

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
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GRAS Notice for *Clostridium* Protein

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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”) hereby 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 <i>Clostridium</i> 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 <i>Clostridium</i> 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

Superbrewed Food
November, 2022

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.

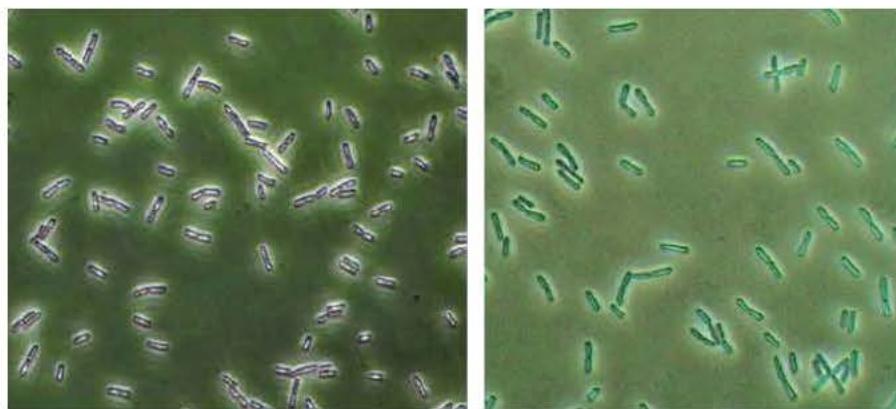
The spore differentiation pathway includes asymmetric cell division via forespore to endospore to mature spore shedding (Dürre, 2014). The parent cell in which the spore is created, is not viable. Thus, spore-forming cells must complete the time-consuming differentiation pathway and proceed through germination 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 diffusion 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 <i>Clostridium</i> Protein						
Parameter	Unit	Analytical Data				
		Lot DNII 210225	Lot DNII 210325	Lot DNII 210401	Lot DNII 210610	Lot DNII 210803
<i>C. tyrobutyricum</i>	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.

Superbrewed Food
November, 2022



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.

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 <i>Clostridium</i> 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 <i>C. tyrobutyricum</i> 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
<i>E. coli</i> 0157-H7	Absent/25 g	AOAC RI 020801
<i>Salmonella</i>	Absent/25 g	AOAC 2017.06

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

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

²Based 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 <i>Clostridium</i> 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 <i>C. tyrobutyricum</i> 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	-
<i>E. coli</i> 0157-H7	/25 g	Absent	Absent	Absent	Absent	Absent	Absent	-
<i>Salmonella</i>	/25 g	Absent	Absent	Absent	Absent	Absent	Absent	-

Abbreviations: CFU = colony forming units;

¹Carbohydrates 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 <i>Clostridium</i> 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 <i>Clostridium</i> 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 <i>Clostridium</i> 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 <i>Clostridium</i> 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;

¹Lot 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 <i>Clostridium</i> 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/kg¹, respectively. Spermine levels in cooked SBs, pork and cow liver were reported to be 21.1, 50.4 and 157.6 mg/kg¹, 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 <i>Clostridium</i> 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 <i>Clostridium</i> 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	90 th Percentile	%	n	Mean	90 th 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 <i>Clostridium</i> 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	90 th Percentile	%	n	Mean	90 th 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 <i>Clostridium</i> Protein)		
Reference	Ingredient	Food Uses
GRN No. 904 (U.S. FDA, 2021h)	<i>Fusarium</i> 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)

PART 4. §170.240. SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with *Clostridium* protein.

PART 5. §170.245. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Not applicable.

PART 6. §170.250. NARRATIVE AND SAFETY INFORMATION

6.1 Introduction

The determination that Superbrewed Food's *Clostridium* protein is GRAS under the intended conditions of use is on the basis of scientific procedures. A weight of evidence approach can be taken to support the safety of *Clostridium* protein comprised of the following: (1) characterization data on the source microorganism; (2) compositional and *in vitro* digestibility data; (3) comparison of the amino acid sequence of the protein to other proteins known to be allergenic; and (4) toxicological testing using Superbrewed Food's protein product.

6.2 Safety of the *C. tyrobutyricum* Source

6.2.1 Presence of the Viable Cells in *Clostridium* Protein

Clostridium protein is dried killed cells obtained from *C. tyrobutyricum* using a corn-derived sugar feedstock. Analysis of 5 representative lots of *Clostridium* protein identified viable cell counts of ranging from 160 to 2,390 CFU/mL (see Part 2). *C. tyrobutyricum* ASM#19 is a mutant which does not form spores and the low numbers of viable cells detected in *Clostridium* protein are not expected to survive and proliferate under the conditions of intended use. Thus, any residual viable cells in the *Clostridium* protein will not pose a safety concern.

6.2.2 Identity

The genus *Clostridium* consists of a heterogeneous set of species which are not phylogenetically coherent. Many species were assigned to the genus only on the basis of their being gram positive, spore forming, and anaerobic organisms. Phylogenetic and comparative analyses indicate that of the >150 *Clostridium* species identified, fewer than half are part of cluster 1, a distinct cluster in the 16S rRNA tree which are generally regarded as the true representatives of the genus *Clostridium* and includes *C. tyrobutyricum* (Lawson and Rainey, 2016; Udaondo *et al.*, 2017). *Clostridium* cluster 1 is recognized as *Clostridium sensu stricto* and the species assigned to this genus are metabolically and physiologically diverse species capable of utilizing carbohydrates and peptones to produce organic acids and alcohols. The G+C content of cluster I species varies from between 22 and 37 mol%, and the 16S rRNA gene sequence similarities range from 92 to 99% (Rainey *et al.*, 1993; Wiegel *et al.*, 2006).

6.2.3 Natural Occurrence

Clostridia species, including *C. tyrobutyricum* are widely found in anaerobic environments and can ferment carbohydrates as well as proteins (Driehuis and Oude Elferink, 2000). *C. tyrobutyricum* is present naturally in the gut of humans and animals, and is considered symbiont. Studies indicate that it is one of the earliest colonizers of the infant gut and can be detected in 10 to 20% of the adult population (Mountzouris *et al.*, 2002; Stoeva *et al.*, 2021). Examples of *C. tyrobutyricum* strains isolated from patient samples from the Icahn School of Medicine at Mount Sinai are reported as part of a BioProject (Icahn School of Medicine, 2020). Moreover, there are reports of the potential health benefits of *C. tyrobutyricum* when part of the gut naturally or through consumption of probiotics (Hrncirova *et al.*, 2019; Xiao *et al.*, 2021; Yang *et al.*, 2022).

Additionally, *C. tyrobutyricum* is commonly associated with spoilage of dairy products as described further below, but is not associated with food-borne illness (EFSA, 2005). It is a normal component of milk and cheese, and transforms lactic acid into butyric acid, acetic acid, carbon dioxide and hydrogen gases. It is typically considered a spoilage microorganism, and is one of the *Clostridia* species responsible for a defect known as “late-blowing” in semi-hard cheeses such as Gouda and Provolone, where gas production results in holes, fissures and bursting of the cheese (EFSA, 2005; Ghoddusi and Sherburn, 2010; Brändle *et al.*, 2016).

C. tyrobutyricum has also been identified in silages and manure used for cattle feeding (Jonsson, 1990; Driehuis and Oude Elferink, 2000; Cremonesi *et al.*, 2012). It is recognized as one of the most important *Clostridial* species to contribute to spoilage of silages on account of its ability to ferment lactate. Spores from *Clostridia* in silage can survive passage through the gastrointestinal (GI) tract and be transferred to feces, resulting in its presence in manure. Moreover, fecal contamination of the udder can result in the transfer of spores to the milk which can negatively impact quality (Driehuis and Oude Elferink, 2000).

Overall, the presence of *C. tyrobutyricum* naturally in dairy products supports the species generally being considered non-toxigenic and non-pathogenic (see Section 6.2.4). Notably, *C. tyrobutyricum* ASM#19 was selected by Superbrewed Food for commercialization on the basis that it does not form spores (see 4 2.2.1).

6.2.4 Potential Toxigenicity and Pathogenicity

Botulism, caused by botulinum neurotoxin (BoNT) is most frequently associated with *Clostridium botulinum* but can occasionally arise from *Clostridium butyricum* (Peck, 2002; Cassir *et al.*, 2016). The disease can occur when BoNT-producing *Clostridium* species colonize the intestine or wounds of animals or humans and subsequently produce the toxin, or alternatively, when contaminated foods are ingested in which the toxin has already been formed.

While *Clostridia* are part of the commensal microbiota, epidemiology studies have implicated some species with human disease, particularly necrotizing enterocolitis in premature infants (EFSA, 2014; Cassir *et al.*, 2016; Schönherr-Hellec *et al.*, 2018). Likewise, *Clostridia* are associated with necrotizing enteritis in animals including poultry, pigs, dogs and ruminants (Popoff *et al.*, 1985; Bousseboua *et al.*, 1989; Gohari *et al.*, 2015). *Clostridium perfringens* is normally the cause of necrotizing enterocolitis but there are rare instances of *C. butyricum* being implicated. For example, Caya and Truant (2000) reported the diagnosis of 53 infant pediatric patients with clostridial bacteremia, of which 50% of cases were associated with *C. perfringens* and 25.9% cases with *C. butyricum*. Recently, a systematic characterization of necrotizing enterocolitis and control strains conducted by Schönherr-Hellec *et al.* (2018) suggested the existence of a specific signature associated with pathogenicity and that a unifying causative mechanism for development of the disease may be activation of an innate immune response.

There are no reports in the published literature associating *C. tyrobutyricum* strains with botulinum neurotoxin production or pathogenicity in humans or animals. These data are consistent with the WGS analysis conducted by Superbrewed Food on *C. tyrobutyricum* ASM#19 which confirms the absence of any genes encoding for toxins commonly associated with *Clostridium* species or any known virulence

factors (see Part 2.2). Overall, the available information indicates *C. tyrobutyricum* ASM#19 is not associated with toxigenicity or pathogenicity.

6.2.5 Overall Conclusions on the Safety of the Source Microorganism

Pariza and Cook (2010) recognized in their guidelines for assessing the safety of enzyme preparations for use in food processing, that the primary consideration when assessing the microbial source is the toxigenic potential, especially the production of toxins that are active via the oral route. Pathogenic potential of the source microorganism (or production strain) is normally less of a concern because of the absence of viable cells or transmissible DNA that might code for pathogenic traits. As mentioned in Part 2, only low levels of viable cells are detected in *Clostridium* protein and these are not expected to survive or proliferate under the conditions of intended use. *C. tyrobutyricum* ASM#19 has been unambiguously identified at species level and no markers for pathogenicity or toxigenicity, and no acquired antibiotic resistance genes were detected by WGS analysis. The results of phenotypic testing demonstrated that *C. tyrobutyricum* ASM#19 is susceptible to antibiotics of veterinary and pharmaceutical relevance. The findings of the genome-wide analysis and physiological evaluation of *C. tyrobutyricum* ASM#19 are consistent with the published literature in which no reports of the species being associated with pathogenicity or toxigenicity in humans or animals were identified.

Taken together, it may be concluded that *C. tyrobutyricum* ASM #19 does not pose a safety concern for humans when used as the source of *Clostridium* protein.

6.3 Nutritional Considerations

6.3.1 Amino Acid Composition

The amino acid composition of *Clostridium* protein is provided in Table 6.1, alongside typical values reported for whey protein, casein, pea protein, mycoprotein and mung bean isolate, which are 5 existing counterparts for which Superbrewed Food's ingredient may be considered a direct replacement under the conditions of use in beverages and conventional foods. The essential amino acid requirements for adults set by the WHO also are provided in the table for comparison (WHO, 2002). *Clostridium* protein is a source of all essential amino acids (*italics, bold*), meeting or exceeding the requirements laid down by the WHO for protein sources on a g/100 g protein basis for all individual amino acids. Relative to whey protein, casein and pea protein, *Clostridium* protein exhibits similar or higher levels of essential amino acids on a per product basis. Unlike these vegetable based protein counterparts, *Clostridium* protein is a source of tryptophan (0.7 g/100 g product). Additionally, the total essential amino acids contents for whey protein, casein and pea protein on a protein basis was estimated by Gorissen *et al.* (2018) to be 43, 34 and 30%, respectively. By comparison, based on the analytical data for the 5 lots of *Clostridium* protein, the total essential amino acids content on a protein basis (crude protein content of 85%), the total essential amino acids content was calculated to be 52%². Taken together, these data indicate that *Clostridium* protein has the potential to act as a source of essential amino acids for humans and there

²Calculation: sum of essential amino acids (including cystine as the oxidized form of cysteine)/mean crude protein content. For all of the plant proteins and *Clostridium* protein, crude protein was calculated as N content x 6.25.

are no anticipated adverse impacts on the total dietary intakes of these amino acids under the proposed conditions of use as an alternative to existing fungal- or vegetable-derived proteins.

Table 6.1: Comparison of Amino Acid Profiles for <i>Clostridium</i> Protein, Whey Protein, Casein and Pea Protein Isolate							
Amino Acid	g/100 g of Product						Adult Requirement ³
	<i>Clostridium</i> Protein ¹	Whey Protein ²	Casein ²	Pea Protein ²	Mycoprotein	Mung Bean Isolate	
Protein	85.0	72-84	67-78	77-88	42-50		(100)
<i>Histidine</i>	1.4	1.4	2.2	1.6	0.4	2.9	1.5
<i>Isoleucine</i>	6.3	3.8	3.0	2.3	0.6	4.9	3.0
<i>Leucine</i>	6.7	8.6	7.8	5.7	1.0	8.6	5.9
<i>Lysine</i>	9.0	7.1	5.9	4.7	0.9	7.1	4.5
<i>Methionine</i>	2.6	1.8	2.2	0.3	0.2	1.3	1.6
<i>Cysteine</i> ⁴	0.7	0.8	0.1	0.2	-	0.3	0.6
<i>Phenylalanine</i>	3.8	2.5	3.1	3.7	0.5	6.9	3.8
<i>Tyrosine</i> ⁴	3.2	2.4	3.4	2.6	0.2	3.2	
<i>Threonine</i>	4.2	5.4	3.5	2.5	0.6	12.3	2.3
<i>Tryptophan</i>	0.7	-	-	-	0.2	0.95	0.6
<i>Valine</i>	5.7	3.5	3.0	-	0.6	5.5	3.9
Alanine	6.6	4.2	2.0	-	-	4.0	-
Arginine	4.2	1.7	2.1	5.9	-	7.8	-
Aspartic acid	10.3	-	-	-	-	-	-
Glutamic acid (+ Glutamine)	10.8	-	-	-	-	(18.3)	
Glycine	4.3	1.5	1.2	2.8	-	3.4	-
Proline	2.6	4.8	6.5	3.1	-	4.4	-
Serine	3.8	4.0	3.4	3.6	-	5.3	-

Abbreviations: “-” = not measured (*Clostridium* protein, whey protein and casein) or not set (adult requirement); amino acids in **italics** are the essential amino acids (IOM, 2005);

¹Mean results of analytical data provided in Section 5 for 5 lots of *Clostridium* protein – the crude protein as-is is reported as a range for the 5 lots tested, where the mean is calculated to be 85.0 g/100g;

²Values reported by Gorissen *et al.*, 2018;

³Based on reported FAO/WHO adult essential amino acid requirements (WHO, 2002) reported as g/100 g protein recognizing that *Clostridium* protein, whey protein, casein and pea protein amino acid values are provided as g/100 raw material where the protein content varies as indicated in the table;

⁴Considered to be conditionally essential by the IOM but included in **italics** for completeness (IOM, 2005).

