Do not work alone in the laboratory or animal rooms after hours or on weekends.
9. Have an eye wash fountain and foot-pedaled faucet available for hand washing.
10. No eating and drinking in the laboratory when someone works with toxins.
11. In a very visible location, list phone numbers where therapeutic antitoxin can be obtained in case of emergency. THIS IS VERY IMPORTANT!
12. Reduce clutter in the laboratory to a minimum and place equipment and other materials in their proper place after use.

References

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3. Hauschild, A.H.W., R. Hilsheimer, K.F. Weiss, and R.B. Burke. 1988. *Clostridium botulinum* in honey, syrups, and dry infant cereals. *J. Food Prot.* 51:892-894.

III. Amplified ELISA Procedure for Detection of Botulinal Toxins A, B, E, and F from Culture. Contact Joseph L. Ferreira (404 253-2216) for questions about method.

These toxins can be detected using an amplified ELISA procedure that has a detection limit of approximately 10 MLD/mL. Toxic cultures may be more antigenic than purified toxins and the level of detection using the ELISA may be more sensitive than the mouse bioassay. Both TPGY and CMM are tested since more toxin may be generated in one medium compared to the other and the mouse bioassay, which is needed for confirmation of ELISA tests, also utilizes these media.

A. Equipment and Materials

1. Microplate, Dynex Immulon II U-bottom, cat. No. 3655
2. Microtiter pipettors to deliver from 0.1- 2.0, 2-20, and 50-200 µl.
3. Multichannel pipettor, 8 or 12 place 50-200 µl
4. Pipets, disposable 1,5,10 ml
5. Glass test tubes 13X100 mm, 15X150 mm
6. Incubator, 35°C
7. Refrigerated centrifuge
8. Microplate washer
9. Microplate shaker
10. Microplate reader (read 490 and 630 nm reference)
11. Microtiter plate seals
12. Multichannel pipet reservoirs

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

1. Tryptone-peptone-glucose-yeast extract broth (TPGY).
2. Cooked meat medium (CMM).

3. 0.05M bicarbonate buffer: 0.8g Na_2CO_3 + 1.47g $NaHCO_3$ in 500 ml distilled H_2O, pH 9.6.
4. 1% Casein buffer: Add 10.0g vitamin-free casein + 7.65 g NaCl, 0.724g Na_2HPO_4 (anhydrous), 0.21g KH_2PO_4 to 900 ml H_2O, and 3 ml of 1 M NaOH. Heat with stirring to ~ 80°C to dissolve casein. Check pH and adjust to 7.9 with 1 M NaOH, q.s. to 1 liter. Sterilize at 121°C for 20 min. Final pH is ~7.4-7.6.
5. Goat type A or E, rabbit type B, or horse F antitoxin.
6. Goat type A, B, E, or F biotinylated antitoxin
7. Tris buffered NaCl-0.005% Tween 20 (TBST): 6.04g Tris base, 8.76g NaCl, Distilled H_2O 900 ml, dissolve Tris and NaCl, pH adjust to 7.5 at 25°C with 2 M HCl, add 50 µl of Tween-20 and q.s. to 1 liter.
8. Extravidin-alkaline phosphatase conjugate (Sigma)
9. Amplified ELISA substrate system (GibCo)
10. 0.3 M H_2SO_4: dilute concentrated acid (MW 98, specific gravity 1.84, purity 96-98%) by adding 1 ml to 59 ml of distilled H_2O.
11. Botulinal complex toxin standards A, B, E, and F. (Metabiologics Inc., Madison, WI)

C. Amplified ELISA Procedure

1. Preparation of samples. Food samples or anaerobic isolates picked from agar plates are inoculated into TPGY (without trypsin) and CMM as recommended in Chapter 17 of the Bacteriological Analytical Manual (2001). TPGY broth and cooked meat media are incubated for 5 days at 26°C and 35°C respectively.

Cultures are centrifuged at 7,000 × g and 4°C for 30 min, supernatant pH is adjusted to 7.4-7.6 using 1 N NaOH or 1N HCl. Samples and controls are analyzed in duplicate for TPGY and for CMM. Analyze undiluted and 1:5 dilutions of each culture supernatant. 1:5= 0.2 ml culture + 0.8 ml casein buffer.

2. Preparation of microtiter plates. Coat each well of the microtiter plate with 100 µl of appropriate dilution of goat type A, E, or F or rabbit type B antitoxin diluted in bicarbonate buffer. Prepare the number of needed microtiter plate wells to test the sample. Dilute the stock antitoxins according to the accompanying directions. Store plate with coating buffer overnight at 4°C with plastic seal cover on top of plate to prevent drying.
3. ELISA analysis of culture media.
    a. Remove plate from 4°C storage and wash plate 5 times in Tris buffered saline (TBST) with 45 second hold between each aspiration. Use a commercial plate washer or other mechanical device; avoid using a squeeze bottle to wash.
    b. Block plate in casein buffer with by filling all wells to the top of the plate (~300 µl/well) and incubate for 60-90 min at 35°C. Prepare the sample and control dilutions while the plate is being blocked.

        Negative controls: Duplicate wells with all reagents except toxin (undiluted sterile CMM and TPGY broth).

Positive controls: Test standard toxins type A, B, E, and F diluted in sterile TPGY and CMM (pH 7.6) at a concentration of 2 ng/ml (~2-60 LD_{50}/ng depending on toxin type).

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

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

e. Wash plate 5 times in TBST as above.

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

g. Wash plate 5 times in TBST as above.

h. Add the streptavidin-alkaline phosphatase conjugate diluted 1:10,000 in casein buffer (100 µl/well), and incubate for 60 min at 35°C.

i. Wash 5 times in TBST with a final 10 minute soak (the last buffer wash is not aspirated). After 10 minute soak, discard the wash and tamp the plate several times on a paper towel to remove wash buffer.

j. Add 50 µl of the GIBCO substrate solution, incubate 12.5 min at room temperature on plate shaker (~100 rpm) then add 50 µl of the GIBCO amplifier and incubate for approximately an

additional 10 min. without shaking. The plate should be taken to the plate reader immediately after addition of the amplifier reagent and be ready to read the reactions. Read absorbance at 490 nm with 630 nm subtraction (reference filter) to account for plate absorbance. The analysis can be stopped at any time (2-15 min) after addition of the amplifier when positive controls give appropriate sensitivity (absorbance ≥ 1.0) and negative controls are acceptable (absorbance not greater than ~ 0.30). The reaction can be stopped with 50 µl of 0.3 M H_2SO_4 and the absorbance read up to two hours later.

Results: A positive test is an absorbance value that is >0.20 above the absorbance observed in the negative controls (sterile uninoculated TPGY broth or CMM).

D. Confirmation of positive ELISA samples.
The ELISA is used for screening culture media that may contain type A, B, E, and/or F botulinal toxins. Samples that are positive using the ELISA must be confirmed using the mouse bioassay.

Flow Diagram for Amp-ELISA

Day 1

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

Day 2

1. Wash plates, block, put on toxic samples and controls, 2 hr incubate.
2. Wash, put on biotinylated IgG's, 1 hr incubate.