GRAS Notice for *Clostridium* Protein

Submitted to:

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

Prepared by:

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New Castle
DE 19720

November, 2022

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LIST OF ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
AI(s)	Adequate Intake(s)
AMR	Antimicrobial Resistance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
BAM	Bacteriological Analytical Manual
BLAST	Basic Local Alignment Search Tool
BoNT	Botulinum Neurotoxin
bp	base pairs
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
cGMP	current Good Manufacturing Practices
CLSI	Clinical and Laboratory Standards Institute
Crispr	Clustered Regularly Interspaced Short Palindromic Repeats
dDDH	digital DNA-DNA Hybridization
DIAAS	Digestible Indispensable Amino Acid Score
DM	Dry Matter
DON	Deoxynivalenol
DNA	Deoxyribonucleic Acid
DRF	Dose Range Finding
DRV(s)	Dietary Reference Value(s)
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EFSA	European Food Safety Authority
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agricultural Organization
FARRP	Food Allergy Research and Resource Program
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FOB	Functional Observational Battery
FSMA	Food Safety Modernization Act
GC	Guanine-Cytosine
GI	Gastrointestinal
GLP	Good Laboratory Practice
GRAS	Generally Recognized as Safe
HACCP	Hazards and Critical Control Points
HDL	High-Density Lipoprotein
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
IOM	Institute of Medicine
LUC	Large Unstained Cells
MCV	Mean Corpuscular Volume
MICs	Minimum Inhibitory Concentrations
NHANES	National Center for Health Statistics' National Health and Nutrition Examination Survey

NCBI	National Center for Biotechnology Information
ND	Not Detected
NGS	Next Generation Sequencing
NIH	National Institutes of Health
NOAEL	No Observed Adverse Effect Level
ODS	Office of Dietary Supplements
OECD	Organization of Economic Cooperation and Development
PCR	Polymerase Chain Reaction
PDCAAS	Protein Digestibility Corrected Amino Acid Score
RAST	Rapid Annotations using Subsystems Technology
RDA(s)	Recommended Daily Allowance(s)
RDI	Recommended Daily Intake
RH	Relative Humidity
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
RTE	Ready-To-Eat
RTD	Ready-To-Drink
SBs	Soybeans
TG	Technical Guidance
TYGS	Type (Strain) Genome Server
UL(s)	Tolerable Upper Limit(s)
U.S.	United States
WGS	Whole Genome Sequencing
WHO	World Health Organization
y	Year

GRAS Notice for *Clostridium* Protein

PART 1. §170.225. SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through §170.285, Superbrewed Food, Inc. (hereafter referred to as "Superbrewed Food") herby informs the United States (U.S.) Food and Drug Administration (FDA) that they are submitting a Generally Recognized As Safe (GRAS) notice for *Clostridium* protein.

1.1 Name and Address of Organization

Superbrewed Food, Inc.
239 Lisa Drive
New Castle
DE 19720

1.2 Name of the Notified Substance

The notified substance is *Clostridium* protein.

1.3 Conditions of Use

Clostridium protein, as manufactured by Superbrewed Food, is intended for use as a direct protein replacement of animal-, fungal- or vegetable-based protein currently used in foods and beverages, and as a supplement to the protein occurring naturally in existing food products as described in Table 1.1.

Table 1.1: Summary of the Individual Proposed Food Uses and Maximum Use Levels for *Clostridium* Protein in Conventional Foods and Beverage Products

Food Category	Food Uses	Maximum Use Level (%) in Final Product
Grain products and pastas	Cereal and granola bars	20
	Meal replacement bars, nutritional bars, energy bars (not specifically marketed as high protein)	20
	High protein bars	40
	Biscuits, chips, crackers	10
	Breads, rolls, bagels, muffins	10
	Dried pasta and noodles	10
	Ready-to-eat breakfast cereals (granola, muesli and high protein)	15
	Ready-to-eat breakfast cereals (branded, RTE)	10
Beverages and beverage bases	Non-milk based nutritional beverages and weight control drinks (RTD)	15
	Non-milk based protein beverages (RTD)	20
	Protein powders (non-milk based)	90 (powder before reconstitution)
	Protein-enriched fruit and vegetable smoothies and juices	20
Dairy products	Milk-based nutritional beverages and weight control drinks	10
	Milk-based protein beverages (RTD)	10

Table 1.1: Summary of the Individual Proposed Food Uses and Maximum Use Levels for *Clostridium* Protein in Conventional Foods and Beverage Products		
Food Category	**Food Uses**	**Maximum Use Level (%) in Final Product**
	Protein powders (milk-based)	90 (powder before reconstitution)
	Chocolate milks, hot chocolate, latte coffees and related drinks	8.5
Dairy product analogs	Non-dairy cheeses, cream cheeses, spreads and dips	20
	Non-dairy cream and sour cream (liquid and powder)	5 (liquid); powder (20)
	Non-dairy yogurts and drinkable yogurts	15
	Non-dairy ice-cream, refrigerated desserts, frozen desserts, whipped toppings	10
	Imitation milks	10
	Non-milk coffee whiteners/creamers	10 (powder) 1 (liquid)
Prepared meals and soups	Ready meals	15
	Soups (creamed vegetable soups only)	10
Plant protein products including meat/poultry analogs	Meat substitutes (meat-free burgers, meatless chicken nuggets, meat-free sausages, meat-free lunch meat etc.)	40
Fats and oils	Salad dressings, spreads, sauces	5
	High protein sauces	15
Confectionary	Gummies, chewy candies, hard candies, marshmallows	20

Abbreviations: RTD = ready to drink; RTE = ready to eat.

1.4 Basis for the Conclusion of GRAS Status

Pursuant to 21 CFR §170.30(a) and (b), the GRAS status of *Clostridium* protein for the intended uses as a source of protein in conventional food and beverages as described herein, has been concluded on the basis of scientific procedures.

1.5 Premarket Exemption Status

Superbrewed Food hereby informs the U.S. FDA of the view that *Clostridium* protein is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FFDCA) based on the 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 of 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:

Superbrewed Food, Inc.
239 Lisa Drive
New Castle
DE 19720

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

In Superbrewed Food's view, all data and information presented in Parts 2 through 7 of this notice do not contain any trade secret, commercial or financial information that is privileged or confidential, and therefore all data and information presented herein are exempt from the Freedom of Information Act, 5 U.S.C. 552.

1.8 Certification

Superbrewed Food hereby certifies that to the best of its 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 Superbrewed Food and pertinent to the evaluation of the safety and GRAS status of *Clostridium* protein for use as a source of protein in conventional food and beverages, as described herein.

Signed,

11/29/2022

_ _____ _____

Bryan P. Tracy, CEO
Superbrewed Food, Inc.
239 Lisa Drive
New Castle
DE 19720, USA

Date

PART 2. §170.230. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

Clostridium protein is the dried killed cells obtained from *C. tyrobutyricum* ASM#19 fermentation using a corn-derived sugar feedstock. The taxonomic classification of *C. tyrobutyricum* ASM#19 is as follows:

Domain: Bacteria

Phylum: Firmicutes
Class: Clostridia
Order: Clostridiales
Family: Clostridiaceae
Genus: *Clostridium*
Species: *Clostridium tyrobutyricum*
Full scientific name: *Clostridium tyrobutyricum* van Beynum and Pette 1935
Designation: ASM#19

The strain has not been genetically modified.

2.2 Characterization of the Source

2.2.1 Origin of the Microbial Source

The source of *Clostridium* protein is *C. tyrobutyricum* strain ASM#19, subsequently referred to as ASM#19. The parent *C. tyrobutyricum* strain was isolated from litter samples taken from a chicken house in Delaware by Superbrewed Food. The litter samples were treated with chloroform to kill all of the vegetative cells, leaving only the spores in the samples. The spores were then revived on rich medium and single colonies were picked for identification. Based on 16S ribonucleic acid (RNA) sequencing, isolate CSS-A7, also referred to as CLC, was found to display 99% similarity to *C. tyrobutyricum*. This strain was deposited internally at Superbrewed Food.