The IOM has established RDAs for the essential amino acids which are summarized in Table 6.2 on a mg/kg body weight/day basis (IOM, 2005). In the intakes assessment (see Section 3.1), among the individual population groups, infants and young children were identified as having the highest mean and 90th percentile consumer-only intakes of *Clostridium* protein at 0.60 and 1.37 g/kg body weight/day, respectively. These mean and 90th percentile intakes were used to estimate the intake of individual essential amino acids on a per body weight basis under the conditions of intended use of *Clostridium* protein and the values are also presented in Table 6.2. For all essential amino acids, mean consumption of *Clostridium* protein by infants and young children was observed to make a significant contribution (as

least 40%) or exceed the RDAs. Likewise, the RDAs for all essential amino acids were met or exceeded by infants and young children that were high level consumers of *Clostridium* protein. It is anticipated that formulators of *Clostridium* protein-containing foods will take into account the amino acid profiles and nutritional recommendations of the target population during formulation. Thus, *Clostridium* protein is expected to contribute to, and not adversely impact, essential amino acid intakes under the proposed conditions of use as an alternative protein source in the specified conventional foods and beverages.

Table 6.2: Comparison of the RDAs for Amino Acids and Estimated Intakes from a Serving and from All Proposed Food Uses of <i>Clostridium</i> Protein			
Amino Acid	RDA for Adults (mg/kg body weight/day)	Amino Acid Intakes Based on Highest Estimated Mean Intakes from All Food Uses (mg/kg body weight/day)¹	Amino Acid Intakes Based on Highest Estimated 90th Percentile Intakes from All Food Uses (mg/kg body weight/day)²
Histidine	14	8.4	19.2
Isoleucine	19	37.8	86.3
Leucine	42	40.2	91.8
Lysine	38	54.0	123.3
Methionine	19	15.6	35.6
Cysteine		4.2	9.6
Phenylalanine	33	22.8	52.1
Tyrosine		19.2	43.8
Threonine	20	25.2	57.5
Tryptophan	5	4.2	9.6
Valine	4	34.2	78.1

Abbreviations: RDA = recommended daily allowance [presented for adults 19 years or older (IOM, 2005)];

¹Calculated as: 0.60 (g/kg body weight/day) x [AA content (g/100 g mean value; Table 6.5)/100] x 1000 = AA (mg/kg body weight/day);

²Calculated as: 1.37 (g/kg body weight/day) x [AA content (g/100 g mean value; Table 6.5)/100] x 1000 = AA (mg/kg body weight/day).

6.3.2 In vitro Protein Digestibility

The *in vitro* digestibility of *Clostridium* protein was assessed by Superbrewed Food using the commercial Megazyme assay kit (Medallion Labs). The protein digestibility score obtained from the assay was used in conjunction with the essential amino acid profile as well as the protein and moisture contents of *Clostridium* protein to calculate the Protein Digestibility Corrected Amino Acid Score (PDCAAS) value. The *in vitro* digestibility of *Clostridium* protein was 96.4% relative to 98.9% for casein under the experimental conditions of the kit. The commercial *in vitro* digestibility kit is reported by the manufacturers to yield results which correlate well with traditional *in vivo* digestion models in rats. Thus, the results of the *in vitro* digestibility study indicate that *Clostridium* protein is a highly digestible source of protein.

6.3.3 Protein Quality Evaluation

Protein quality evaluation is the process of measuring the effectiveness of a food protein source to meet the metabolic demand for amino acids and nitrogen. Correctly determined, measurement of the quality of a protein source provides a means of predicting the overall efficiency of protein utilization. This

allows recommendations to be made for intakes of the protein source to ensure safe use that meets the metabolic demands of the target population (WHO, 2002).

The quality of the protein relates to its amino acid profile and to its bioavailability, i.e., the proportion of the protein that can be absorbed from the diet and utilized. Protein utilization is normally evaluated in terms of both digestibility and biological value, with the latter reflecting the effectiveness of the absorbed amino acid profile in meeting the metabolic requirement.

The FAO/WHO Expert Consultation on Protein Quality Evaluation in 1989 recommended the use of the PDCAAS to evaluate the quality of a protein source for humans (FAO, 1991). In 1993, the PDCAAS method was adopted by the U.S. FDA as the “preferred best method” for predicting protein quality and continues to be used (21 CFR §101.9; U.S. FDA, 2021f).

The PDCAAS method is based on the principle that protein quality can be predicted from the digestibility and amino acid composition of a protein source. In practice, PDCAAS relates the first limiting essential amino acid of a protein source to the content of the same amino acid in a reference pattern of essential amino acids (referred to as “amino acid score”) adjusting for digestibility:

$$\text{Amino acid score} = \frac{\text{mg of amino acid (limiting) in 1 g of test protein}}{\text{mg of amino acid (limiting) in reference pattern}}$$

$$\text{PDCAAS \%} = \text{digestibility} \times \text{amino acid score}$$

The *in vitro* digestibility results for *Clostridium* protein obtained using the commercial Megazyme kit can also be used to calculate PDCAAS (Medallion Labs, 2021). The crude protein content of *Clostridium* protein as determined by the Dumas (combustion) method was used to adjust the reported amino acid contents on a product basis to a protein basis (crude protein content 82.9 g/100 g). The reference patterns of essential amino acids used to calculate the amino acid scores were those for 2 to 5 year-old pre-school children as recommended by the FAO/WHO in 1991, and also those for 6 month old infants and 3 to 10 year old children as recommended by the FAO/WHO in 2013 (FAO, 1991 and 2013). Historically, the reference pattern for 2 to 5 year old pre-school children has been used to determine scoring patterns for all foods except infant formulas but more recently this has been replaced by the separate patterns for 6 month old infants, covering foods for young children (6 months to 3 years of age) and 3 to 10 year old children covering foods for older children, adolescents and adults.

The calculated amino acid scores based on the reference pattern for 2 to 5 year old children are presented in Table 6.3. The limiting amino acid in *Clostridium* protein was calculated to be tryptophan with a score of 0.7. From the amino acid score of 0.75 and *in vitro* digestibility of 96.4%, a PDCAAS of 72% was calculated.

Table 6.3: Calculation of Amino Acid Scores for <i>Clostridium</i> Protein (FAO, 1991)			
Essential Amino Acids for Human Nutrition	<i>Clostridium</i> Protein Total Amino Acid Content (mg/g protein) ¹	FAO/WHO Recommended Values (mg/g protein; 1991) ²	Calculated Amino Acid Scores for <i>Clostridium</i> Protein ³
Histidine	17	19	0.88
Isoleucine	76	28	2.70
Leucine	81	66	1.22

Table 6.3: Calculation of Amino Acid Scores for <i>Clostridium</i> Protein (FAO, 1991)			
Essential Amino Acids for Human Nutrition	<i>Clostridium</i> Protein Total Amino Acid Content (mg/g protein) ¹	FAO/WHO Recommended Values (mg/g protein; 1991) ²	Calculated Amino Acid Scores for <i>Clostridium</i> Protein ³
Lysine	109	58	1.88
Methionine + cysteine	38	2	2.36
Phenylalanine + tyrosine	85	63	1.34
Threonine	50	34	1.48
Tryptophan	82	11	0.75
Valine	67	35	1.92

Abbreviations: FAO = Food and Agricultural Organization;

¹Amino acid profile of Lot DNII 210225 used in the *in vitro* digestibility study;

²FAO reference values are those reported in 1991 in order to comply with the U.S. FDA requirements for calculating the PDCAAS value, rather than the updated values reported by the FAO/WHO in 2011;

³Calculation: (mg amino acid in 1 g protein)/(mg amino acid in 1 g protein in reference sample).

The calculated amino acid scores based on the reference patterns for young children (based on 6 month old infant) and for older children, adolescents and adults (based on 3 to 10 year old children) are presented in Table 6.4. The limiting amino acid in *Clostridium* protein was calculated to be histidine for both assessments with scores of 0.84 and 1.05, respectively. From the amino acid score for young children of 0.84 and *in vitro* digestibility of 96.4%, a PDCAAS of 81% was calculated. Similarly, from the amino acid score for older children, adolescents and adults of 1.05 and *in vitro* digestibility of 96.4%, a PDCAAS of 101% was calculated.

Table 6.4: Calculation of Amino Acid Scores for <i>Clostridium</i> Protein (FAO, 2013)					
Essential Amino Acids for Human Nutrition	<i>Clostridium</i> Protein Total Amino Acid Content (mg/g protein) ¹	FAO/WHO Recommended Values (mg/g protein; 2013) for Children 6 Months to 3 Years ²	Calculated Amino Acid Scores for <i>Clostridium</i> Protein ³	FAO/WHO Recommended Values (mg/g protein; 2013) for Older Children, Adolescents and Adults ²	Calculated Amino Acid Scores for <i>Clostridium</i> Protein ³
Histidine	17	20	0.84	16	1.05
Isoleucine	76	32	2.36	30	2.52
Leucine	81	66	1.22	61	1.32
Lysine	109	57	1.91	48	2.27
Methionine + Cysteine	38	27	1.43	23	1.67
Phenylalanine + Tyrosine	85	52	1.63	41	2.07
Threonine	50	31	1.63	25	2.02
Tryptophan	82	8.5	0.97	6.6	1.24
Valine	67	43	1.56	40	1.68

Abbreviations: FAO = Food and Agricultural Organization; WHO = World Health Organization;

¹Amino acid profile of Lot DNII 210225 used in the *in vitro* digestibility study;

²FAO reference values are the updated values reported in 2013 noting that for regulatory purposes, the pattern for young children (6 months to 3 years) is recommended for foods for all populations groups except infant formula – for children data are from the 6-month old and for older children, adolescents and adults data are from 3 to 10 year old children;

³Calculation: (mg amino acid in 1 g protein)/(mg amino acid in 1 g protein in reference pattern).

The PDCAAS of some animal- and vegetable-derived protein sources are presented in Table 6.5 and compared with the values of 72% and 101% calculated for *Clostridium* protein, respectively based on the amino acid reference patterns for 2 to 5 year old pre-school children (FAO, 1991) and 6 month to 3 year old children (FAO, 2013). The PDCAAS for pea protein concentrate and *Clostridium* protein calculated using the FAO/WHO reference amino acid pattern recommendations from 1991 are comparable (73 and 72%, respectively) and the limiting amino acid in both of these sources is tryptophan. Conversely, using the FAO/WHO reference amino acid pattern recommendations from 2013, the PDCAAS for whey protein isolate and *Clostridium* protein are similar (97 and 101%, respectively) and the limiting amino acid in both of these sources is histidine.

Table 6.5: PDCAAS for Selected Foods (Taken from: Joint FAO/WHO Expert Consultation on Protein Quality, 1991; Mathai et al., 2017)		
Product	PDCAAS (% untruncated; 1991) (Limiting Amino Acids)	PDCAAS (%; 2013) (Limiting Amino Acids)¹
Casein	100 ²	-
Egg white	100 ²	-
Whey protein isolate	99 (aromatic amino acids) ³	97 (histidine) ³
Milk protein concentrate	100 (threonine) ³	121 (sulfur-containing amino acids) ³
Pea protein concentrate	73 (tryptophan) ³	71 (sulfur-containing amino acids) ³
Soy protein isolate	93 (sulfur-containing amino acids) ³	86 (sulfur-containing amino acids) ³
Beef	92 ²	-
Kidney beans (canned)	68 ²	-
Lentils (canned)	52 ²	-
<i>Clostridium</i> protein	72 (tryptophan)	101 (histidine)

Abbreviations: FAO = Food and Agricultural Organization; WHO = World Health Organization;

¹Based on reference pattern for 6 month old infants to 3 year-old children (used to score foods for older children, adolescents and adults);

²Taken from the Joint FAO/WHO Expert Consultation on Protein Quality Evaluation (1991);

³As reported by Mathai et al. (2017).

Taken together, the available data indicate that *Clostridium* protein is a good quality protein source that is not expected to be nutritionally disadvantageous when used as a direct replacement for animal-, fungal- and vegetable-derived proteins in the proposed range of conventional foods and beverages.

6.3.4 Nucleic Acids Content

The crude protein content of *Clostridium* protein, calculated as N x 6.25, incorporates both the true protein content and also non-protein nitrogen, such as nucleic acids, cell wall components (peptidoglycans), vitamins, amines and ammonia. Of these, the primary contributor to the non-protein nitrogen content of *Clostridium* protein is expected to be the nucleic acid content. A maximum limit on

nucleic acids of 4 g/100 g is set for *Clostridium* protein of which around 16 or 17% will be nitrogen, a value not dissimilar to that of protein (Kay and Vrede, 2008). On this basis, the non-protein nitrogen from nucleic acids will represent no more than 5% of the 12.8 g nitrogen content of *Clostridium* protein containing 80 g/100 g crude protein³.