ASM#19 is an asporogenous strain that was generated by natural evolution from CLC. For selection of the asporogenous mutant, a successive vegetative transfer of CLC was performed, maintaining the culture in vegetative growth by transferring at least daily on a rich medium for a number of passages. Since asporogenous strains tend to grow faster than sporogenous strains, eventually, after a sufficient number of passages they take over the culture. The culture was plated and single colonies were selected to check for asporogenous phenotype. ASM#19 was isolated and confirmed to be of asporogenous phenotype, i.e., unable to survive chloroform treatment.

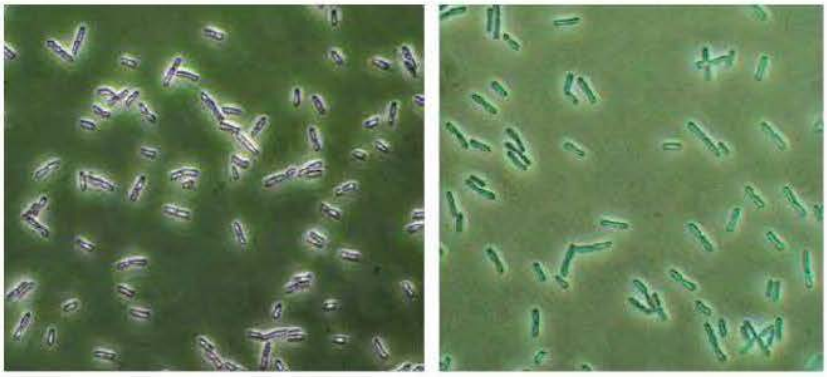
The spore differentiation pathway includes asymmetric cell division via forespore to endospore to mature spore shedding (Dürre, 2014). The parent cell in which the spore is created, is not viable. Thus, spore-forming cells must complete the time-consuming differentiation pathway and proceed through germination in order to go back into vegetative growth. A culture passaged ten to hundreds of generations of growth can eventually enrich for natural asporogenous phenotypes. ASM#19 is one such phenotype, out of many, that Superbrewed Food isolated as an individual culture.

ASM#19 has been deposited in the CABI culture collection under the reference number SD20/06.

2.2.2 Description of the Production Strain

C. tyrobutyricum ASM#19 is a rod-shaped, gram-positive bacterium that grows under anaerobic condition and produces butyric acid, acetic acid and hydrogen gas as the major fermentation products from glucose. The typical morphology of *C. tyrobutyricum* when grown on a glucose-based media with 0.05% yeast extract to late exponential phase and examined by light microscopy is presented in Figure 2.1. The strain is comprised of long, regular, straight rods of uniform size, and the cells are actively dividing and motile.

Figure 2.1: Light Microscopy (Microphotographs) of *C. tyrobutyricum* ASM#19 during Late Exponential Growth, at 400X Magnification



2.2.3 Biochemical Characterization

The ability of *C. tyrobutyricum* ASM#19 to grow on different carbon sources was evaluated and the typical fermentation characteristics are presented in Table 2.1. *C. tyrobutyricum* ASM#19 was grown on glucose in a pH bottom-controlled fermentation with ammonium hydroxide at pH 5.5. Under these conditions ASM#19 primarily produces butyric acid with some acetic acid, ethanol, and 2,3-butanediol. In contrast, *C. tyrobutyricum* is unable to grow when xylose is the sole carbon source.

Table 2.1: Fermentation Characteristics using Glucose or Xylose as the Carbon Source

Carbon Source	Fermentation Time	Consumed Substrate	Produced Metabolites (g/L)	
Glucose	39 hours	67.4 g/L	Butyric acid	24.8
			Acetic acid	0.9
			Ethanol	0.5
			2,3-Butanediol	0.4
Xylose	96 hours	0.1 g/L	Butyric acid	0.2
			Acetic acid	0.2
			2,3-Butanediol	0.0

2.2.4 Genetic Sequencing and Characterization

The strain has been identified as *C. tyrobutyricum* ASM#19 by whole genome sequence (WGS) and 16S ribosomal RNA (rRNA) gene sequence analysis.

2.2.4.1 16S RNA Sequencing ASM#19

The sequence of 16S rRNA for *C. tyrobutyricum* ASM#19 was amplified by polymerase chain reaction (PCR) using Unibac forward and reverse primers, and sequenced. The sequence was compared to the National Center for Biotechnology Information (NCBI) refseq_rna NCBI Transcript Reference Sequences database using Microbial Nucleotide Basic Local Alignment Search Tool (BLASTN). The match with the highest score (99.423% percent identity) was *C. tyrobutyricum* strain ATCC 25755, which according to American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collections, is the same strain as DSM 2637. There was an 8 nucleotide difference between ASM#19 and ATCC 25755, confirming that ASM#19 is a closely related but distinct strain of *C. tyrobutyricum*.

2.2.4.2 Whole Genome Sequence Analysis of ASM#19

Genomic deoxyribonucleic acid (DNA) was isolated from a pure culture of *C. tyrobutyricum* ASM#19 and the DNA libraries were prepared. The libraries were sequenced by Next Generation Sequencing (NGS) on an Illumina Miseq using a Miseq V2-500 cycle kit (Illumina) to generate 2 x 250 paired-end reads. The data was de-multiplexed on Basespace, the Illumina server, to generate FASTQ files for the sample. The data was then further analyzed using CLC-Bio (Qiagen version 12.0.3). The genome size of *C. tyrobutyricum* ASM#19 was 3,063,565 base pairs (bp) and the guanine-cytosine (GC) content was 31.0%.

Using the whole genome sequence of *C. tyrobutyricum* ASM#19, bioinformatic analysis was conducted to investigate the genetic basis of its asporogenous phenotype. The analysis involved alignment of all the genes involved in sporulation (*ca*. 38 genes) and revealed a single nucleotide mutation in the *spo0A* gene (a known regulator for sporulation/differentiation), a single nucleotide mutation from a C to a T, which generated a stop codon within the coding region. This mutation truncated the predicted *Spo0A* protein from 271 residues to 158 residues, which is expected to result in a non-functional protein. The lack of a functional *spo0A* gene should prevent differentiation in ASM#19 thereby explaining the loss of the spore-forming phenotype.

2.2.4.3 Strain Identification by Whole Genome Digital DNA-DNA Hybridization (dDDH)

A dDDH analysis (pair-wise alignment between two specific, known genomes) was conducted between the full genomes of *C. tyrobutyricum* ASM#19 and *C. tyrobutyricum* DSM 2637. The dDDH value obtained with three different formulas varied between 93.2% - 98.9%, clearly exceeding the value of 70% which is considered as the cut-off value for identity at species level (Meier-Kolthoff *et al*., 2013). A similar analysis was conducted by submission of the complete genome sequence of ASM#19 to the Type (Strain) Genome Server (TYGS) at DSMZ (Meier-Kolthoff and Göker, 2019). This confirms that *C. tyrobutyricum* ASM#19 is the same species as *C. tyrobutyricum* DSM 2637.

2.2.5 Antibiotic Susceptibility

C. tyrobutyricum ASM#19 was phenotypically and genotypically screened for antimicrobial resistance/susceptibility. Phenotypic testing was conducted on the strain to determine the minimum inhibitory concentrations (MICs) against a selected range of antimicrobials of relevance to human and veterinary medicine. The results were compared with the microbial cut-off values reported by the Clinical and Laboratory Standards Institute (CLSI) for anaerobes, by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for gram positive anaerobes, and by the European Food Safety Authority (EFSA, 2018) for "other" gram positive bacteria, respectively. For those antimicrobials for which breakpoints are available, the reported MIC values for *C. tyrobutyricum* ASM#19 were higher than the microbial cut-off values established by CLSI and/or EUCAST and/or EFSA for vancomycin, erythromycin, tetracycline, chloramphenicol, clindamycin and ampicillin. Gentamycin and kanamycin were not tested on the basis that these antibiotics were unable to function under anaerobic conditions.