Estimated Background Intakes of Nucleic Acid Levels in Common Foods

Nucleic acids occur widely in vegetable- and animal-derived foods in the form of RNA, DNA, nucleotides and free nucleic acid bases. Examples of common foods known to be rich in nucleic acids include liver, fish roe, vegetables and mushrooms, and the RNA and DNA contents of selected examples of such foods are presented in Table 6.6. Liver was found to contain in the region of 2.2 to 3.2 g/100 g dry matter of RNA and 1.5 to 2.0 g/100 g dry matter of DNA depending on the source, equating to a total content (RNA + DNA) of 4.0 to 4.7 g/100 g dry matter. Other relatively high sources included chestnut mushrooms and oyster fungi with levels of 2.1 to 2.4 g/100 g dry matter RNA and 0.14 g/100 g dry matter DNA, equal to around 2.3 to 2.6 g/100 g dry matter in total (RNA + DNA).

Food	RNA [g/100 g DM; (mean)]	DNA [g/100 g DM; (mean)]	Total RNA + DNA [g/100 g DM]¹
Liver (pig)	3.12-3.55 (3.21)	1.44-1.81 (1.48)	4.69
Liver (calf)	2.12-2.30 (2.29)	1.71-2.02 (1.73)	4.02
Liver (beef)	2.14-2.28 (2.21)	1.89-2.00 (1.95)	4.16
Herring roe	1.53	0.06	1.59
Trout (smoked)	0.47	0.10	0.57
Cod	0.47	0.03	0.5
Tuna	0.17	0.08	0.25
Broccoli	2.06	0.51	2.57
Cauliflower	1.45	0.2	1.65
Spinach	1.40	0.26	1.66
Cabbage	1.46	0.2	1.66
Peas	0.50	0.16	0.66
Yeast (baking)	6.62	0.60	7.22
Chestnut mushrooms	2.11	0.14	2.25
Oyster fungi	2.41	0.14	2.55
Potatoes	0.14	0.1	0.24
Onion	0.26	0.17	0.43
Avocado	0.15	0.06	0.21
Lentils	0.38-0.39 (0.39)	0.7-0.8 (0.8)	1.19
Kidney beans	0.47	0.1	0.57
Wheat	0.23	0.06	0.29
Rye	1.1-1.4 (0.13)	0.6-0.8 (0.7)	0.29
Oats	0.3	0	0.3
Corn	0.41	0.11	0.52

Abbreviations: DM = dry matter; DNA = deoxyribonucleic acid; RNA = ribonucleic acid;

³ Calculation: 80% protein is equivalent to 12.8 g/100 g N (80/6.25); 4% nucleic acids containing 17% N will contribute 0.68 g/100 g N (4x0.17). Percentage contribution to total N content: (0.64/12.8) x 100 = 5.3%.

¹Calculation: total RNA and DNA calculated based on mean values (where applicable).

The typical serving sizes and nucleic acids (RNA + DNA) contents, reported on a dry matter basis, were used to estimate the intakes per serving of these components from common foods in the normal diet of the U.S. population. The estimated nucleic acids intakes are presented in Table 6.7.

A 28 g serving of liver (as-is; equivalent to 12 g on a dry matter basis) was calculated to provide around 0.5 g RNA + DNA/serving, with vegetables such as broccoli, cauliflower and cabbage estimated to provide around 0.23, 0.11 and 0.13 g RNA + DNA/80 g serving (as-is; equivalent to 6 to 9 g on a dry matter basis), respectively. Mushrooms in particular, are high in nucleic acids with chestnut or oyster mushrooms estimated to contain around 0.72 g or 0.82 g RNA + DNA/serving (80 g as-is; 32 g on a dry matter basis), respectively. By comparison, a 30 g serving of non-dairy cheese containing 20% *Clostridium* protein with a nucleic acids content at the maximum specified limit of 4 g/100 g, will provide around 0.24 g nucleic acids/serving which is similar to that of a serving of broccoli but less than that in a serving of liver or mushrooms.

Table 6.7: Estimated Intakes of Nucleic Acids Per Serving from Common Foods			
Food	Total RNA + DNA [g/100 g DM of Food]	Serving Size [g as-is (DM)] ¹	Estimated Total RNA + DNA Intake (g/Serving) ²
Non-dairy cheese containing 20% <i>Clostridium</i> protein	4.0 (as-is)	30 (as-is)	0.24
Liver (pig)	4.69	28 (12)	0.55
Liver (calf)	4.02	28 (12)	0.47
Liver (beef)	4.16	28 (12)	0.49
Herring roe	1.59	14 (4.5)	0.07
Trout (smoked)	0.57	150 (32)	0.18
Cod	0.5	220 (46)	0.23
Tuna	0.25	150 (51)	0.13
Broccoli	2.57	80 (9)	0.23
Cauliflower	1.65	80 (6)	0.11
Spinach	1.66	80 (8)	0.13
Cabbage	1.66	80 (8)	0.13
Peas	0.66	80 (17)	0.11
Chestnut mushrooms	2.25	80 (32)	0.72
Oyster fungi	2.55	80 (32)	0.82
Potatoes	0.24	300 (84)	0.20
Onion	0.43	15 (1.5)	0.01
Avocado	0.21	80 (22)	0.05
Lentils	1.19	80 (28)	0.33
Kidney beans	0.57	80 (30)	0.17
Wheat (in bread)	0.29	10 (9)	0.03
Rye (in bread)	0.29	10 (9)	0.03
Oats	0.3	50 (13)	0.01
Corn	0.52	105 (25)	0.13

Abbreviations: DM = dry matter; DNA = deoxyribonucleic acid; RNA = ribonucleic acid;

¹DM serving (g): typical serving sizes as-is and adjusted for reported water content based on example foods in FoodData Central (USDA, 2019; <http://fdc.nal.usda.gov/>) – for wheat/rye, it is assumed 10 g of flour might be present in a serving of bread;

²Calculation: (RNA + DNA per 100 g DM)/100 x serving size (g DM).

Potential Impact of Clostridium Protein on Background Intakes of Nucleic Acids from the Diet

Background intakes of nucleic acids by individuals consuming diets rich in vegetables and meat were also estimated based on a typical plate of food comprising beef liver, onions and mushrooms accompanied by broccoli, spinach and potatoes as well as bread. Using the reported nucleic acids content (as RNA and DNA) and serving sizes reported in Table 6.8 for each component of the meal, the overall intake of nucleic acid was calculated to be 1.79 g/sitting (meal). Replacing calf liver with a meat substitute (e.g., patty) containing 40% *Clostridium* protein will increase the nucleic acid intakes for the meal from 1.79 to 2.28 g/meal (approx. 28% increase).

Under the proposed conditions of use of *Clostridium* protein, the highest mean and 90th percentile consumer-only intakes were estimated to be 14.7 and 33.2 g/person/day, respectively for male teenagers. The exposure by mean- and high-level (90th percentile) consumers to nucleic acids when

present in *Clostridium* protein at the maximum permitted amount of 4 g/100 g is calculated to be 0.59 and 1.33 g/person/day, respectively. These intakes reflect worst-case daily intakes rather than for one meal, but corroborate the above calculations that *Clostridium* protein has the potential to increase exposure to nucleic acids from the diet when replacing meat and similar protein products. The impact of *Clostridium* protein on nucleic acid intake however, is not additive (i.e., does not equate to background levels + *Clostridium* protein contribution) on the basis that the primary function is to replace rather than supplement other protein sources in the diet.

Table 6.8: Estimated Intakes of Nucleic Acids in a Typical Meal			
Food	Total RNA + DNA [g/100 g DM of Food]	Serving Size (g DM)¹	Estimated Total RNA + DNA Intake (g/Serving)²
<i>Meat substitute containing 40% Clostridium protein</i>	4.0	60	0.96
Liver (calf)	4.02	12	0.47
Broccoli	2.57	9	0.23
Spinach	1.66	8	0.13
Chestnut mushrooms	2.25	32	0.72
Potatoes	0.24	84	0.20
Onion	0.43	1.5	0.01
Wheat (in bread)	0.29	9	0.03
Total	-	-	1.79 (per meal)

Abbreviations: DM = dry matter; DNA = deoxyribonucleic acid; RNA = ribonucleic acid.

¹DM serving (g): typical serving sizes as-is and adjusted for reported water content based on example foods in FoodData Central (USDA, 2019; <http://fdc.nal.usda.gov/>) – for wheat/rye, it is assumed 10 g of flour might be present in a serving of bread;

²Calculation: (RNA + DNA per 100 g DM)/100 x serving size (g DM).

Nucleic Acids Levels in Microbial Proteins

Foods derived from rapidly growing cells, such as bacterial and fungal proteins are characterized by relatively high nucleic acid contents, primarily in the form of RNA (Jonas *et al.* 2001; Nalage *et al.*, 2016). In general, bacterial protein products are reported to contain 8 to 12 g/100 g nucleic acids and fungal protein products between 7 and 10 g/100 g, primarily in the form of RNA (Nasseri *et al.*, 2011; Nalage *et al.*, 2016; Ritala *et al.*, 2017; Bratosin *et al.*, 2021). The level of nucleic acids⁴ in a representative lot of *Clostridium* protein (Lot CMRE191010) produced without specific treatment to reduce the nucleic acid content was reported to be 78.2 g/kg. Heating *Clostridium* protein under appropriate conditions activates endogenous ribonucleases which degrade the RNA and reduce the level to not more than 4 g/100 g, with the values for 5 representative lots of *Clostridium* protein reported to vary from 1.6 to 2.9 g/100 g (see Table 2.6; Ritala *et al.*, 2017)⁵. By comparison, the two microbial proteins with GRAS

⁴ Measured using the in-house test method described in Mydland *et al.* (2008) involving hydrolysis of the nucleic acid containing components to the individual purines which are then analyzed individually and summed to give the total content.

⁵ Heat-treatment is a widely recognized procedure for the reduction of nucleic acids content in foods (Ritala *et al.* 2017). Endogenous RNA degraded enzymes (ribonucleases) are activated which degrade the RNA. The degraded RNA components diffuse out of the cells but biomass loss can also occur. Superbrewed Food has optimized the

notified status for use as protein sources in conventional foods and beverages in the U.S., Quorn™ (*F. venenatum* protein; GRN No. 91; U.S. FDA, 2002) and *F. flavolapsis* protein (GRN No. 904; U.S. FDA, 2021h) are subject to similar processing to *Clostridium* protein to degrade RNA (Ritala *et al.*, 2017) and a maximum limit for nucleic acids of 2 g/100 g is set. Although the level in *Clostridium* protein is higher than that in these *Fusarium* protein counterparts, as indicated above, the levels in bacterial proteins are naturally higher than fungal proteins.

Comparison of the Estimated Intakes of Nucleic Acids from Microbial Proteins on a Per Serving Basis

Clostridium protein may be used as a meat substitute at levels of up to 40% by weight in the ready-to-eat product (see Section 1.3). The anticipated exposure to nucleic acids from typical food uses of *Clostridium* protein and its existing counterpart, mycoprotein, are presented in Table 6.9. A typical 60 g serving of a meatless patty will therefore, contain up to 0.96 g of nucleic acids from *Clostridium* protein. By comparison, the properties of mycoprotein (marketed as Quorn™) are such that it is widely sold as a ground product comprising 94% of the fungal protein⁶. An individual consuming the recommended 110 g serving of the ground product as part of a meal, will be exposed to up to 2.1 g nucleic acids, which is around twice that provided by a vegan patty containing 40% *Clostridium* protein. Notably, there are also a range of processed products containing around 40% mycoprotein which is more comparable with the maximum use level of *Clostridium* protein in meat substitutes. Thus, as a substitute for mycoprotein, *Clostridium* protein is not likely to significantly impact potential intakes of nucleic acids from the diet.

Table 6.9: Estimated Intakes of Nucleic Acids Per Serving from Microbial Proteins			
Food	Total RNA + DNA [g/100 g of Food]	Serving Size (g)	Estimated Total RNA + DNA Intake (g/Serving)
Meat substitute containing 40% <i>Clostridium</i> protein	4.0	60	0.96
Quorn Meatless grounds (as sold; 94% mycoprotein)	2.0	110	2.1

Abbreviations: DM = dry matter; DNA = deoxyribonucleic acid; RNA = ribonucleic acid.

6.3.5 Mineral Content

Estimated Intakes of Mineral from Clostridium Protein vs. Recommended Dietary Levels

The IOM has established Adequate Intakes (AIs) and RDAs, as well as ULs for various minerals which are summarized in Table 6.10 (IOM, 2011b). Under the conditions of intended use of *Clostridium* protein as a protein source in the range of specified conventional foods and beverages, male teenagers were identified as having the highest mean and 90th percentile consumer-only intakes at 14.7 and 33.2 g/person/day, respectively. Mineral intakes by male teenagers consuming the mean or high-level amounts of *Clostridium* protein per day were estimated based on the reported composition of the ingredient and compared to the AI or RDA for each element (Table 6.10). Except for manganese, molybdenum and selenium, the estimated intakes of each individual mineral from *Clostridium* protein

processing of *Clostridium* protein to minimize the nucleic acids content but at the same time balance the loss in biomass.

⁶ Example: <https://www.walmart.com/ip/12-Pack-Quorn-Meatless-and-Soy-Free-Grounds-12-Oz/660374309>

under the intended conditions of use equated to no more than 10% of the AI/RDA for mean consumers and no more than 25% for high-level consumers (90th percentile). For manganese, molybdenum and selenium, *Clostridium* protein was estimated to contribute 36, 47 and 36%, respectively of the AI/RDA in mean consumers, and 74, 100 and 73%, respectively of the AI/RDA in high-level users (90th percentile). However, compared to the tolerable upper limit (UL) for manganese, molybdenum and selenium, the contribution by mean users was calculated to be 9, 1 and 5%, respectively, and 19, 2 and 10%, respectively for high-level users (90th percentile). Thus, under this worst-case intake scenario, although *Clostridium* protein makes a significant contribution to the daily requirements for manganese, molybdenum and selenium, the overall intakes from all food uses is not expected to present a safety concern on the basis that the contribution to the ULs is no more than 20% for any element.