Considering that *C. tyrobutyricum* ASM#19 displayed a MIC value for ampicillin higher than the microbial cut-off values as reported by CLSI, EUCAST and EFSA, the genome was subjected to a targeted search for ampicillin resistance conveying genes. Three representative Class D beta-lactamase family proteins from 3 different strains of *Clostridia* (*C. beijerinckii, C. botulinum, C. difficile*) were chosen and Basic Local Alignment Search Tool (BLAST) searches were conducted for each of these against the full genome sequence of *C. tyrobutyricum* ASM#19. The percent identity was below 27% for all these genes, confirming that ampicillin resistance in *C. tyrobutyricum* ASM#19 is not derived from a beta-lactamase gene, but is an intrinsic property of the strain.

The WGS was interrogated for the presence of genes coding for, or contributing to, resistance to antimicrobials relevant to human and veterinary medicine. Comparisons were made against 2 databases, CARD (McArthur *et al*., 2013; Alcock *et al*., 2020) and Resfinder (Zankari *et al*., 2012). The outcome of the analysis was that no acquired antimicrobial resistance (AMR) genes were identified for any of the antimicrobials evaluated by phenotypic testing. In one database, a gene encoding for fluoroquinolone resistance was identified but further analysis confirmed that this gene was not associated with mobile genetic elements. The results of the phenotypic testing together with the outcome of the search of the WGS, indicate that *C. tyrobutyricum* ASM#19 displays some intrinsic resistance to certain antimicrobials, i.e., natural resistance to certain antibiotics but that no resistance has been acquired in plasmids or other mobile genetic elements. Thus, it may be concluded that *C. tyrobutyricum* ASM#19 will not contribute to the pool of antibiotic resistance in the environment. Further analysis for the potential presence of transferable AMR genes is described in Section 2.2.8 below.

2.2.6 Antimicrobial Production

Following *C. tyrobutyricum* ASM#19 fermentation, the broth from the fermentation and a representative sample of *C. tyrobutyricum* fermentation solubles, i.e., the broth after filtration to remove the cells and concentration, were tested for inhibitory activity against the reference strains *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633. These reference strains are known to be susceptible to a range of antimicrobials using the disc diffraction assay. No zones of inhibition were observed indicating that *C. tyrobutyricum* ASM#19 does not produce antimicrobials.

2.2.7 Genome Annotation, Plasmids and Virulence Factors

Following the sequencing and assembly of the *C. tyrobutyricum* ASM#19 genome, contigs were processed and analyzed using Rapid Annotations using Subsystems Technology (RAST) (Aziz *et al*., 2008; Overbeek *et al*., 2014). RAST analysis revealed 3,166 open reading frames, 69 Clustered Regularly Interspaced Short Palindromic Repeats (Crispr) spacers and 53 RNA sequences (tRNA and rRNA). No transposable elements or plasmids were identified in *C. tyrobutyricum* ASM#19. Six regions contained intact or incomplete prophage sequence and were further investigated as described in Section 2.2.8.

BLAST analysis of the annotated 3,166 open reading frames derived from the genome of *C. tyrobutyricum* ASM#19 against virulence factors from known Clostridial pathogens (*C. botulinum. C. perfringens, C. novyi, C.tetani, and C.baratii*) did not reveal any matches to genes with similarity to the genes encoding for toxin production in these strains. Significant matches were considered as those with at least 80% similarity (at protein level or equivalent) and 60% coverage of the query sequence (EFSA, 2019).

Overall, query cover from the BLAST analysis was found to be low (<30% in most cases) and the percent identity was below 42%. The query coverage was high for 3 toxins (>60%) but their similarity was <70% compared to the known pathogenic strains, i.e., less than the threshold set by EFSA for further investigation of a gene which may encode for toxigenicity (EFSA, 2018). A 43% identity in amino acid sequence was identified between hemolysin type iii from *C. novyi* to the corresponding protein in *C. tyrobutyricum* ASM#19, with 75% coverage of the protein length and a $6e^{-38}$ E-value. Hemolysins are toxin family proteins, known for their hemolytic activities. Hemolysin type iii is known to be connected to virulence in *Vibrio parahaemolyticus* and in *Bacillus cereus*, but it is also found in *Lactobacillus* species and in *Pediococcus acidilactici* which have long and established histories of safe use in food and feed. Considering the gene is found in both pathogenic and non-pathogenic species, a further definitive test was conducted to identify any hemolytic activity in *C. tyrobutyricum* ASM#19. The strain was streaked on CDC blood agar plates with in-house *C. perfringens* as a positive control and *E. coli* BL21 as a negative control. No hemolysis was observed in the assay and together with the information obtained from BLAST searches, indicate that the *C. tyrobutyricum* ASM#19 is non-toxigenic and non-pathogenic.

2.2.8 Absence of Transferable Antibiotic Resistance Genes

As mentioned above (Section 2.2.7), possible prophage sequences were identified in the RAST analysis conducted on the WGS of *C. tyrobutyricum* ASM#19. This finding is not unexpected; Howard-Varona *et al*. (2017) state that approximately 74% of Firmicutes contain prophages. To further characterize these, the web-based tool Phaster (https://phaster.ca/) (Zhou *et al*., 2011; Arndt *et al*., 2016) was used to scan the full genome of *C. tyrobutyricum* ASM#19 for phages and prophages. Using Phaster, 6 possible hits were identified of which 3 comprised full prophage sequences, and 3 were likely to be incomplete prophages. The main safety concern associated with prophages is the possibility that they are integrated into the bacterial genome and become activated under certain conditions, entering the lytic cycle. Any genes encoding for antimicrobial resistance or virulence associated with these prophages may potentially be released into the environment and be acquired by other bacteria. Although many phages have a limited host range, often infecting only a limited number of strains within one species, some can infect a wider range of species and their ability to evolve to infect new hosts is not well

understood (Koskella and Meaden, 2013). Thus, in order to evaluate the potential for transfer of AMR genes identified in *C. tyrobutyricum* ASM#19 to other microbes, the relative locations of the prophage sequences and these genes (fluoroquinolone resistant gyrB and DNA topoisomerase IV subunit B) was investigated. The sequences of these two AMR genes were not in the vicinity of the prophage sequences of the genome. It is therefore considered highly unlikely that the prophage sequences present in *C. tyrobutyricum* ASM#19 could transfer antimicrobial resistance to other bacteria.

2.2.9 Presence of Viable Cells in *Clostridium* Protein

At the end of the fermentation process, *C. tyrobutyricum* (an obligate anaerobe) is heated under conditions, including exposure to oxygen, which will inactivate (kill) the cells. The downstream processing also includes a drying step which will further minimize the potential for any viable cells in *Clostridium* protein. Using a culture-based method developed by Superbrewed Food, the viable cells in 5 representative lots of *Clostridium protein* were measured (see Table 2.2). Only low levels of cells were detected, i.e., 160 to 2,390 CFU/mL in the 5 lots tested. These lots analyzed were produced at Superbrewed Food's pilot-plant facility in Delaware and it is anticipated that sterilization and flushing techniques will be improved on a commercial scale leading to viable cell counts that are at the lower end of this range and display less variability. Notably, *C. tyrobutyricum* cells are non-spore forming and are not expected to survive in the presence of air/oxygen. Overall, it may be concluded that any residual levels in the *Clostridium* protein will not pose a safety concern.

Table 2.2: Levels of Viable Cells in 5 Representative Lots of *Clostridium* Protein

Parameter	Unit	Analytical Data				
		Lot DNII 210225	Lot DNII 210325	Lot DNII 210401	Lot DNII 210610	Lot DNII 210803
C. tyrobutyricum	CFU/g	160	2,390	425	477	587

Abbreviations: CFU = colony forming units.

2.3 Raw Materials and Processing Aids

All raw materials and processing aids used to generate the feedstock for fermentation are commonly used by the dry grind ethanol fermentation process for the production of potable alcohol. Only raw materials and processing aids are permitted by federal regulation or have GRAS status for use under comparable conditions in fermentation processes to potable alcohol are used (see Table 2.3).

Table 2.3: Raw Materials Used to Generate the Feedstock

Raw Material	Function	Regulatory Status
Corn	Nutrient source	Food-grade corn suitable for the production of potable alcohol and low in Se.
Alpha-amylase	Processing aid	GRAS status for use as an enzyme in starch processing and in the production of potable alcohol. Complies with current FAO/WHO and FCC recommended specifications for food-grade enzymes.
Glucoamylase	Processing aid	GRAS status for use as an enzyme starch processing and the manufacture of potable alcohol. Complies with current FAO/WHO and FCC recommended specifications for food-grade enzymes.

Abbreviations: FAO = Food and Agricultural Organization; FCC = Food Chemical Codex; WHO = World Health Organization.

Likewise, all raw materials used in the anaerobic fermentation process, or compositionally similar substances, have an established history of use as ingredients in food in the U.S. and are commonly used by the fermentation industry (see Table 2.4).

Table 2.4: List of Raw Materials Used in the Anaerobic Fermentation Process (Optimized Process)

Raw Material	Function	Regulatory Status
Sugar feedstock	Nutrient	Derived from food-grade corn (see Table 2.3)
Manganese sulfate monohydrate	Mineral source	21 CFR §184.1461 – GRAS at levels consistent with cGMP as an ingredient in nutrient supplements, baked goods, non-alcoholic beverages, dairy product analogs, fish products, meat product and poultry products; also permitted for use in infant formula (U.S. FDA, 2021a)
Ammonium iron(II) sulfate hexahydrate	Mineral source	Ammonium sulfate: 21 CFR §184.1143 – GRAS for use at levels consistent with cGMP as a dough strengthener, firming agent and processing aid; levels not to exceed 0.15% in baked goods and 0.1% in gelatins and puddings (U.S. FDA, 2021b) Iron sulfate: 21 CFR §184.1315 – GRAS for use at levels consistent with cGMP as an ingredient in nutrient supplements and processing aids; also permitted for use in infant formula (U.S. FDA, 2021c)
Ammonium hydroxide	pH adjustment	21 CFR §184.1139 – GRAS for use at levels consistent with cGMP as a leavening agent, pH control agent, surface finishing agent and boiler water additive (U.S. FDA, 2021d)
Hydrochloric acid	pH adjustment	21 CFR §182.1057 – GRAS for use at levels consistent with cGMP as a buffer and neutralizing agent (U.S. FDA, 2021e)

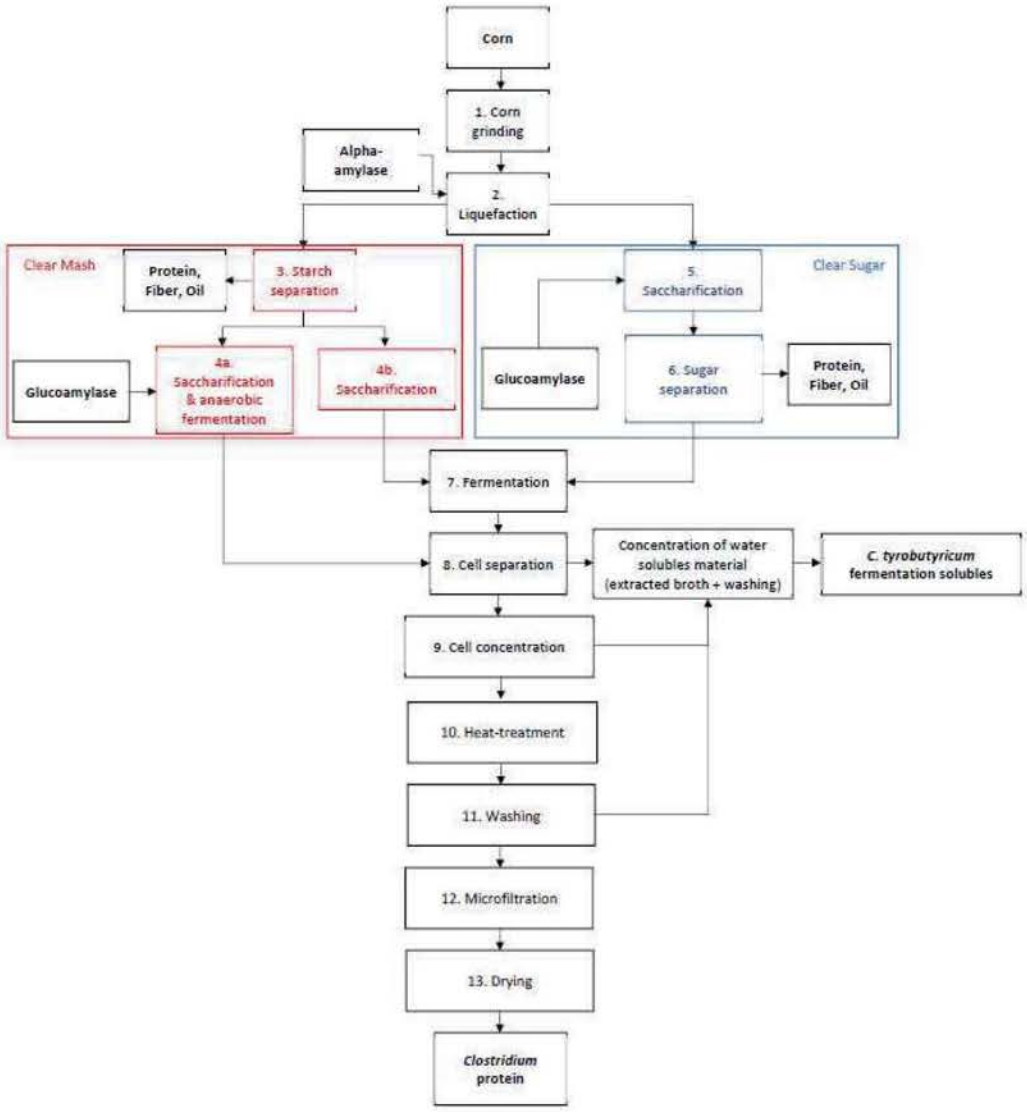
Abbreviations: cGMP = current good manufacturing practice.

The master cell lines of *C. tyrobutyricum* are stored at -80°C in cryovials. A new tube from the master cell bank is used for each fermentation and there is no repeated passage of the strain after fermentation. The cell bank is sampled every 3 to 4 months and 16S rRNA sequencing conducted to confirm the identity as ASM#19.

2.4 Description of the Manufacturing Process

An overview of the manufacturing process to *Clostridium* protein is provided in Figure 2.2. The process involves the following stages: (1) grinding and liquefaction of corn; (2) processing of the liquefied corn to yield one of two possible feedstocks, referred to as "Clear Sugar" and "Clear Mash", respectively; (3) anaerobic fermentation using *C. tyrobutyricum* ASM#19; (4) separation, washing and drying of the *C. tyrobutyricum* cells to yield *Clostridium* protein. Each of the stages in the process are described in turn below.

Figure 4.1: Flow-Chart of the Manufacturing Process to *Clostridium* Protein



Stage 1: Corn Grinding and Liquefaction [Steps 1 and 2]

Hammer ground corn is used to form a mash slurry which is subject to liquefaction using alpha-amylase. This liquefied material is used to generate either the "Clear Sugar" or "Clear Mash" feedstocks.

Stage 2a: Generation of the Clear Mash Feedstock [Steps 3 and 4a or 4b]

The hydrolyzed starch (sugars) fraction of the liquefied corn from step 2 (above) is separated from the protein, fiber and oil fractions and subject to further hydrolysis using glucoamylase. The hydrolysis can

take place simultaneously with (Step 4a), or before (Step 4b), anaerobic fermentation using *C. tyrobutyricum*.

Stage 2b: Generation of the Clear Sugar Feedstock [Steps 5 and 6]

The liquefied corn from Step 2 can be saccharified using amylase and glucoamylase before being separated from the protein, fiber and oil fractions.

Notably, the Clear Sugar and Clear Mash feedstocks are essentially glucose feedstocks produced by the same processes (i.e., conversion of hydrolyzed starch to glucose, and isolation of the sugars fraction from the protein, fiber and oil fractions) but where these steps are conducted in a different order.