Table 6.10: Comparison of Mineral Requirements and Estimated Intakes from the Proposed Food Uses of <i>Clostridium</i> Protein by Male Teenagers						
Mineral	AIs or RDAs (mg/day) ¹	ULs (mg/day)	Mineral Intakes from <i>Clostridium</i> Protein by Male Teenagers			
			Mean Intakes (14.7 g/person/day)		High User Intakes (33.2 g/person/day)	
			Estimated Intake (mg) ²	% Contribution to AI or RDA ³	Estimated Intake (mg) ⁴	% Contribution to AI or RDA ⁵
Calcium	1,300*	3,000	2.4	0.2	5.4	0.4
Phosphorus	1,250	4,000	54	4	121	10
Magnesium	410*	350 ⁶	6.2	2	14	3
Potassium	3,000	-	4.7	0.2	11	0.4
Sodium	1,500	-	17	1	37	3
Iron	11*	45	1.1	10	2.5	23
Zinc	11*	34	0.8	7	1.8	16
Copper	0.89*	8	<0.01	1	<0.03	3
Manganese	2.2	9	0.8	36	1.7	74
Molybdenum	0.043*	1.7	0.02	47	0.04	100
Selenium	0.055*	0.4	0.02	36	0.04	73

Abbreviations: AI = adequate intake; ND = not determined; RDA = recommended daily allowance; UL = tolerable upper limit; y = year;

¹Reported as AIs except for those minerals with an "*" which are reported as RDAs;

²Calculated as: mean mineral content (mg/kg; see Part 2) x (14.7 g/100 g);

³Calculated as: mineral content (mg) in 14.7 g *Clostridium* protein/RDA (mg/day) for males (14-18 y) x 100;

⁴Calculated as: mean mineral content (mg/kg; see Part 2) x (33.2 g/100 g);

⁵Calculated as: mineral content (mg) in 33.2 g *Clostridium* protein/RDA (mg/day) for males (14-18 y) x 100;

⁶The UL for magnesium is for the pharmaceutical supplement use only and does not include use in food or water.

Superbrewed Food has investigated the source of selenium internally and determined that the most likely source is the corn used to generate the sugar feedstock for *C. tyrobutyricum* fermentation (Finley *et al.*, 1996). To ensure the levels of selenium are controlled in *Clostridium* protein, Superbrewed Food sources only low selenium corn.

Comparison of Manganese, Molybdenum and Selenium Contents in Common Foods with *Clostridium* Protein

Clostridium protein contains around 52 mg/kg of manganese, 1.3 mg/kg of molybdenum and 1.3 mg/kg of selenium. The amount of each trace element provided by *Clostridium* protein when present at the maximum proposed use level of 20% in a 30 g portion of non-dairy cheese was estimated and the results are presented in Table 6.11. A serving of non-dairy cheese containing *Clostridium* protein will provide 0.3 mg of manganese, 8 µg of molybdenum and 8 µg of selenium vs. DRVs of 2.3 mg for manganese, 45 µg for molybdenum and 55 µg for selenium. Thus, a typical serving of non-dairy cheese containing *Clostridium* protein is a good source⁷ of manganese, molybdenum and selenium, providing around 13, 18 and 15%, respectively of DRVs.

Examples of the manganese, molybdenum and selenium contents per serving of various foods commonly consumed by the general U.S. population are also presented in Table 6.11. The amounts of manganese, molybdenum and selenium per 30 g serving of a non-dairy cheese containing 20% by weight of *Clostridium* protein are similar to, or fall below, those provided by a serving of ground beef (molybdenum and selenium), tuna (selenium), soybeans (manganese), milk (molybdenum and selenium) and whole wheat bread (manganese and selenium).

Overall, under the conditions of intended use, consumption of *Clostridium* protein as a direct replacement for animal-, fungal- and vegetable-derived proteins in the diet is not expected to be nutritionally disadvantageous in terms of the mineral content.

Table 6.11: Manganese, Molybdenum and Selenium Contents Per Serving of Selected Foods (Taken from NIH, 2021a, b and c)				
Food	Serving size	Manganese (mg/serving)	Molybdenum (µg/serving)	Selenium (µg/serving)
Non-dairy cheese containing 20% <i>Clostridium</i> protein	30 g	0.3	7.6	7.5
Ground beef	85 g	0	8	18
Soybeans (boiled)	0.5 cup	0.7	-	-
Milk (1% fat)	1 cup	0	22	8
Tuna (yellowfin, cooked/canned)	85 g	0.0	5	92
Bread (wholewheat)	2 slices	1.4	2	30

6.3.6 Vitamin Intakes

The IOM has established RDAs for riboflavin, folate, vitamin B6 and vitamin B12 which are summarized in Table 6.12 (IOM, 2011c). Under the conditions of intended use of *Clostridium* protein as a protein source in the range of specified conventional foods and beverages, male teenagers were identified to have the highest mean and 90th percentile consumer-only intakes at 14.7 and 33.2 g/person/day, respectively. B vitamin intakes by male teenagers consuming mean or high-level (90th percentile)

⁷ A food is considered a “good source of” a vitamin, mineral or other nutritional substance when it contains 10 to 19% of the Recommended Daily Intake (RDI) or Daily Reference Value (DRV) per reference amount customarily consumed (21 CFR §101.54; U.S. FDA, 2021i).

amounts of *Clostridium* protein per day were estimated based on the reported composition of the ingredient and compared to the RDA (Table 6.12). The estimated intakes of folate and vitamin B6 from *Clostridium* protein under the conditions of intended use fall well below the RDA for that B vitamin (<10%) for both mean- and high-level users. The contribution of *Clostridium* protein to the RDA for riboflavin was estimated to be 23 and 46%, respectively for mean- and high-level users. Likewise, *Clostridium* protein was estimated to contribute 78 and 178%, respectively of the RDA of vitamin B12 for mean- and high-level users. No tolerable upper limit has been established for riboflavin or vitamin B12 because of a lack of suitable data (IOM, 2011d). The nutritional implications of the exposure to B vitamin under the conditions of intended use of *Clostridium* protein are therefore, evaluated by comparison to the riboflavin and vitamin B12 content of other common foods below.

Table 6.12: Comparison of B Vitamin Requirements and Estimated Intakes from the Proposed Food Uses of <i>Clostridium</i> Protein by Male Teenagers					
Vitamin	RDAs¹	Estimated Intake²	% Contribution to AI or RDA³	Estimated Intake⁴	% Contribution to AI or RDA⁵
Riboflavin (vitamin B2)	1.3 mg/day	0.3 mg/day	23%	0.6 mg/day	46%
Folate (vitamin B9)	400 µg/day	15 µg/day	3%	33 µg/day	8%
Vitamin B6 (pyridoxine)	1.3 mg/day	41 µg/day	3%	93 µg/day	7%
Cyanocobalamin (vitamin B12)	2.4 µg/day	1.4 µg/day	78%	3.2 µg/day	178%

Abbreviations: RDA = recommended daily allowance; y = year;

¹RDAs as reported by the IOM (2011c);

²Calculated as: mean vitamin content (mg/100g or µg/100g; see Part 2) x (14.7 g/100 g);

³Calculated as: vitamin content (mg or µg) in 14.7 g *Clostridium* protein/RDA (mg/day or µg/day) for males (14-18 y) x 100;

⁴Calculated as: mean vitamin content (mg/day or µg/day; see Part 2) x (33.2 g/100 g);

⁵Calculated as: mineral vitamin (mg or µg) in 33.2 g *Clostridium* protein/RDA (mg/day or µg/day for males (14-18 y) x 100.

Comparison of B Vitamin Content of Microbial Proteins

As previously mentioned, two microbial proteins have GRAS notified status for use as protein sources in conventional foods and beverages in the U.S., Quorn™ (*F. venenatum* protein; GRN No. 91; U.S. FDA, 2002) and *F. flavolapsis* protein (GRN No. 904; U.S. FDA, 2021h). Publicly available data from Marlow Foods (2020) indicates that the levels of riboflavin, vitamin B6, folate and vitamin B12 in Quorn™ are around 0.26 mg/100 g, 0.1 mg/100 g, 114 µg/100 g and 0.71 µg/100 g, respectively compared to 1.8 mg/100 g, 0.28 mg/100 g, 99.6 µg/100 g and 9.6 µg/kg for *Clostridium* protein. *F. flavolapsis* protein was reported to contain 0.1 mg/100 g vitamin B6 and <0.440 µg/100 g of vitamin B12 (GRN No. 904; U.S. FDA, 2021h). These findings are consistent with the published literature in which microbial proteins in general are reported to contain B vitamins and bacterial proteins in particular, vitamin B12 (Nalage *et al.*, 2016; Ritala *et al.*, 2017). Thus, the B vitamin content of *Clostridium* protein can be considered substantially equivalent to microbial protein counterparts in the diet.

Comparison of the Riboflavin and Vitamin B12 of Common Foods with Clostridium Protein

Clostridium protein contains around 1.8 mg/100 g of riboflavin and 9.6 µg of vitamin B12. The amount of riboflavin and vitamin B12 provided by *Clostridium* protein when present at the maximum proposed use level of 20% in a 30 g portion of non-dairy cheese was estimated and the results are presented in Table 6.13. A serving of non-dairy cheese containing *Clostridium* protein will contain 0.1 mg of riboflavin and 0.6 µg of vitamin B12 per serving vs. DRVs of 1.3 mg and 2.4 µg for these vitamins, respectively. Thus, a typical serving of non-dairy cheese containing *Clostridium* protein is a high source⁸ of vitamin B12, providing around 25% of the DRV.

Examples of the riboflavin and vitamin B12 contents of various foods commonly consumed by the general U.S. population are also presented in Table 6.13. A typical serving of non-dairy cheese containing *Clostridium* protein at 20% by weight contains a similar amount of riboflavin and less vitamin B12 than an equivalent serving of Swiss cheese (0.1 mg and 0.6 µg/serving vs. 0.09 mg and 0.9 µg/serving). The amount of riboflavin and vitamin B12 provided by a serving of non-dairy cheese containing *Clostridium* protein at 20% by weight falls well below the amount contained in a typical serving of lamb liver or beef steak [0.1 mg and 0.6 µg/serving vs. 1.5 mg and 16 µg/serving (lamb liver) and 0.6 mg and 3.6 µg/serving (beef steak)].

Overall, under the conditions of intended use, consumption of *Clostridium* protein as a direct replacement for animal-, fungal- and vegetable-derived proteins in the diet is not expected to be nutritionally disadvantageous in terms of the B vitamin content.

Table 6.13: Riboflavin and Vitamin B12 Content of Selected Protein Products					
Food	Amount		Amount (µg) per Serving		
	Riboflavin (mg/100 g)	Vitamin B12 (µg/100 g)	Serving Size (g)	Riboflavin (mg)	Vitamin B12 (µg)
<i>Clostridium</i> protein	1.8	9.6	30	0.1	0.6
Liver (lamb, fried) [FDC ID: 17368]	5.3	57.5	28	1.5	16
Beef steak [FDC ID: 1098182]	0.3	1.9	187 (medium steak)	0.6	3.6
Mung bean isolate [GRN No. 684; U.S. FDA, 2017]	0.06-0.13	<2-10	30	0.02-0.04	3
Swiss cheese [FDC ID: 746767]	0.3	3	30	0.09	0.9

6.3.7 Organic Acids

Under the conditions of intended use of *Clostridium* protein as a protein source in the range of specified conventional foods and beverages, male teenagers were identified to have the highest mean and 90th percentile consumer-only intakes at 14.7 and 33.2 g/person/day, respectively. The mean-level intakes

⁸ A food is considered a “high source of” a vitamin, mineral or other nutritional substance when it contains 20% or more of the Recommended Daily Intake (RDI) or Daily Reference Value (DRV) per reference amount customarily consumed (21 CFR §101.54; U.S. FDA, 2021i).

equate to an exposure by male teenagers to ammonium butyrate, ammonium acetate and ammonium lactate of 0.08, 0.07 and 0.03 mg/person/day, respectively based on the mean values reported in Part 2. Similarly, the high-level intakes to an exposure by male teenagers to ammonium butyrate, ammonium acetate and ammonium lactate of 0.2, 0.2 and 0.07 g/person/day, respectively.

Butyric acid, tributyrin, acetic acid and its sodium and calcium salts, triacetin, and lactic acid and its sodium, potassium and calcium salts have a long and established history of use as flavors and technical additives in food (U.S. FDA, 2021j to s). Furthermore, various ammonium salts are GRAS for use as additives in food, including ammonium carbonate (21 CFR §184.1137; U.S. FDA, 2021t), ammonium chloride (21 CFR §184.1138; U.S. FDA, 2021u) and ammonium hydroxide (21 CFR §184.1139; U.S. FDA, 2021d). Considering the wide scope of use of butyrate, acetate and lactate in the acid and salt form in foods, no safety concerns are anticipated from the presence of residual levels of these substances in *Clostridium* protein.

6.4 Allergenicity

An evaluation of the potential allergenic risk of *Clostridium* protein to humans was conducted at the University of Nebraska-Lincoln following the Codex criteria (Codex Alimentarius, 2009). As a first step, a literature search was conducted in order to identify any reports of *C. tyrobutyricum* being associated with allergenicity. No cases of allergenicity were identified.

DNA sequences for *C. tyrobutyricum* ASM#19 and the encoded gene sequences were predicted using the GLIMMER 2 software at John Hopkins University. There were an estimated 3,220 predicted proteins which were then compared to 2,171 allergen and putative allergen sequences in the AllergenOnline.org version 20 database using FASTA version 36.3.8 in batch mode. The AllergenOnline.org database at the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska was started in 2004–2005. It is a public, peer-reviewed database of allergens developed from the amino acid sequences of proteins in the NCBI protein database (Goodman *et al.*, 2005 and 2016) and is updated annually. The AllergenOnline.org version 20 sequences represent 873 protein-taxonomic proteins from 392 allergenic sources. Current Codex recommended criteria for cross-reactivity states that proteins with >35% amino acid sequence identity over 80 or more amino acids could represent proteins that might lead to IgE cross-reactivity and potential allergic reactions for those with significant pre-existing allergies. Where the sequences of *Clostridium* protein displayed alignments which met the criteria, the individual sequence matched allergens were also compared to the NCBI protein database (2020) using BLASTP version 2.9.0+ (2019) to consider the relevance of the alignment. Expectation-scores (*E*-scores) reflect the measure of the relatedness among protein sequences and can help to separate aligned sequences which potentially occur randomly from those that may share structurally relevant similarities. Small *E*-scores in the region of 1×10^{-7} or less, reflect a likely functional similarity and can be suggestive of a biologically-relevant relationship for allergy or potential cross-reactivity. Conversely, large *E*-scores of 1.0 or more are typically associated with similarities in alignments which are not biologically relevant (Henikoff and Henikoff, 1992 and 1996; Pearson, 2000, 2014 and 2016). For the analysis of *C. tyrobutyricum*, the *E*-score threshold used was 10 and the calculated *E*-scores were recorded to understand the relevance of the matches. The sequences in *C. tyrobutyricum* identified to meet this criteria were compiled to show the best FASTA match to an allergen in AllergenOnline.org version 20

including the sequence identity, alignment length, and *E*-score as well as results from the BLASTP to NCBI proteins.