Stage 3: C. tyrobutyricum Fermentation [Step 7]

A series of fermentation steps take place using the Clear Mash (Stage 2a) or the Clear Sugar (Stage 2b). The pH and temperature are monitored continuously to ensure the efficiency of the fermentation. The fermentation is anaerobic and oxygen is excluded from the process. Consumption of sugars is monitored during the fermentation processes. Cell concentration is monitored throughout the main fermentation and the growth of any contaminating lactic acid bacteria, primarily arising from the corn-derived feedstock, is measured by following the lactate concentration.

Stage 4: Separation, Washing and Drying [Steps 8 to 13]

At the end of the fermentation, the *C. tyrobutyricum* cell mass is separated from the fermentation broth by microfiltration. The cell mass is concentrated and heated to kill the cells. A further heat-treatment is performed in order to reduce the nucleic acids levels in *Clostridium* protein to <4 g/100 g (final product).

The cells are dried using a dual-drum dryer to achieve a moisture content of not more than 10%. The resultant *Clostridium* protein is an off-white powder which is packaged into 50 lb polypropylene bags or super sacks lined with polyethylene.

Production Controls

The manufacture of *Clostridium* protein for use as a commercial feed product will be conducted in accordance with current Good Manufacturing Practice (cGMP) for human food and a Hazard Analysis Critical Control Point (HACCP) plan will be in place. It will also comply with the relevant requirements of the Food Safety Modernization Act (FSMA) and Bioterrorism Act (2002).

2.5 Product Specification and Analytical Data

2.5.1 Product Specifications

Appropriate compositional food-grade specifications have been established for *Clostridium* protein and are presented in Table 2.5. All parameters are determined using internationally recognized procedures or validated methods.

Table 2.5: Proposed Product Specifications for *Clostridium* Protein		
Parameter	**Specification**	**Method of Analysis**
Physical Characteristics and Composition		
Appearance	Off-white powder	Visual inspection
Moisture	≤10 g/100 g	AOAC 925.09 (Gravimetry)
Crude protein	≥80 g/100 g	AOAC 990.03; AOAC 992.15 (Combustion; N x 6.25)
Crude fat	≤3 g/100 g	AOAC 996.06 modified (GC-FID)
Ash	≤6 g/100 g	AOAC 942.05 (Combustion)
Carbohydrates	≤8 g/100 g	Calculation [100-(crude protein + fat + moisture + ash)][1]
Ammoniacal N	≤1 g/100 g	AOAC 941.04 (Titrimetry)
Nucleic acids	≤4 g/100 g	Superbrewed Food Internal Protocol[2]
Heavy Metals		
Lead	≤0.3 mg/kg	J. AOAC vol. 90 (2007) pp. 844-856 modified (ICP-MS)
Cadmium	≤0.05 mg/kg	J. AOAC vol. 90 (2007) pp. 844-856 modified (ICP-MS)
Arsenic	≤0.1 mg/kg	J. AOAC vol. 90 (2007) pp. 844-856 modified (ICP-MS)
Mercury	≤0.05 mg/kg	J. AOAC vol. 90 (2007) pp. 844-856 modified (ICP-MS)
Microbiology		
Viable *C. tyrobutyricum* cells	≤2,500 CFU/g	Culture-based method (internal validated procedure)
Aerobic plate count	≤10,000 CFU/g	AOAC 966.23
Yeast and mold	≤100 CFU/g	BAM Chap. 18 modified
E. coli 0157-H7	Absent/25 g	AOAC RI 020801
Salmonella	Absent/25 g	AOAC 2017.06

Abbreviations: AOAC = Association of Official Analytical Chemists; BAM = Bacteriological Analytical Manual; CFU = colony forming units;

[1]Crude protein is based on N x 6.25 and will include N arising from nucleic acids;

[2]Based on methods described in Mydland *et al.* (2008).

2.5.2 Conformance with Product Specifications

Analytical data for 5 non-consecutive lots of *Clostridium* protein are summarized in Table 2.6. The data were collected on lots manufactured at pilot-scale but the raw materials and processes reflect the planned commercial manufacture. On this basis, the data are considered representative of the commercial article. The analytical results demonstrate that *Clostridium* protein can be manufactured in conformance with the proposed specifications and exhibits acceptable lot to lot variation.

Table 2.6: Results of Analysis of 5 Representative Lots of *Clostridium* Protein								
Parameter	**Unit**	**Spec.**	**Analytical Data**					**Mean**
			Lot DNII 210225	**Lot DNII 210325**	**Lot DNII 210401**	**Lot DNII 210610**	**Lot DNII 210803**	
Date of Manufacture			Feb 25, 2021	Mar 25, 2021	Apr 1, 2021	June 10, 2021	Aug 3, 2021	-
Physical Characteristics and Composition								
Appearance	-	Off-white powder	Off-white powder	Off-white powder	Off-white powder	Off-white powder	Off-white powder	-
Moisture	g/100 g	≤10	6.5	5.2	5.2	3.2	3.8	4.8
Crude protein (N x 6.25)	g/100 g	≥80	82.9	84.6	84.4	88.2	85.1	85.0
Fat (total as triglycerides)	g/100 g	≤3	1.7	1.4	1.0	1.5	1.1	1.3
Ash	g/100 g	≤6	2.6	3.8	3.7	2.2	4.3	3.3
Carbohydrates[1]	g/100 g	≤8	6.4	5.0	5.8	4.9	5.7	5.6
Ammonia N	g/100 g	≤1	<0.2	<0.2	<0.2	<0.2	<0.2	-
Nucleic acids (RNA+DNA)	g/100 g	≤4	2.7	1.7	2.6	1.6	2.9	2.3
Heavy Metals								
Lead	mg/kg	≤0.3	0.026	0.240	0.046	0.131	0.023	0.0932
Cadmium	mg/kg	≤0.05	0.015	0.012	0.015	<0.010	0.013	0.0138
Arsenic	mg/kg	≤0.1	0.032	0.074	0.097	0.023	0.076	0.0604
Mercury	mg/kg	≤0.05	0.016	<0.010	<0.010	<0.010	<0.010	-
Microorganisms								
Viable *C. tyrobutyricum* cells	CFU/g	≤2,500	160	2,390	425	477	587	-
Aerobic plate count	CFU/g	≤10,000	3,900	<10	<10	640	2,500	-
Yeast	CFU/g	≤100	<10	<10	10	<10	<10	-
Mold			10	10	10	40	10	-
E. coli 0157-H7	/25 g	Absent	Absent	Absent	Absent	Absent	Absent	-
Salmonella	/25 g	Absent	Absent	Absent	Absent	Absent	Absent	-

Abbreviations: CFU = colony forming units;
[1]Carbohydrates content (by difference) = 100 – (crude protein + fat + moisture + ash).

2.5.3 Amino Acid Profile

The amino acid profiles of 5 non-consecutive representative lots of *Clostridium* protein are summarized in Table 2.7. The highest amounts of individual amino acids identified in *Clostridium* protein were lysine (8.31 to 9.38% mean 9.04%), aspartic acid (9.53 to 10.66%; mean 10.26%) and glutamic acid (10.02 to 11.34%; mean 10.8%). *Clostridium* protein contained only low levels of tryptophan (0.65 to 0.73%;

mean 0.69%) and the sulfur-containing amino acids, cystine (0.66 to 0.86%; mean 0.72%) and free cysteine (<0.10%). Consistent results were obtained across the 5 lots tested with the amount of any individual amino acid varying by less than 1%.

Table 2.7: Amino Acid Profiles of 5 Representative Lots of *Clostridium* Protein							
Parameter	**Unit**	**Analytical Data**					**Mean**
		Lot DNII 210225	**Lot DNII 210325**	**Lot DNII 210401**	**Lot DNII 210610**	**Lot DNII 210803**	
Histidine	%	1.37	1.34	1.30	1.53	1.41	1.39
Isoleucine	%	6.27	6.49	5.95	6.80	6.17	6.34
Leucine	%	6.67	6.89	6.24	7.03	6.72	6.71
Lysine	%	9.12	9.30	8.31	9.38	9.08	9.04
Methionine	%	2.49	2.55	2.23	3.04	2.55	2.57
Cystine	%	0.70	0.67	0.66	0.86	0.73	0.72
Cysteine (free)	%	<0.10	<0.10	<0.10	<0.10	<0.10	-
Phenylalanine	%	3.80	3.90	3.57	4.05	3.85	3.83
Tyrosine	%	3.22	3.22	3.04	3.43	3.27	3.24
Threonine	%	4.18	4.41	3.80	4.13	4.37	4.18
Tryptophan	%	0.68	0.65	0.70	0.73	0.68	0.69
Valine	%	5.56	5.86	5.51	6.06	5.62	5.72
Alanine	%	6.50	6.89	6.11	6.61	6.65	6.55
Arginine	%	4.28	4.31	3.84	4.50	4.23	4.23
Aspartic acid	%	10.18	10.66	9.53	10.49	10.43	10.26
Glutamic acid	%	10.86	11.34	10.02	11.17	10.83	10.84
Glycine	%	4.32	4.41	3.95	4.38	4.34	4.28
Proline	%	2.60	2.67	2.40	2.71	2.63	2.60
Serine	%	3.79	3.93	3.48	3.81	3.91	3.78
Total	***%***	***86.59***	***89.49***	***80.64***	***90.71***	***87.47***	***86.98***

2.5.4 Dietary Fiber and Sugar Contents

The dietary fiber and sugar contents of 5 non-consecutive representative lots of *Clostridium* protein are presented in Table 2.8. Dietary fiber content varied from 11.0 to 14.5% across the 5 lots tested with a mean value of 12.3%. There were no detectable sugars in any of the lots tested. The cell wall of gram-positive bacteria such as *C. tyrobutyricum* mainly consists of peptidoglycans, which are glycan strands cross-linked by short-chain peptides (Vollmer *et al.*, 2008; Dörr *et al.*, 2019). These cell wall components are expected to be the main contributors to the fiber content but on account of their N content, to also form part of the crude protein calculation (N x 6.25). As a consequence, the carbohydrates content calculated by difference (4.9 to 6.4 g/100 g; mean 5.6 g/100 g) is lower than the dietary fiber content.

Table 2.8: Sugar Profile and Dietary Fiber Content of 5 Representative Lots of *Clostridium* Protein							
Parameter	**Unit**	**Analytical Data**					**Mean**
		Lot DNII 210225	**Lot DNII 210325**	**Lot DNII 210401**	**Lot DNII 210610**	**Lot DNII 210803**	
Dietary fiber	g/100 g	12.9	11.3	11.7	11.0	14.5	12.3
Total sugars	g/100 g	<0.35	<0.35	<0.35	<0.35	<0.35	-

2.5.5 Mineral Profile

The mineral profiles of 5 non-consecutive representative lots of *Clostridium* protein are presented in Table 2.9. The mineral content of *Clostridium* protein arises from inherent components of the cells and also by carry-over of nutrients and processing aids used in the manufacturing process. The fermentation broth is separated from the cells of *C. tyrobutyricum* after fermentation by filtration, and the cells are then washed with water to further reduce the carry-over of media components including iron, manganese and sulfate ions.

Mean levels of the macro minerals calcium, phosphorus, magnesium, potassium and sodium were 162 mg/kg, 3,650 mg/kg, 420 mg/kg, 318 mg/kg and 1,100 mg/kg, respectively across the 5 lots tested. Sulfur and chloride were identified at mean levels of 7,400 and 282 mg/kg, respectively. Manganese and iron mean contents across the 5 lots of *Clostridium* protein were 52.2 and 76.2 mg/kg, respectively.

Table 2.9: Mineral Profiles of 5 Representative Lots of *Clostridium* Protein

Parameter	Unit	Analytical Data					Mean
		Lot DNII 210225	Lot DNII 210325	Lot DNII 210401	Lot DNII 210610	Lot DNII 210803	
Calcium	mg/kg	110	80	40	50	530	162
Phosphorus	mg/kg	4,270	2,710	3,080	2,720	5,470	3,650
Magnesium	mg/kg	630	330	120	230	790	420
Potassium	mg/kg	270	190	460	270	400	318
Sodium	mg/kg	1,280	1,110	840	1,320	950	1,100
Iron	mg/kg	116	34	30	67	134	76.2
Zinc	mg/kg	50	38	21	88	78	55
Copper	mg/kg	<1	<1	<1	<1	<1	<1
Manganese	mg/kg	90	51	13	38	69	52.2
Molybdenum	mg/kg	2.4	0.60	1.27	0.51	1.53	1.26
Selenium	mg/kg	5.83	0.089	0.13	0.10	0.11	1.25
Sulfur	mg/kg	7,100	7,000	7,400	8,300	7,200	7,400
Chloride	mg/kg	320	180	380	230	300	282
Total	***mg/kg***	***14,244***	***11,724***	***12,385***	***13,314***	***15,923***	***13,518***

2.5.6 Vitamins Profile

The vitamin profiles of 3 non-consecutive representative lots of *Clostridium* protein were determined and the results are presented in Table 2.10. Microorganisms mainly contain B vitamins and bacterial cells in particular, are normally rich in vitamin B12 (Nalage *et al.*, 2016). Mean levels of folate, vitamin B2 and vitamin B12 were 99.6 µg/100 g, 1.79 mg/100 g and 9.6 µg/100 g, respectively across the 3 lots of *Clostridium* protein tested.

Table 2.10: Vitamins Profile of 3 Representative Lots of *Clostridium* Protein					
Parameter	**Unit**	**Analytical Data**			**Mean**
		Lot DNII 210225	**Lot DNII 210610**	**Lot DNII 210803**	
Vitamin D2	IU/100 g	<4	<4	<4	<4
Vitamin D3	IU/100 g	<4	<4	<4	<4
Total folate expressed as folic acid equivalents	µg/100 g	87.2	37.6	174.0	99.6
Vitamin B1 (Thiamin)	mg/100 g	0.07	0.04	0.19	0.10
Vitamin B2 (Riboflavin)	mg/100 g	1.07	2.28	2.02	1.79
Vitamin B3 (Niacin)	mg/100 g	0.51	1.52	1.23	1.09
Vitamin B5 (Pantothenic acid)	mg/100 g	0.45	0.12	0.44	0.33
Vitamin B7 (Biotin)	mg/100 g	0.33	0.11	0.11	0.18
Vitamin B6 (Pyridoxine)[1]	µg/100 g	815	14.5	6.5	278.7
Vitamin B12 (Cyanocobalamin)	µg/100 g	13.9	7.20	7.61	9.57
Vitamin K1	µg/100 g	ND	ND	ND	-
Vitamin K2 (MK-4)	µg/100 g	ND	<6.5	ND	-
Vitamin K2 (MK-7)	µg/100 g	0.05	10.0	ND	-

Abbreviations: ND = not detected;

[1]Lot DNII 210225, and Lots DNII210610 and 210803, respectively were tested at separate contract laboratories and different test methods were used which may account for the variation in results obtained.

2.5.7 Organic Acids and 2,3-Butanediol

C. tyrobutyricum fermentation produces butyric and acetic acids, and potentially also small amounts of 2,3-butanediol and 2-hydroxybutyric acid. Contamination of the corn used to generate the sugar-feedstock with lactic acid bacteria can also result in the formation of low levels of lactic acid. Ammonium hydroxide is used to control the pH of the fermentation and therefore, the organic acids (butyrate, acetate, 2-hydroxybutyrate and lactate) are present as the ammonium salts. The fermentation broth containing the fermentation metabolites is separated from the cells of *C. tyrobutyricum* by filtration during the manufacturing process to *Clostridium* protein, and the cells are then washed with water to further reduce the carry-over of fermentation metabolites before drying. Thus, only low levels of residual organic acids and 2,3-butanediol are expected to be present in *Clostridium* protein, as evidenced by analysis for 5 non-consecutive representative lots of *Clostridium* protein (see Table 2.11).