Evaluation of *C. tyrobutyricum* predicted protein sequences against the AllergenOnline.org version 20 database identified 23 alignments that were 80 residues or longer with a sequence identity >35%. However, the sequence identity values noted for *C. tyrobutyricum* were much higher in identity to other microbial proteins from sources not known to be allergenic than to established allergens. Comparison of the *Clostridium* protein sequences to the NCBI protein database demonstrated that the sequences identified are not unique and are common to bacterial species. Moreover, against the criteria in AllergenOnline.org for assigning proteins as allergens, the *C. tyrobutyricum* sequences were not considered potent and highly unique sequences.

Overall, while the amino acid sequence identity of 23 of the proteins in *C. tyrobutyricum* display matches above the minimum criteria set by Codex (2003 and 2009) for possible cross-reactivity, these matches were considered by the study authors to be due to random sequence identities and not to be above the levels that might be classified as potential allergens. Considering that the results of the bioinformatics analysis indicate that *Clostridium* protein is of low allergenic risk to humans via the oral route, no further testing was conducted. These conclusions are consistent with the low oral allergenic risk generally associated with microbial species.

6.5 Absorption, Distribution, Metabolism and Excretion (ADME)

In humans, ingested protein is digested by hydrolytic enzymes produced by the stomach, pancreas and small intestine. The stomach releases gastric juices containing hydrochloric acid and the enzyme, pepsin, which initiate the breakdown of the protein into smaller oligopeptides (Vahdatpour *et al.*, 2016). The cleaved peptides formed by gastric digestion are further hydrolyzed by pancreatic enzymes, such as trypsin, chymotrypsin, and carboxypeptidases (Goodman, 2010). At the intestinal mucosal membrane, further hydrolysis of oligopeptides occurs via an array of brush border peptidases, which break down oligopeptides into free amino acids and di- and tri-peptides (Miner-Williams *et al.*, 2014; Vahdatpour *et al.*, 2016; van der Wielen *et al.*, 2017). With very few exceptions (i.e., neonates in the first few days of life) larger peptides and intact proteins are not systemically absorbed, with estimates of >95% absorption occurring as individual amino acids and <5% as di- and tri-peptides (Washabau, 2013; Vahdatpour *et al.*, 2016; Moughan and Wolfe, 2019).

The resultant mixture of free amino acids and small peptides are transported into the mucosal cells by a number of specific carrier systems for individual amino acids and di- and tri-peptides (Gilbert *et al.*, 2008). Individual amino acids are absorbed via sodium-dependent and independent amino acid transporters, whereas short peptides are absorbed through a proton coupled peptide transporter (PEPT1; Cho *et al.*, 2013). Once absorbed, peptides may be hydrolyzed by epithelial intracellular peptidases or, if resistant, released intact across the basolateral membrane into the circulation (Miner-Williams *et al.*, 2014). Absorbed amino acids pass to the liver, where a portion of the amino acids are used either for catabolic reactions to yield energy (Wu *et al.*, 2005) or for protein synthesis (Gorissen *et al.*, 2020). The remainder pass through into the systemic circulation and are utilized by the peripheral tissues (Trommelen *et al.*, 2021).

As such, the current data supports that regardless of the protein consumed, the human systemic circulation would encounter primarily individual amino acids and a small amount of short di- and tri-peptides, which are then utilized by the body through common biological pathways.

6.6 Toxicological Studies using *Clostridium* Protein

Clostridium protein was subjected to a standard battery of toxicity studies, consisting of two dose range finding (DRF) studies in rats, a 90-day dietary feeding study in rats, a bacterial reverse mutation test and an *in vitro* mammalian micronucleus assay (Jonaitis *et al.*, 2022). These studies were conducted in accordance with the recommendations laid down in Chapter III of the U.S. FDA Redbook (U.S. FDA, 2000). Genotoxicity studies are not routinely conducted on novel proteins but considering the absence of any significant history of use of *Clostridia*, or products derived thereof, as food ingredients, the genotoxic potential was considered pertinent to the safety evaluation.

With the exception of a preliminary DRF study in rats, the test articles used in the toxicity studies were representative of the product to be marketed and for which analytical data are provided herein. The preliminary DRF study in rats was conducted on *Clostridium* protein which was manufactured by the same process described in Section 2.4 but was not subjected to heat treatment to reduce the nucleic acid levels. This “crude” *Clostridium* protein ingredient was compositionally equivalent to the GRAS substance except for the presence of higher levels of nucleic acids, i.e., *ca.* 8 g/100 g vs. a maximum limit of 4 g/100 g, where the mean value across 5 representative lots was 2.3 g/100 g (see Part 2).

Apart from the preliminary DRF study conducted in rats using crude *Clostridium* protein, the findings of the toxicological studies are published and form pivotal evidence of the safety of the ingredient for the intended use in the specified conventional foods and beverages.

6.6.1 Preliminary DRF Study using Crude *Clostridium* Protein

A preliminary DRF study was undertaken by Superbrewed Food to evaluate the palatability and general toxicity of crude *Clostridium* protein (Experimental Lot CMRE191010Ti; 87.8 g crude protein and 7.8 g nucleic acids/100 g). The study was not performed in full compliance with Good Laboratory Practice (GLP) but was conducted in a GLP-compliant facility and the study design followed the general principles outlined in Organization of Economic Cooperation and Development (OECD) Technical Guidance (TG) 407 and the U.S. FDA Redbook Section IV.C.4.a.

Groups of CRL Sprague-Dawley CD® IGS rats (5/sex/group; 8 weeks old, 244 to 249 g males and 221 to 225 g females) were fed an open standard diet containing 0 (control), 4.75, 9.5 or 19% crude *Clostridium* protein for 14 days. The dietary treatments containing graded levels of crude *Clostridium* protein were prepared by partial replacement of casein in the basal (control) diet. The protein content of *Clostridium* protein was taken into account in formulating the experimental diets, but no adjustments were made for other nutritional components in the test article. Food and water were available *ad libitum* throughout the study.

The animals were observed twice daily for mortality, and body weights and food consumption were measured on Days 0, 3, 7, 10 and 14. On Day 0, prior to the first treatment with crude *Clostridium* protein, and approximately weekly thereafter, a detailed clinical observation was conducted while

handling the animal. Potential signs noted included, but were not limited to, changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling), or bizarre behavior (e.g., self-mutilation, walking backwards) were also recorded if present. At the end of the study, animals in the high-use level (19% *Clostridium* protein) and control group were subjected to gross necropsy, including examination of the external surface of the body, as well as all orifices, musculoskeletal system, the cranial, thoracic, abdominal, and pelvic cavities, and their associated organs and tissues. All gross lesions were recorded. Organ weights of the adrenal glands, kidneys, spleen, brain, liver, thymus, testes or ovaries and heart were recorded. Histopathological examination of the adrenal glands, kidneys, liver, heart, spleen, brain, thymus, uterus, ovaries, oviducts as well as all gross lesions was performed. Due to observed clinical findings of potential toxicological interest, the hindlimbs of selected animals were also evaluated microscopically.

All animals survived until the end of the study period. No significant differences were noted in food consumption of rats fed crude *Clostridium* protein compared to controls. Weekly body weights of male rats fed crude *Clostridium* protein-containing diets were generally comparable to the controls except for a decrease in those fed 19% crude *Clostridium* protein on Day 10 and Day 14 ($P<0.05$ or 0.01). A significant increase in daily body weight gain ($P<0.05$) was observed in male rats fed 4.75% crude *Clostridium* protein in the diet on Days 3 to 7 but significant decreases were observed in those fed 9.5 or 19% crude *Clostridium* protein during the final interval of the study (Days 10 to 14) and the overall study (Days 0 to 14) ($P<0.05$ or 0.01). These changes were reflected in a significant decrease in food efficiency over the same study interval and overall ($P<0.05$ or 0.01). Body weights, daily body weight gains and food efficiency for female rats were comparable among treatment groups throughout the study, although numerical reductions in body weight parameters were observed in animals fed diets containing 19% crude *Clostridium* protein. These changes in performance parameters were attributed by the study authors to test substance-related observations noted in the hind limbs of all treatment groups.

There were potentially adverse test substance-related observations during the final days of the 14-day study in male and female rats fed diets containing crude *Clostridium* protein at all treatment levels. The observations included swelling of the hindlimbs and plantar surface, diminished weight bearing, associated with non-adverse signs of callousing, flaking, and dry skin of the hindlimbs. Corresponding clinical observations of hypotonic gait, ataxic gait, impaired locomotion, impaired surface righting, and hyperkeratosis were also recorded. Piloerection was observed with minimal incidence in male rats receiving diets containing 9.5 or 19% crude *Clostridium* protein.

There were no gross observations noted among rats selected for histopathological examination. In male rats receiving diets containing 19% crude *Clostridium* protein, organ weight changes included significantly decreased ($P<0.05$) absolute kidney-to-body weight ratios and significantly increased ($P<0.05$) relative weights for the brain. All other absolute and organ weights for male rats in this group were comparable to controls. In female rats fed diets containing 19% crude *Clostridium* protein, there was a statistically significant increase ($P<0.05$) in the adrenals-to-body weight and adrenal to brain ratios. Spleen to body weight ratio was significantly increased ($P<0.05$) in female rats receiving crude *Clostridium* protein at any dietary level when compared with controls. Similarly, kidney to body weight

ratio was significantly increased ($P<0.05$) in female rats receiving diets containing 9.5 or 19% crude *Clostridium* protein relative to controls.

Microscopic findings in the group fed 19% crude *Clostridium* protein included marked chronic-active inflammation characterized by variably-dense accumulations of inflammatory cells within the synovial membrane and/or intra-articular and periarticular soft tissues (mononuclear cells were often predominant with smaller neutrophilic aggregates), edema in the synovial membrane and/or intra-articular and periarticular soft tissues, fibrovascular tissue expansion in and around joints, vascular congestion, scattered foci of hemorrhage, and synoviocyte hypertrophy and hyperplasia. Occasionally, areas of inflammation surrounded foci of amorphous eosinophilic material or tissue drop-out. Edema fluid varied from being clear to pale-basophilic (myxomatous). These microscopic findings correlated with the clinical observations of diminished hindlimb weight bearing. Additionally, findings in the hindlimbs included minimal hyperkeratosis of the plantar epithelium which correlated with the clinical observations of dry, flaking and callous involving the hindpaw plantar surface.

Except for artefactual changes associated with tissue processing, articular cartilage, bones, and bone marrow were histologically unremarkable. Although the described constellation of microscopic findings in the hindlimbs are consistent with gouty arthritis/synovitis, the characteristic gross finding of pale nodules around joints was not reported. Furthermore, microscopic findings such as a foreign body giant cell response and needle shaped yellow-brown urate crystals also characteristic of gout, were not observed using standard light microscopy. Urate crystals were not observed with polarized microscopy. However, several studies have shown that formalin-fixation of tissues for greater than 12 hours can result in dissolution of urate crystals, precluding a definitive diagnosis of urate tophi via standard histopathologic evaluation (Shidham *et al.*, 1998 and 2001). In addition to the clinical and organ weight findings (particularly kidney weights), concurrent findings such as hyperuricemia and clinicopathologic/histopathologic evidence of kidney damage may help support an overall clinical diagnosis of gout in treated animals.

Under the conditions of the study, dietary concentrations of crude *Clostridium* protein above 4.75%, corresponding to 3,951 mg/kg body weight/day for males and 3,504 mg/kg body weight/day for females were not tolerated.

As mentioned in Section 6.3.4, nucleic acids occur widely in vegetable-, fungal- and animal-derived foods in the form of RNA, DNA, nucleotides and free nucleic acid bases. Microbial (single cell) proteins are characterized by their relatively high nucleic acid contents, primarily in the form of RNA (Jonas *et al.*, 2001; Nalage *et al.* 2016). On ingestion by animals and humans, nucleic acids will be sequentially cleaved by intestinal enzymes to form purines which are absorbed, metabolized and principally excreted in the urine as uric acid (Giesecke and Tiemeyer, 1982; PAG, 1983; Jonas *et al.*, 2001). Abnormally high concentrations of serum uric acid, or hyperuricemia, causing uric acid precipitation can present as gout and inflammatory arthritis, with increased concentrations of uric acid in the urine potentially resulting in the formation of renal calculi (Kamel and Kramer, 1979; PAG, 1983; Delimaris, 2013; Lockyer and Stanner, 2016; Jakše *et al.*, 2019). On this basis, one of the primary safety considerations in developing microbial proteins for use as food ingredients is the potential elicitation of gout or kidney stones. The

adverse findings observed in the DRF study in rats fed diets containing 9.5 or 19% crude *Clostridium* protein can therefore be attributed to the nucleic acid content.

The nucleic acid content of crude *Clostridium* protein was analyzed and found to be 7.8 g/100 g. The exposure by rats to nucleic acid from its presence in crude *Clostridium* protein was estimated for each treatment group and the results are summarized in Table 6.14. During the 14-day DRF study, diets containing nucleic acids at 308 and 273 mg/kg body weight/day appeared to be well-tolerated by male and female rats, respectively, with effects associated with purine compounds derived from RNA breakdown observed at 616 and 547 mg nucleic acids/kg body weight/day for male and female rats, respectively.

Table 6.14: Nucleic Acid Content of the <i>Clostridium</i> Protein-Containing Treatment Diets		
Crude <i>Clostridium</i> Protein Dietary Concentration (%)	Nucleic Acid Content (% of Diet)	Nucleic Acid Content (mg/kg body weight/day)
4.75	0.37	Males: 308 Females: 273
9.5	0.74	Males: 616 Females: 547
19	1.48	Males: 1,233 Females: 1,093

As described in Part 2, Superbrewed Food incorporates a heat-treatment step into the manufacture of *Clostridium* protein in order to reduce the levels of nucleic acids to no more than 4 g/100 g. A follow-up 14-day DRF study was conducted using *Clostridium* protein meeting these specifications and representative of product to be marketed. The study is described below.