Table 2.11: Organic Acids and 2,3-Butanediol Content of 5 Representative Lots of *Clostridium* Protein							
Parameter	**Unit**	**Analytical Data**					**Mean**
		Lot DNII 210225	**Lot DNII 210325**	**Lot DNII 210401**	**Lot DNII 210610**	**Lot DNII 210803**	
Ammonium butyrate	g/kg	1.8	2.6	13.1	3.8	7.4	5.7
Ammonium acetate	g/kg	4.3	2.7	9.4	2.3	5.6	4.9
Ammonium lactate	g/kg	0.6	0.9	5.0	0.6	3.4	2.1
Ammonium 3-hydroxybutyrate	g/kg	ND	ND	ND	ND	ND	-
2,3-butanediol	g/kg	ND	0.1	0.1	0.2	ND	-

Abbreviations: ND = not detected.

2.5.8 Biogenic Amines

The levels of biogenic amines in 5 non-consecutive representative lots of *Clostridium* protein are presented in Table 2.12. Histidine and agmatine sulfate were not identified above detection limits in any of the 5 lots of *Clostridium* protein tested. Putrescine levels ranged from 10.1 to 62.0 mg/kg with a mean value of 27.6 mg/kg. The occurrence of putrescine in foods was evaluated by EFSA in 2011 and mean levels were found to range from 3.2 to 3.4 mg/kg in alcoholic beverages, 4.6 to 12 mg/kg in fermented fish products, 25.3 to 64.7 mg/kg in dairy products and 84.2 to 84.6 mg/kg in fermented sausages (EFSA, 2011).

Measurable levels of cadaverine also were observed, ranging from 0.6 to 1.5 mg/kg and with a mean value of 1.1 mg/kg for the 5 lots of *Clostridium* protein. By comparison, EFSA (2011) reported mean levels of cadaverine in various fermented products on the market in the EU to range from 14.0 to 17.0 mg/kg in fermented fish meat, 37.4 to 38 mg/kg in fermented sausages, 17.2 to 17.5 mg/kg in ripened meat products, 72 to 109 mg/kg in cheese, 26 to 35.4 mg/kg in fermented vegetables, and 180 to 182 mg/kg in fish sauce.

In addition, spermidine and spermine were identified in the 5 lots of *Clostridium* protein analyzed, ranging from 71.0 to 98.6 mg/kg (mean 79.1 mg/kg) and 19.6 to 44.6 mg/kg (mean 27.0 mg/kg), respectively. These polyamines are of less concern from a human health perspective compared to other biogenic amines such as histamine and cadaverine (Atiya Ali *et al.*, 2011; EFSA, 2011) and occur widely in foods, especially vegetable and meat products. An analysis of foods consumed by the Swedish population reported spermidine levels in cooked soybeans (SBs), peas, pear, red beans and chicken meat of 51.1, 61.1, 65.0, 19.5 and 17.6 mg/kg[1], respectively. Spermine levels in cooked SBs, pork and cow liver were reported to be 21.1, 50.4 and 157.6 mg/kg[1], respectively.

[1] Calculated from the amounts per specified portion size reported by Atiya Ali *et al.* (2011).

Overall, the levels of biogenic amines in *Clostridium* protein are comparable or lower than the levels reported in commonly consumed foods and do not pose a safety concern under the conditions of intended use.

Table 2.12: Biogenic Amines Profile of 5 Representative Lots of *Clostridium* Protein

Parameter	Unit	Analytical Data					Mean
		Lot DNII210225	Lot DNII210325	Lot DNII210401	Lot DNII210610	Lot DNII210803	
Tyramine	mg/kg	<0.4	<0.4	8.3	<0.4	2.5	-
Putrescine	mg/kg	17.6	62.0	15.1	33.0	10.1	27.6
Cadaverine	mg/kg	0.6	1.4	1.5	1.1	0.9	1.09
Histamine	mg/kg	<2.0	<2.0	<2.0	<2.0	<2.0	-
Spermidine	mg/kg	73.2	78.7	98.6	71.0	74.1	79.12
Spermine	mg/kg	26.4	20.3	24.3	19.6	44.6	27.03
Agmatine sulfate	mg/kg	<5.0	<5.0	<5.0	<5.0	<5.0	-

2.5.9 Mycotoxins

Considering that the feedstock for fermentation is corn, mycotoxin testing was conducted on 5 non-consecutive representative lots of *Clostridium* protein. With the exception of deoxynivalenol (DON) at 10 µg/kg in Lot DNII210401, no mycotoxins were identified above detection limits in any of the lots tested. The U.S. FDA has set an advisory level for DON of 1 mg/kg in finished wheat products that may potentially be consumed by humans (U.S. FDA, 2010). Thus, the low level of DON (10 µg/kg) detected in *Clostridium* protein is not expected to pose a safety concern under the conditions of intended use.

2.6 Stability Data

Superbrewed Food recommends storing *Clostridium* protein at <25°C in the original packaging under dry conditions and protected from direct sunlight. The shelf-life is supported by a real-time study conducted on 2 representative lots of *Clostridium* protein stored at 25°C and 60% relative humidity (RH) in clear polypropylene bags reflective of the commercial packaging. The results after 12-months of storage are presented in Table 2.13. Over the 12-month storage period, *Clostridium* protein conformed to the product specifications with no observed growth in microorganisms or formation of biogenic amines.

Table 2.13: Stability Study Results for 2 Representative Lots of *Clostridium* Protein under Real-Time Conditions (25°C, 60% RH)					
Parameter	**Unit**	**Specification**	**Analytical Results**		
			T=0 Months	**T=3 Months**	**T=After 12 Months**
Lot DNII210325					
Appearance	Visual inspection	Off-white powder	Off-white	Off white, no clumps	White/cream
Odor	Sensory inspection	-	No smell	No smell	No smell
Moisture	g/100 g	≤10	4.2	5.4	8.6
Crude protein	g/100 g	≥80	85.7	84.3	85.4
Yeast	CFU/g	≤100	200	<10	<10
Mold	CFU/g		10	10	10
E. coli	/25 g	Absent	Absent	Absent	Absent
Salmonella	/25 g	Absent	Absent	Absent	Absent
Tyramine	mg/kg	-	<5	13	<0.4
Putrescine	mg/kg	-	39	53	38.2
Cadaverine	mg/kg	-	<5	<5	1.93
Histamine	mg/kg	-	<5	<5	<2
Lot DNII210401					
Appearance	Visual inspection	Off-white powder	Cream	Cream, no clumps	White/cream
Odor	Sensory inspection	-	No smell	No smell	No smell
Moisture	g/100 g	≤10	3.6	5.9	8.0
Crude protein	g/100 g	≥80	86.2	84.8	86.3
Yeast	CFU/g	≤100	<10	<10	<10
Mold	CFU/g		9	10	10
E. coli	/25 g	Absent	Absent	Absent	Absent
Salmonella	/25 g	Absent	Absent	Absent	Absent
Tyramine	mg/kg	-	<5	11	9.96
Putrescine	mg/kg	-	<5	17	6.53
Cadaverine	mg/kg	-	<5	<5	1.88
Histamine	mg/kg	-	<5	<5	<2

Abbreviations: CFU = colony forming units.

PART 3. §170.235. DIETARY EXPOSURE

3.1 Estimated Intake of *Clostridium* Protein

Estimates for the intake of *Clostridium* protein were based on the proposed food-uses and use-levels in conjunction with the food consumption data included in the National Health and Examination Surveys (NHANES) conducted in 2017-2018 (CDC, 2022a and b; USDA, 2018). The percentage of users was high among all population groups evaluated in the current intake assessment; between 50 and 89% of the population groups consisted of users of those food products in which *Clostridium* protein is proposed for use.

Among the total population (all ages), the mean and 90th percentile consumer-only intakes of *Clostridium* protein were determined to be 10.5 and 25.7 g/person/day, respectively. Of the individual population groups, male teenagers were determined to have the greatest mean and 90th percentile consumer-only intakes of *Clostridium* protein on an absolute basis, at 14.7 and 33.2 g/person/day, respectively, while infants and young children had the lowest mean and 90th percentile consumer-only intakes of 7.0 and 16.3 g/person/day, respectively. It should be noted that *Clostridium* protein is not intended for use in products directly marketed to infants and young children and exposure will only arise from consumption of conventional foods and beverages.