6.6.2 DRF Study using *Clostridium* Protein (Jonaitis *et al.*, 2022)

A DRF study was performed in male rats using *Clostridium* protein (Lot SM200904; 82.6 g crude protein and 2.79 g nucleic acids/100 g). The study was not GLP-compliant but was conducted in a GLP-compliant facility and the study design followed the general principles outlined in OECD TG 407 and the U.S. FDA Redbook Section IV.C.4.a. Groups of male Sprague Dawley rats (5/group; 6 to 8 weeks of age and 185 to 232 g) were administered *Clostridium* protein at concentrations of 0 (control), 2.5, 5.0, 7.5, or 10.0% for 14 days. The dietary treatments containing graded levels of *Clostridium* protein were prepared by partial replacement of casein in the basal (control) diet, and the control group of animals received standard AIN rodent feed pellets. The experimental diets were formulated to take into account the proximate and mineral profile of *Clostridium* protein. Food and water were provided *ad libitum* for the entire duration of the study period.

Animals were observed daily for clinical signs of toxicity and mortality, whereas body weights were recorded on Days 0, 4, 8, 11 and 14. Daily food intake was calculated on the basis of mean food consumption over 4-day periods. Locomotor activity was assessed on Day 14 and urine was collected on Day 15 for routine urinalysis. Prior to necropsy on Day 15, blood was taken from the retro-orbital sinus and analyzed for routine hematology and clinical chemistry parameters, as well as for uric acid concentration. Absolute and relative organ weights were recorded for the brain, adrenal glands, heart,

kidneys, liver, spleen and thymus. These organs were subject to gross pathology and histopathology examination. Histopathology of the left hind paw was also performed as a marker for joint effects.

All animals survived until the end of the study and no abnormalities in general condition or signs of clinical toxicity were observed. There were no significant differences noted in food consumption, final body weights or body weight gain of rats fed diets containing *Clostridium* protein relative to controls. There were a number of statistically significant findings noted in hematological parameters. Statistically significant ($P \leq 0.05$) reductions in white blood cells, lymphocytes, monocytes, basophils and large unstained cell (LUC) counts were observed in rats fed diets containing 10% *Clostridium* protein. Basophils were also significantly ($P \leq 0.05$) decreased in rats receiving diets containing 5.0 or 7.5% *Clostridium* protein. Furthermore, mean corpuscular volume (MCV) was significantly ($P \leq 0.05$) increased in rats fed the 10.0% *Clostridium* protein-containing diets. While statistically significant differences were reported, all hematology findings remained within historical control data for rats of this age and strain. Other statistically significant variations in hematology parameters were observed but there was no apparent *Clostridium* protein treatment level-related response and the changes were not of a magnitude to be considered toxicologically relevant. Likewise, a few statistically significant variations were observed in clinical chemistry parameters in rats fed 5.0 or 7.5% *Clostridium* protein-containing diets but these changes were considered by the study investigators to have arisen as a result of slightly high or low control values rather than to be treatment-related. A minimal increase in serum calcium concentration was noted in rats fed diets containing 10.0% *Clostridium* protein but the changes remained within historical control data for this age and strain of animals. There were no treatment level-related responses, and the magnitude of the changes were not considered to be of toxicological significance. Serum uric acid concentrations were decreased in rats fed diets containing 2.5% *Clostridium* protein but there was no treatment level-related response.

Inclusion of 5.0 or 7.5% *Clostridium* protein in the diet had no effect on urine parameters in rats but, a slight increase in protein concentrations was observed in the urine of animals fed the 10.0% *Clostridium* protein-containing diets. Additionally, slightly lower urinary volume was observed in this high-level treatment group, but the severity of the change was not considered to be toxicologically relevant.

No gross lesions were observed between rats fed 10.0% *Clostridium* protein in the diet and controls. Similarly, no treatment-related findings were reported by microscopic examination with all findings falling within normal ranges of background pathology encountered in this age and strain of rat. Hind paw histology was normal.

Overall, *Clostridium* protein administered in the diet for 14-days was well-tolerated by male rats at levels of up to 10.0%. Although hematology (white blood cell parameters and mean corpuscular volume), clinical chemistry (calcium) and urine (protein presence) changes were observed at 10.0% *Clostridium* protein in the diet, only lower basophil counts were observed at 5.0 or 7.5% inclusion levels, and all parameters fell within the range of historical controls. Based on these findings, it was concluded that the same dietary *Clostridium* protein levels of 2.0, 5.0, 7.5 or 10.0%, corresponding to 2,099, 4,153, 6,372 or 8,457 mg/kg body weight/day in male rats was appropriate for use in the 90-day feeding study.

The nucleic acid content of the lot of *Clostridium* protein used in the DRF study was determined analytically to be 2.8 g/100 g. The exposure by rats to nucleic acid from its presence in *Clostridium*

protein was estimated for each treatment group and the results are summarized in Table 6.15. During the 14-day DRF study, diets containing up to 236 mg nucleic acids/kg body weight/day were well-tolerated by male rats. These levels are consistent with the findings of the 14-day DRF study conducted using crude *Clostridium* protein (see Section 6.6.1) in which effects related to purine-toxicity were not observed in male or female rats fed diets containing 308 and 273 mg/kg body weight/day, respectively.

Table 6.15: Nucleic Acid Content of the <i>Clostridium</i> Protein-Containing Treatment Diets		
Crude <i>Clostridium</i> Protein Dietary Concentration (%)	Nucleic Acid Content (% of Diet)	Nucleic Acid Content (mg/kg body weight/day)
2.5	0.07	Males: 59
5.0	0.14	Males: 116
7.5	0.21	Males: 178
10.0	0.28	Males: 236

6.6.3 Subchronic Dietary Feeding Study (Jonaitis *et al.*, 2022)

A 90-day dietary feeding study was conducted in rats using *Clostridium* protein (Lot DNII210225; 82.9 g crude protein and 2.68 g nucleic acids/100 g). The study was GLP-compliant and carried out in accordance with OECD TG 408 and U.S. FDA Redbook Chapter IV.C.4.a. Groups of male and female Crl:CD Sprague Dawley rats (10/sex/group; at least 6 weeks of age and 139 to 255 g) were administered diets containing 0 (control), 5.0, 7.5, or 10.0% *Clostridium* protein for 90 days. The dietary treatments containing graded levels of *Clostridium* protein were prepared by partial replacement of casein in the basal (control) diet, where animals in the control group received standard AIN rodent feed pellets. The experimental diets were formulated to take into account the proximate and mineral profile of *Clostridium* protein (i.e., to minimize nutritional differences among experimental treatments).

Animals were assessed daily for mortality and clinical signs of toxicity. Body weights and feed consumption were recorded on a weekly basis on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85 and 91. Daily food intake was calculated on the basis of weekly food consumption values. Water intake was monitored by visual inspection of bottles throughout the study period. Arena observations were recorded weekly. During Week 13, ophthalmic examinations (control and 10% *Clostridium* protein group), functional observational battery (FOB⁹; week 13) and estrous stage determinations (day of scheduled necropsy for females) were conducted. At interim periods (Days 30 and 54) and at the end of the study (Day 91), blood was collected from all groups and analyzed for routine hematology and clinical chemistry parameters (Days 54 and 91), blood coagulation (Day 91) and uric acid levels (Day 91). At the end of the study, urine samples were also collected from all groups and subject to routine urinalysis measurements. All surviving animals were euthanized and subjected to a full *post-mortem* examination, including evaluation of the carcass and musculoskeletal system, all external surfaces and orifices, cranial cavity and external surfaces of the brain, and thoracic, abdominal, and pelvic cavities. Absolute and relative organ weights including brain, heart, kidneys, stomach, large and small intestines, liver, lung, reproductive organs, thyroid, lymph nodes, spleen, pancreas, amongst other glands and tissues, were collected, weighed, and subjected to macroscopic examination. Tissues collected from the control and

⁹ FOB: hearing ability, pupillary reflex and static righting reflex; fore- and hind-limb grip strength; locomotor activity

the 10.0% *Clostridium* protein groups were also subject to full microscopic histopathological examination.

The experimental diets used in the animal studies were nutritionally balanced by adjusting for the compositional profile of *Clostridium* protein. Consequently, there were minimal differences in protein, ash, fat or mineral profiles between the dietary treatments (generally <0.2% variation between parameters). The only exception was crude fiber which was observed to increase from 2.2 g/100 g (control diet) to 3.3 g/100 (10% *Clostridium* protein in the diet).

No treatment-related mortality or clinical signs of toxicity were observed during the study period. There were no significant differences in food consumption, final body weights or body weight gains of rats fed diets containing *Clostridium* protein compared to controls. The findings from ophthalmic examination and functional observations were considered normal, and no toxicologically significant or *Clostridium* protein-related findings were noted.

At the end of the study, there were no significant hematological effects observed in males fed diets containing *Clostridium* protein. In females, statistically significant ($P \leq 0.05$) decreases in neutrophils (mid and high dose groups) and monocyte counts (all dose groups) were observed after 90 days. The interim hematology measurements did not indicate any statistically significant differences in monocyte or neutrophil counts at Day 30 in any of the male or female dose groups. However, a statistically significant ($P \leq 0.05$) reduction in neutrophils was measured in all dosed males at Day 54 without any significant differences observed in any female groups. At this same time point (Day 54), female rats of the mid and high dose groups had significantly ($P \leq 0.05$) reduced monocytes, while male rats did not. At the end of the study, no significant differences were observed in males for these endpoints. The laboratory historical values for female Sprague Dawley rats for neutrophil count¹⁰ was $0.52 \times 10^9/\text{L} \pm 0.22$ (0.3 to 0.85; based on N=426). For monocyte counts, the historical values¹¹ were $0.072 \times 10^9/\text{L} \pm 0.03$ (0.042 to 0.102; based on N=426). After 90 days, in female rats, the measured values for neutrophil counts were 0.46 and $0.43 \times 10^9/\text{L}$ for the mid- and high-treatment level groups (7.5 and 10% *Clostridium* protein), respectively. Whereas measured values for monocyte counts were 0.10, 0.06, and $0.09 \times 10^9/\text{L}$ for the low- mid- and high-treatment level groups (5.0, 7.5 and 10% *Clostridium* protein), respectively. After 90 days, both neutrophil and monocyte counts were within the standard deviation of the reference ranges for neutrophil and monocyte counts for females. These findings were not considered to be adverse, primarily because no concurrent findings were measured in male rats at any dietary treatment level and measurements were subject to high inter-group variability. For example, in the female rat control group, the mean neutrophil count was $0.89 \times 10^9/\text{L}$ with a standard deviation spanning 0.43 to 1.35. With the more robust neutrophil count historical control range of 0.3 to 0.85, it can be seen that the control group actually had comparatively higher neutrophil counts than would be expected, leading to statistically significant differences in the protein-fed groups. The neutrophil counts of the *Clostridium* protein-fed females were relatively comparable between groups and were all well within the normal control standard deviation range. A similar occurrence was observed with monocyte

¹⁰ Originally, the historical control values for neutrophil counts at the time of the study were $0.7 \times 10^9/\text{L} \pm 0.38$; however, this was based only on 16 animals.

¹¹ Originally, the historical control values for monocytes at the time of the study were $0.2 \times 10^9/\text{L} \pm 0.07$; however, this was based only on 46 animals.

counts, wherein the control group had relatively higher than expected counts, while *Clostridium* protein-fed females had values that were well-within the historical control range. The monocyte differences in females also lacked dose-dependence. These data further support that these statistically significant findings were not an indication of an adverse effect.

Inclusion of *Clostridium* protein in the diet of the rats had no statistically significant effect on any serum chemistry parameters on Day 30, however significant effects were observed on Day 54 and persisted until the end of the study (Day 90). The reduction of total bilirubin in female rats of mid and high dose groups (2.44 and 2.87 $\mu\text{mol/L}$, respectively) remained within the reference range for this strain, sex, and age of rat ($2.9 \mu\text{mol/L} \pm 0.48$). The lack of any associated adverse physiological effects and absence of a concurrent reduction in bilirubin in male rats in any group, further supports that the decrease in bilirubin was an incidental finding and not biologically significant. On Day 90 females of the high dose group had statistically significant ($P \leq 0.05$) decreased levels of thyroid stimulating hormone when compared to controls. In absence of a histopathological correlation (see below), these clinical pathology changes were not considered to be adverse. In male rats, the only clinical chemistry value that was statistically significant ($P \leq 0.05$) compared to control values, was reduced high-density lipoprotein (HDL) cholesterol of the mid- and high-treatment level groups (7.5 and 10.0% *Clostridium* protein). This finding was not considered to be biologically relevant or an adverse effect, as the mean values in all groups were all within a standard deviation of each other, there were no concurrent changes in any of the other lipids or triglyceride levels, and nor were there any effects seen at interim evaluations, or any such effects observed in female rats. As such, in the absence of any other evidence to indicate adverse health effects from the consumption of *Clostridium* protein, these findings were not considered to be biologically relevant.

Other statistically significant ($P \leq 0.05$) hematology or serum chemistry findings were observed but occurred sporadically at interim evaluations or only in low- or mid-treatment level groups of one sex. As these statistical findings did not persist throughout the study (i.e., not statistically significantly at the final evaluation), were not treatment level-dependent, and/or were observed in only one sex, they were not considered to be toxicologically relevant.

No adverse effects on blood coagulation parameters were noted in any of the *Clostridium* protein-fed groups, compared to control animals. There was a statistically significant decrease in serum uric acid concentrations in rats fed 5.0 or 7.5% *Clostridium* protein (low- and mid-level treatments) compared to controls, but no differences between the high treatment level group (10.0% *Clostridium* protein) and the controls. There was no treatment level-related response and the effect was opposite to that expected in the case of toxicity; consequently, the finding was not considered test item-related. Urinalysis parameters were comparable among animals from all groups.