Table 3.1: Summary of the Estimated Daily Intake of *Clostridium* Protein from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group (Years)	All Person Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants and young children	0 to 2	3.5	9.1	50	342	7.0	16.3
Children	3 to 11	10.0	24.8	89	1,036	11.3	26.1
Female teenagers	12 to 19	7.8	21.1	82	415	9.5	23.7
Male teenagers	12 to 19	7.8	21.1	77	404	14.7	33.2
Female adults	20 and up	8.2	22.1	84	2,019	9.8	24.5
Male adults	20 and up	8.8	22.4	81	1,846	10.9	26.5
Total population	All ages	8.4	22.4	80	6,062	10.5	25.7

Abbreviations: n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

The estimated total intake of *Clostridium* protein on a body weight basis (g/kg body weight/day) from all proposed food uses in the U.S. population group is summarized in Table 3.2. On a body weight basis, the total population (all ages) mean and 90th percentile consumer-only intakes of *Clostridium* protein were determined to be 0.22 and 0.54 g/kg body weight/day, respectively. Among the individual population groups, infants and young children were identified as having the highest mean and 90th percentile consumer-only intakes of 0.60 and 1.37 g/kg body weight/day, respectively. Male adults had

the lowest mean and 90th percentile consumer-only intakes of 0.13 and 0.31 g/kg body weight/day, respectively.

Table 3.2: Summary of the Estimated Daily per Kilogram Body Weight Intake of *Clostridium* Protein from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group (Years)	All Person Intake (g/kg body weight/day)		Consumer-Only Intake (g/kg body weight/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants and young children	0 to 2	0.3	0.7	50	342	0.60	1.37
Children	3 to 11	0.4	1.0	89	1,036	0.44	1.04
Female teenagers	12 to 19	0.1	0.4	82	415	0.16	0.39
Male teenagers	12 to 19	0.1	0.4	77	404	0.22	0.55
Female adults	20 and up	0.1	0.3	84	2,019	0.14	0.37
Male adults	20 and up	0.1	0.3	81	1,846	0.13	0.31
Total population	All ages	0.2	0.5	80	6,062	0.22	0.54

Abbreviations: n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

This type of intake methodology is generally considered to be "worst-case" as a result of several conservative consumption made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. Short-term surveys also tend to over-estimate the intakes of food products consumed relatively infrequently.

A common or usual name for labeling purposes will be established for *Clostridium* protein in accordance with the general principles laid down by 21 CFR §102.5 and as appropriate, in consultation with the Office of Nutrition and Food Labeling (ONFL). Superbrewed Food anticipates using a common name along the lines of "**Postbiotics Cultured Protein**"" to identify the microbial origin of the product. It is recognized that Quorn™ which was successfully notified as GRAS for use as a protein source in 2002 has the common name "mycoprotein" for labeling purposes (*F. venenatum* protein; GRN No. 91; U.S. FDA, 2002).

3.2 Estimated Protein Intakes by the U.S. Population and Contribution by *Clostridium* Protein

3.2.1 Protein Intakes by the General Population

The U.S. FDA has established a Dietary Reference Value (DRV) for protein of 50 g/day for adults and children over the age of 4 years (U.S. FDA, 2021f). Similarly, the Institute of Medicine (IOM) sets the Recommended Daily Allowances (RDAs) for protein at 52 g/person/day for males between 14 and 18 years of age, 56 g/day for adult males of 19 years of age and over, and 46 g/person/day for females 14

years of age or over (IOM, 2011a). These values are based on consumption of 0.8 g protein/kg body weight/day for reference body weights of a given life stage group.

In practice, protein intakes are higher than the RDAs established by the IOM. Berryman *et al*. (2018) conducted a systematic analysis of dietary protein intakes and trends during 2-year cycles of NHANES (2001-2014; n = 57,980; ≥ 2 years old) by the U.S. population. The results were calculated on an absolute basis and body weight basis for individual population groups considering also sex and ethnicity. Estimated mean protein intakes ranged from 55.3 ± 0.9 g/person/day (young children aged 2-3 years) to 88.2 ± 1.1 g/person/day (adults aged 19-3- years) across all demographics. On a body weight basis, estimated mean protein intakes ranged from 1.10 ± 0.01 g/kg body weight/day (adults aged ≥ 71 years) to 3.63 ± 0.07 g/kg body weight/day (children aged 2-3 years) across all demographics.

Under the proposed conditions of use, the highest mean and 90th percentile consumer-only intakes of *Clostridium* protein were estimated to be 14.7 and 33.1 g/person/day (0.22 and 0.55 g/kg body weight/day), respectively for male teenagers. Infants and young children consumed the greatest amount of *Clostridium* protein on a per body weight basis, with the highest mean and 90th percentile consumer-only intakes of 0.60 and 1.37 g/kg body weight/day, respectively.

Comparison of the estimated consumer-only intakes of *Clostridium* protein with the RDAs for the different life stage groups, as well as the protein intakes estimated by Berryman *et al*. (2018) indicates that under this worst-case modeling scenario, the ingredient represents a substantive fraction of the daily protein requirements of individuals. However, in practice, *Clostridium* protein will be a direct replacement for other plant- and animal-derived proteins such as mycoprotein, whey protein, casein and pea protein for use under equivalent conditions (i.e., similar food groups and use levels). In this respect, *Clostridium* protein will contribute to, but not alter, total daily protein intakes from all sources by the U.S. population.

Furthermore, the National Institute of Health (NIH) Office of Dietary Supplements (ODS) has published an overview of dietary supplements used for exercise and athletic performance and which includes the use of protein supplements (NIH, 2022). Based on a review of numerous clinical trials, the NIH concluded that no safety concerns were associated with daily recommended intakes of protein of up to 2.0 g/kg body weight/day, equivalent to around 140 g/day for a 70 kg individual. Thus, under these worst-case scenario conditions of intake, *Clostridium* protein may make a significant contribution to protein intakes but is unlikely to pose a safety concern in the proposed food uses and at the proposed use levels.

3.2.2 Protein Intakes from Existing Counterparts

Numerous vegetable-, fungal- and animal-derived protein isolates and their hydrolysates have been the subject of GRAS notifications, including mycoprotein, pea protein and mung bean protein (see Table 3.3), and whey protein isolate is affirmed as GRAS under 21 CFR §184.1979c (U.S. FDA, 2021g). These fungal- and vegetable-derived proteins are intended for use in a comparable range of products to *Clostridium* protein and consumers will have access to a range of products containing different protein products. The majority of the population's intake of protein is, and will remain, unprocessed foods such as meat, dairy products and legumes (Kim *et al*., 2019; Shan *et al*., 2019).

Table 3.3: Examples of Food Uses for Fungal- and Vegetable-Derived Proteins (Existing Counterparts for *Clostridium* Protein)		
Reference	**Ingredient**	**Food Uses**
GRN No. 904 (U.S. FDA, 2021h)	*Fusarium* protein	Plant protein products including meat and poultry analogs, dairy product analogs, milk products, beverages and beverage bases, breakfast cereals, fruit juices and vegetable juices, grain products and pastas, baked goods and baking mixes, soups and soup mixes, and fats and oils
GRN No. 581 (U.S. FDA, 2016)	Un-hydrolyzed and hydrolyzed pea protein	Smoothies, baked goods, cereals, snacks, dry blend beverage, pre-mix beverage powders, processed meats, ready meals, protein/nutrition bars, ready-to-drink beverages, extruded products, soups/sauce
GRN No. 684 (U.S. FDA, 2017)	Mung bean protein isolate	Baked goods, beverages, breakfast cereals, condiments, dairy product analogs, fruit and water ices, gelatins, puddings, fillings, grain products, milk products, plant protein products, snack foods
GRN No. 386 (U.S. FDA, 2011)	Canola protein isolate and hydrolyzed canola protein isolate	Bakery products, snack foods, beverages, soups, dairy products, protein drinks, processed meat products, meat analogues, nutrition bars
GRN No. 91 (U.S. FDA, 2002)	Mycoprotein	Meat alternative for use in a range of dishes, including frozen entrees as well as for the central component of a meal as a fillet, as pieces, mince, cold cuts etc. (whole food)