There were no treatment-related observations in the gross macroscopic evaluations. All of the recorded macroscopic findings were within the range of background gross observations encountered in rats of this age and strain. Liver weights displayed high physiological variability and the mean values reached statistical significance ($P \leq 0.05$) when expressed relative to body weight in females fed 10.0% *Clostridium* protein and males fed 5.0 or 7.5% *Clostridium* protein, compared to the control group. However, these decreases were very slight in magnitude not treatment level-dependent (in males) and were not

correlated with any macroscopic findings for either sex. As such, it was concluded that the decreased relative liver weights were not related to treatment. No *Clostridium* protein-related findings were revealed during microscopic examination of tissues. All findings were sporadic, without a treatment level-related response pattern or were consistent with the age and strain of rats. Hind gut histology was normal.

Based on the results of this study demonstrating an absence of any adverse effects related to inclusion of *Clostridium* protein in the diet, the highest treatment level of 10.0%, corresponding to 5,558 and 6,671 mg/kg body weight/day for male and female rats, respectively was determined to be the No-Observed-Adverse-Effect-Level (NOAEL). Moreover, the comparable growth performance observed among the dietary treatment groups is consistent with *Clostridium* protein providing a digestible and high-quality protein source for rats.

The nucleic acid content of the lot of *Clostridium* protein used in the 90-day dietary feeding study was determined analytically to be 2.7 g/100 g. The exposure by rats to nucleic acid from its presence in *Clostridium* protein was estimated for each treatment group and the results are summarized in Table 6.16. During the 90-day feeding study, diets containing up to 149 and 179 mg nucleic acids/kg body weight/day in male and female rats, respectively were well-tolerated.

Table 6.16: Nucleic Acid Content of the <i>Clostridium</i> Protein-Containing Treatment Diets		
Crude <i>Clostridium</i> Protein Dietary Concentration (%)	Nucleic Acid Content (% of Diet)	Nucleic Acid Content (mg/kg body weight/day)
5.0	0.13	Males: 73 Females: 84
7.5	0.20	Males: 109 Females: 134
10.0	0.27	Males: 149 Females: 179

6.7 Genotoxicity of *Clostridium* Protein

The genotoxicity potential of *Clostridium* protein was evaluated in a bacterial reverse mutation assay and *in vitro* mammalian micronucleus assay (Jonaitis *et al.*, 2022). The studies are summarized in Table 6.17.

Table 6.17: Summary of Genotoxicity Studies Conducted using <i>Clostridium</i> Protein			
Study	Design	Concentrations	Findings
Bacterial reverse mutation assay [GLP; OECD TG 471]	<i>Salmonella typhimurium</i> tester strains TA98, TA100, TA1535, and TA1537, and <i>Escherichia coli</i> tester strain WP _{2uvrA} -pKM101 Metabolic activation: +/- S9 Treat and wash modification	Expt 1: 0, 52, 164, 512, 800, 1,600, 5,000 µg/plate	Tester train WP _{2uvrA} -pKM101 elicited a dose-dependent response increase in revertants in presence of S9 only; these findings were not observed when the study was repeated
		Expt 2: 0, 275, 492, 878, 1,568, 2,800, and 5,000 µg/mL	<i>Clostridium</i> protein was non-mutagenic under the conditions of the test
<i>In vitro</i> mammalian micronucleus assay [GLP; OECD TG 487]	Peripheral human lymphocytes Metabolic activation: +/- S9 Long-term treatment: 24 hours Short-term treatment: 3 hours	0 (water; solvent control), 16, 31, and 63 µg/mL	<i>Clostridium</i> protein was non-clastogenic or aneugenic under the conditions of the test

Abbreviations: OECD = Organization of Economic Cooperation and Development; TG = test guidelines.

6.8 Critical Evaluation of the Safety Information

The safety of *Clostridium* protein for the intended use as a source of protein in specified conventional foods and beverages is based on scientific procedures using a weight of evidence approach. The source *C. tyrobutyricum* is an asporogenous strain generated by natural evolution from a strain isolated from litter samples taken from a chicken house. There are no reports in the published literature of *C. tyrobutyricum* being associated with toxigenicity and pathogenicity. *C. tyrobutyricum* has been unambiguously identified at species level and no markers for pathogenicity or toxigenicity, and no acquired antibiotic resistance genes were detected by WGS analysis. The results of phenotypic testing demonstrated that the strain is susceptible to antibiotics of pharmaceutical and veterinary relevance. Only low levels of viable cells were detected in *Clostridium* protein, and these are not expected to survive or proliferate under the conditions of intended use.

Clostridium protein comprises a minimum of 80 g/100 g of crude protein, and a maximum of 3 g/100 g of fat, 8 g/100 g of carbohydrates, 6 g/100 g of ash and 10 g/100 g of moisture. *Clostridium* protein is heat-treated to reduce the nucleic acid content to a maximum of 4 g/100 g. Comparison of the amino acid profile of *Clostridium* protein with FAO reference values indicates that the ingredient will contribute to, but not adversely impact, essential amino acid intakes from the diet under the conditions of intended use as a direct replacement for animal-, fungal- and vegetable-derived protein. The *in vitro* digestibility of *Clostridium* protein was high at 96.4% and the % PDCAAS was 75 based on the reference pattern for 2 to 5 year old children (FAO, 1991), 81 based on the updated reference pattern for young children, and 101 based on the updated reference pattern for older children, adolescents and adults (FAO, 2013). Taken together, these data indicate that *Clostridium* protein is a good quality protein source that is not expected to be nutritionally disadvantageous when used as a direct replacement for

existing animal-, fungal- and vegetable-derived proteins in the specified range of conventional foods and beverages.

An important safety consideration in the production of microbial proteins is the high nucleic acid content and the potential elicitation of gout and kidney stones due to the ingestions of purine compounds from the breakdown of RNA in the GI tract which increases uric acid concentrations in the blood. The fungal proteins that have been successfully notified as GRAS in the U.S. for use in foods have a maximum specified limit for nucleic acids of 2 g/100 g. Bacterial proteins are naturally higher in nucleic acids and this accounts for the difference in maximum levels between the fungal proteins and *Clostridium* protein even after heat-treatment to reduce the levels as far as technically feasible. Nucleic acids are naturally present in vegetable- and animal-derived products, with the levels in common foods such as broccoli, mushrooms and liver on a per serving basis demonstrated to be similar or other than the amount provided by a typical serving of a non-dairy cheese containing *Clostridium* protein. Moreover, fungal proteins are widely used as ground products comprising >90% of the microbial product and under the conditions of use are estimated to lead to exposure on a per serving basis almost twice that of a vegan patty containing *Clostridium* protein. Thus, while it is acknowledged that *Clostridium* protein will significantly contribute to nucleic acid intakes from the background diet, under the conditions of intended use, no safety concerns are anticipated. Moreover, although not necessarily well-supported by a body of toxicology data, when a microbial protein is intended for use as a primary source of protein for humans, it is generally recommended that the daily intake of nucleic acids does not exceed 2 g/day. Under the proposed conditions of use of *Clostridium* protein, the highest mean and 90th percentile consumer-only intakes were estimated to be 14.7 and 33.2 g/person/day, respectively. These intakes equate to a nucleic acids exposure of 0.59 and 1.33 g/person/day, respectively for *Clostridium* protein containing the maximum amount of nucleic acids of 4 g/100 g. Thus, these estimates also indicate that the nucleic acid content of *Clostridium* protein will not be a safety concern following long-term consumption of the ingredient by humans.

An assessment of the mineral profile of *Clostridium* protein was conducted by comparing the estimated intakes of each element under the conditions of intended use against the AIs and RDAs established by the IOM. Overall, *Clostridium* protein was determined to make a significant contribution to daily requirements for manganese, molybdenum and selenium but exposure is not expected to be detrimental from a nutritional or safety perspective. Likewise, analysis of the B vitamin content of *Clostridium* protein indicates that the ingredient has the potential to be a high source of vitamin B12 under the conditions of intended use but that these intakes will not be nutritionally disadvantageous.

The results of protein analysis supports that *Clostridium* protein does not pose a realistic risk of food allergy to consumers. These findings are consistent with the low oral allergenic risk generally associated with microbial species.

A battery of toxicity tests were conducted using *Clostridium* protein, specifically a DRF study in rats, a 90-day dietary feeding study in rats and two *in vitro* genotoxicity assays. The methodology used for the toxicity assessment of *Clostridium* protein is consistent with the general principles laid down in the U.S. FDA Redbook Chapter III for the assessment of food ingredients.

The 90-day study in rats was conducted in male and female rats provided 0 (control), 5.0, 7.5 or 10.0% *Clostridium* protein in the diet as a partial replacement for casein. Comparable growth performance was observed among dietary treatment groups consistent with *Clostridium* protein providing a digestible and high-quality protein source for rats. The only statistically significant effects measured in the 90-day feeding study consisted of a small number of hematology and clinical chemistry findings, primarily in females. Reduced neutrophil counts (7.5, and 10.0% *Clostridium* protein) and monocyte counts (5.0, 7.5, and 10% *Clostridium* protein), as well as reduced total bilirubin (7.5 and 10.0% *Clostridium* protein) were observed in female rats only at the end of the study. These findings were not considered adverse due to a lack of concurrent findings in males and the high level of inter group variability. The reduction of total bilirubin in female rats of mid and high doses remained within the reference range for this strain, sex, and age of rat ($2.9 \mu\text{mol/L} \pm 0.48$) and supports that the decrease in bilirubin was an incidental finding and not biologically significant. Based on the results of this study demonstrating an absence of any adverse effects related to inclusion of *Clostridium* protein in the diet, the highest treatment level of 10.0%, corresponding to 5,558 and 6,671 mg/kg body weight/day for male and female rats, respectively was determined to be the NOAEL.

Under the conditions of intended use of *Clostridium* protein in conventional foods and beverages, on a body weight basis, infants and young children were determined to have the highest mean and 90th percentile consumer-only intakes of 600 and 1,370 mg/kg body weight/day, respectively. The amount of *Clostridium* protein fed to female rats in the 90-day study was 4-fold higher than the highest 90th percentile intakes estimated from the proposed food uses of the ingredient.

The nucleic acid content of the lot of *Clostridium* protein used in the 90-day dietary feeding study was determined analytically to be 2.7 g/100 g. Exposure to nucleic acids by rats consuming diets containing 10% *Clostridium* protein was estimated to be 149 and 179 mg/kg body weight/day in male and female rats. As mentioned above, under the conditions of intended use, infants and young children were determined to have the highest mean and 90th percentile consumer-only intakes of 600 and 1,370 mg/kg body weight/day, respectively of *Clostridium* protein, equating to 24 and 55 mg nucleic acids/kg body weight/day for an ingredient containing the maximum amount of 4 g nucleic acids/100 g. The amount of nucleic acids from *Clostridium* protein fed to female rats in the 90-day study was 3-fold higher than the highest 90th percentile intakes estimated from the proposed food uses of the ingredient.

Genotoxicity studies are not normally conducted on novel proteins, but considering the microbial source and the absence of any established history of use of *C. tyrobutyricum* or products derived thereof, as food ingredients, evaluation of the genotoxic potential was considered pertinent to the safety evaluation. Consistent with the WGS and bioinformatics analysis, the results of the *in vitro* genotoxicity tests demonstrate that *Clostridium* protein is non-genotoxic.

It is generally recognized that the standard battery of testing in animals has limitations when applied to macronutrients on the basis that there are practical limitations in deriving NOAELs or tolerability limits. The concentration of the test item generally cannot be incorporated into the diet at sufficiently high levels to derive the conventional 100-fold safety factor allowing for intra- and inter-species variation without resulting in nutritional imbalances which can lead to secondary consequences such as adverse physiological effects (Borzelleca, 1996; Munro *et al.*, 1996; EFSA, 2011). Consequently, the findings of

the 90-day dietary feeding study must be considered in conjunction with the characterization of the *Clostridium* protein and the nutritional composition of the product in order to demonstrate safety under the intended conditions of use as a protein source in conventional foods and beverages. Thus, the results of the 90-day dietary feeding study in rats are considered in conjunction with the characterization data on the strain, as well as the nutritional properties, *in vitro* digestibility data and allergenic potential, to provide a weight of evidence assessment of the safety of *Clostridium* protein under the specified uses in conventional foods and beverages.

6.9 Basis for GRAS Conclusions

Superbrewed Food intends to market *Clostridium* protein as a direct replacement for animal-, fungal- or vegetable-based protein currently used in foods and beverages in the U.S., and as a supplement to the protein occurring naturally in existing food products. *Clostridium* protein is the dried killed cells obtained from *C. tyrobutyricum* fermentation using a corn-derived sugar feedstock. It is an off-white powder comprising a minimum of 80 g/100 g of protein, and a maximum of 3 g/100 g of fat, 5 g/100 g of carbohydrates, 6 g/100 g of ash and 10 g/100 g of moisture.

Finally, the Expert Panel convened on behalf of Superbrewed Food, Inc. independently and collectively, critically evaluated the data and information summarized above and concluded that the intended use of *Clostridium* protein produced in accordance with cGMP and meeting appropriate food-grade specifications, for use as a source of protein in the range of specified conventional foods and beverages, is GRAS based on scientific procedures. It was also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, Superbrewed Food, Inc. has concluded that *Clostridium* protein is GRAS under the intended conditions of use on the basis of scientific procedures and is excluded from the definition of a food additive.

PART 7. §170.255. LIST OF SUPPORTING DATA AND INFORMATION

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Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of *Clostridium* Protein for Use in Foods

INTRODUCTION

Superbrewed Food, Inc. (hereafter referred to as “Superbrewed Food”) convened a panel (the “Expert Panel”) of independent scientists, qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information on *Clostridium* protein, and to determine whether the intended uses of *Clostridium* protein in specified conventional food and beverage products would be Generally Recognized as Safe (GRAS) based on scientific procedures. The food uses of *Clostridium* protein include meat and poultry analogs, dairy analogs, prepared meals, meal replacement products, milk and non-milk-based nutritional beverages, vegetable and fruit-based drinks, baked goods, breakfast cereals, soups, dressings and sauces at levels ranging from 1 to 40% by weight of the ready-to-eat (RTE) or ready-to-drink (RTD) products. In protein powders for formulation into beverages using milk or water for consumption as a supplemental protein source in the diet, use levels will vary up to a maximum of 90% by weight in the powder. The Expert Panel consisted of the below-signed qualified scientific experts: Professor Eric Johnson (University of Wisconsin-Madison), Professor Kelly Swanson (University of Illinois at Urbana-Champaign) and Dr. Ashley Roberts (AR Toxicology, Inc.).

The Expert Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data. This information was presented in a dossier provided by Superbrewed Food [Documentation to Support the Generally Recognized As Safe (GRAS) Status of *Clostridium* Protein for Use in Foods], which included an evaluation of all available scientific data and information, both favorable and unfavorable, relevant to the safety of *Clostridium* protein for the intended food uses. The body of data was prepared in part from a comprehensive search of the scientific literature and also included information characterizing the source and identity of the ingredient, manufacture of the ingredient, product specifications, supporting analytical data, stability data, intended conditions of use, estimated exposure under the conditions of intended use, potential allergenicity and *in vitro* digestibility, as well as studies evaluating the safety of the ingredient. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following independent critical evaluation, the Expert Panel unanimously concluded that *Clostridium* protein, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications, is GRAS based on scientific procedures, under the conditions of intended use in specified conventional foods and beverages as described above. A summary of the basis for the Expert Panel’s conclusion appears below.

SUMMARY AND BASIS FOR GRAS

Superbrewed Food intends to market *Clostridium* protein for use as a direct protein replacement of animal-, fungal- or vegetable-based protein currently used in foods and beverages in the United States (U.S.), and as a supplement to the protein occurring naturally in existing food products. *Clostridium* protein is an off-white powder comprising the dried killed cells obtained from *Clostridium tyrobutyricum* fermentation using a corn-derived sugar feedstock.

The microbial source has been unambiguously characterized as the non-spore forming *C. tyrobutyricum* ASM#19. Whole Genome Sequence (WGS) analysis indicates the absence of any genetic element sequences that code for virulence factors or protein toxins. Phenotypic and WGS analysis together indicate that the strain has not acquired any antimicrobial resistance and is susceptible to antimicrobials of human and veterinary importance. Testing for inhibitory activity of culture supernatants against reference strains also confirms *C. tyrobutyricum* ASM#19 does not produce antimicrobial substances. Consistent with the cells from the fermentation being killed during the manufacturing process, only low levels of viable cells were detected in 5 representative lots of *Clostridium* protein, with levels ranging from 160 to 2,390 CFU/mL.

The raw materials and processing aids used in the commercial production of *Clostridium* protein are food-grade and permitted for use in food. The manufacturing processing involves (1) grinding and liquefaction of corn; (2) further processing of 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; and (4) separation, washing and drying of the killed cells to yield *Clostridium* protein. A heat-treatment step (70°C for 20 minutes) is included in the production process to reduce the nucleic acid levels in *Clostridium* protein to no more than 4 g/100 g. Commercial production of *Clostridium* protein will be in accordance with cGMP and a Hazard Analysis Critical Control Point (HACCP) plan will be in place. The process will also comply with the requirements of the Food Safety and Modernization Act (FSMA).

Appropriate food-grade specifications are established for *Clostridium* protein which include well-defined ranges for the levels of the primary nutrient (protein) and compositional components of potentially toxicological concern (nucleic acids and ammoniacal N). Criteria to control the levels of heavy metals and microbiological contaminants are also included. The results of analysis for 5 lots of *Clostridium* protein considered representative of the commercial material verify that the ingredient can be manufactured in conformance with the compositional and contaminant specifications, and that acceptable lot to lot variability can be achieved. *Clostridium* protein comprises a minimum of 80 g/100 g of protein and maximum of 3 g/100 g of fat, 5 g/100 g of carbohydrates, 6 g/100 g of ash and 10 g/100 g of moisture. The amino acid composition, mineral content and vitamin profile of *Clostridium* protein were also determined analytically. Further analysis revealed that only low levels of residual fermentation metabolites (organic acids and 2,3-butanediol) were present in *Clostridium* protein after separation of the cells. No biogenic amines were identified at amounts which might pose a safety concern and the absence of any detectable levels of mycotoxins was confirmed.

A stability study is ongoing to establish a shelf-life for *Clostridium* protein stored unopened in the original packaging at ambient temperature (<25°C) in the absence of excessive moisture or direct

sunlight. The interim results of the study showed that storage of *Clostridium* protein for 3-months at 25°C and 60% relative humidity (RH) in bags representative of the commercial packaging, was not associated with any detrimental changes in organoleptic properties, composition, microbiological parameters or levels of biogenic amines.

The food uses of *Clostridium* protein include meat and poultry analogs, dairy analogs, prepared meals, meal replacement products, milk and non-milk-based nutritional beverages, vegetable and fruit-based drinks, baked goods, breakfast cereals, soups, dressings and sauces at levels ranging from 1 to 40% by weight of the RTE or RTD products. In protein powders for formulation into beverages using milk or water for consumption as a supplemental protein source in the diet, use levels will vary up to a maximum of 90% by weight in the powder. Under the proposed conditions of use, male teenagers were determined to have highest mean and 90th percentile consumer-only intakes of *Clostridium* protein on an absolute basis, at 14.7 and 33.2 g/person/day, respectively. On a body weight basis, infants and young children were identified to have the highest mean and 90th percentile consumer-only intakes of 600 and 1,370 mg/kg body weight/day, respectively.

Comparison of the estimated consumer-only intakes of *Clostridium* protein with the Recommended Daily Allowances (RDAs) for protein for different life stage groups, as well as consumption estimates for protein intakes in practice by the U.S. population (Berryman *et al.*, 2018), indicates that the ingredient has the potential to represent a substantive fraction of the daily protein requirements for individuals. In practice, *Clostridium* protein will be a direct replacement for animal-, fungal- and vegetable-based proteins such as *Fusarium* protein (or mycoprotein), casein and pea protein in the diet, and therefore, the ingredient will contribute to, but not alter, the total daily protein intakes from all sources by the U.S. population.

A weight of evidence approach can be applied to support the safety of *Clostridium* protein for the intended use as an ingredient in conventional foods and beverages, based on the following: (1) characterization data on the source microorganism; (2) compositional and *in vitro* digestibility data; (3) comparison of the amino acid sequence of the protein to other proteins known to be allergenic; and (4) toxicological testing using Superbrewed Food's product.

Assessment of the safety of the source microorganism can largely be based on the Pariza *et al.* (2015) decision tree for microbial cultures. Although the guidelines are primarily envisaged to assess the safety of microbial cultures for use in fermented food and feed production, or for probiotic use, the principles and concepts described therein can be applied to *C. tyrobutyricum* as a protein source. One notable difference between microbial cultures for the production of fermented foods or probiotics, and those used as protein sources, is the absence of viable cells in the food ingredient. Thus, elements of the decision trees developed by Pariza and Johnson (2001) and Pariza and Cook (2010) for the assessment of enzyme preparations, which rarely contain viable cells from the source, can be applied to *Clostridium* protein.

C. tyrobutyricum occurs naturally in dairy products. There are no reports in the published literature associating *C. tyrobutyricum* with toxin production or pathogenicity in humans or animals. The findings of the genome-wide analysis and physiological evaluation of *C. tyrobutyricum* ASM#19 are consistent with the published literature in which no reports of the species being associated with pathogenicity or

toxigenicity in humans or animals were identified. Taken together, it may be concluded that *C. tyrobutyricum* ASM #19 does not pose a safety concern when used in the manufacture of *Clostridium* protein for use as a food ingredient.

The quality of a protein varies between sources and is principally defined by its amino acid composition and digestibility (Institute of Medicine; IOM. 2019). Besides the protein content, the nutritional value of *Clostridium* protein is also affected by its chemical composition (i.e., nucleic acids, minerals, vitamins and organic acids contents). Comparison of the amino acid profile of *Clostridium* protein with FAO reference values indicates that the ingredient will contribute to, but not adversely impact, essential amino acid intakes from the diet under the conditions of intended use as a direct replacement for animal- and vegetable-derived protein. The *in vitro* digestibility of *Clostridium* protein was high at 96.4% and the % PDCAAS was 75 based on the reference pattern for 2 to 5 year old children (FAO, 1991), 81 based on the updated reference pattern for young children, and 101 based on the updated reference pattern for older children, adolescents and adults (FAO, 2013). Taken together, these data indicate that *Clostridium* protein is a good quality protein source that is not expected to be nutritionally disadvantageous when used as a direct replacement for existing animal-, fungal- and vegetable-based proteins in the specified range of conventional foods and beverages.

An important safety consideration in the production of microbial proteins is the high nucleic acid content and the potential elicitation of gout and kidney stones due to the ingestions of purine compounds from the breakdown of ribonucleic acid (RNA) in the gastrointestinal (GI) tract which increases uric acid concentrations in the blood (PAG, 1974; Gieseke *et al.*, 1982; Jonas *et al.*, 2001). Nucleic acids occur widely in vegetable-, fungal- and animal-derived foods with typical servings of calf liver, chestnut mushrooms and broccoli estimated to provide in the region of 0.49, 0.23 and 0.72 g/serving, respectively. By comparison, a typical serving of non-dairy cheese containing 20% *Clostridium* protein will provide up to 0.24 g/serving which is in the region of that of a serving of broccoli.

Under the proposed conditions of use of *Clostridium* protein, the highest mean and 90th percentile consumer-only intakes were estimated to be 14.7 and 33.2 g/person/day, respectively. These intakes equate to a nucleic acids exposure of 0.59 and 1.33 g/person/day, respectively for *Clostridium* protein containing the maximum amount of nucleic acids of 4 g/100 g. In terms of overall contribution, similar to other microbial proteins, *Clostridium* protein has the potential to increase exposure to nucleic acids when substituting for conventional meat or dairy products in the diet. However, this contribution is not expected to be greater than that of the existing fungal protein counterparts currently on the market.

An assessment of the mineral profile of *Clostridium* protein was conducted by comparing the estimated intakes of each element under the conditions of intended use against the Adequate Intakes (AIs) and RDAs established by the IOM (2019). Overall, *Clostridium* protein was determined to make a significant contribution to daily requirements for manganese, molybdenum and selenium but exposure is not expected to be detrimental from a nutritional or safety perspective. Likewise, analysis of the B vitamin content of *Clostridium* protein indicates that the ingredient has the potential to be a high source of vitamin B12 under the conditions of intended use but intakes will not be nutritionally disadvantageous.

Additionally, it was recognized that residual amounts of butyrate, acetate and lactate carrying over into *Clostridium* protein from the fermentation process are expected to fall well below the levels of these

organic acids and their salts used as additives in food in the U.S. Thus, no safety concerns were anticipated from the presence of low levels of these fermentation metabolites in the food ingredient.

The allergenic potential of *Clostridium* protein was evaluated by *in silico* methods using the criteria described by Codex Alimentarius Commission on foods derived from biotechnology (Codex, 2009; Goodman *et al.*, 2008). The results of protein analysis support that *Clostridium* protein does not pose a realistic risk of food allergy to consumers. These findings are consistent with the low oral allergenic risk generally associated with microbial species.

Although the available evidence on the microbial source indicates it is non-pathogenic and non-toxicogenic, there is insufficient body of knowledge of *Clostridia* species or products derived thereof, in food. On this basis, toxicological testing of *Clostridium* protein was warranted.

A battery of toxicity tests were conducted using *Clostridium* protein, specifically a dose-range-finding (DRF) study in rats, a 90-day dietary feeding study in rats and two *in vitro* genotoxicity assays. The methodology used for the toxicity assessment of *Clostridium* protein is consistent with the general principles laid down in the U.S. FDA Redbook Chapter III for the assessment of food ingredients. The findings of these studies are published and provide pivotal evidence of the safety of the ingredient for the intended use in conventional foods and beverages (Jonaitis *et al.*, 2022).

The 90-day feeding study was conducted in male and female rats provided 0 (control), 5.0, 7.5 or 10.0% *Clostridium* protein in the diet as a partial replacement for casein. Comparable growth performance was observed among dietary treatment groups consistent with *Clostridium* protein providing a digestible and high-quality protein source for rats. Based on the results of this study demonstrating an absence of any adverse effects related to inclusion of *Clostridium* protein in the diet, the highest treatment level of 10.0%, corresponding to 5,558 and 6,671 mg/kg body weight/day for male and female rats, respectively was determined to be the NOAEL.

Under the conditions of intended use of *Clostridium* protein in conventional foods and beverages, on a body weight basis, infants and young children were determined to have the highest mean and 90th percentile consumer-only intakes of 600 and 1,370 mg/kg body weight/day, respectively. The amount of *Clostridium* protein fed to female rats in the 90-day dietary feeding study was 4-fold higher than the highest 90th percentile intakes estimated from the proposed food uses of the ingredient.

The nucleic acid content of the lot of *Clostridium* protein used in the 90-day dietary feeding study was determined analytically to be 2.7 g/100 g. Exposure to nucleic acids by rats consuming diets containing 10% *Clostridium* protein was estimated to be 149 and 179 mg/kg body weight/day in male and female rats, respectively. As mentioned above, under the conditions of intended use, infants and young children were determined to have the highest mean and 90th percentile consumer-only intakes of 600 and 1,370 mg/kg body weight/day, respectively of *Clostridium* protein, equating to 24 and 55 mg nucleic acids/kg body weight/day for an ingredient containing the maximum amount of 4 g nucleic acids/100 g. The amount of nucleic acids from *Clostridium* protein fed to female rats in the 90-day study was 3-fold higher than the highest 90th percentile intakes estimated from the proposed food uses of the ingredient.

Genotoxicity studies are not normally conducted on novel proteins, but considering the microbial source and the absence of any established history of use of *C. tyrobutyricum* or products derived thereof, as

food ingredients, evaluation of the genotoxic potential was considered pertinent to the safety evaluation. Consistent with the WGS and bioinformatics analysis, the results of the *in vitro* genotoxicity tests demonstrate that *Clostridium* protein is non-genotoxic.

It is generally recognized that the standard battery of testing in animals has limitations when applied to macronutrients on the basis that there are practical limitations in deriving NOAELs or tolerability limits. The concentration of the test item generally cannot be incorporated into the diet at sufficiently high levels to derive the conventional 100-fold safety factor allowing for intra- and inter-species variation without resulting in nutritional imbalances which can lead to secondary consequences such as adverse physiological effects (Borzelleca *et al.*, 1996; Munroe *et al.*, 1996; EFSA, 2011b). The findings of the 90-day dietary feeding study in rats was therefore, considered together with characterization data on the strain, the nutritional properties of the ingredient, *in vitro* digestibility data and allergenic potential, to provide a weight of evidence assessment of the safety of *Clostridium* protein under the conditions of intended use.

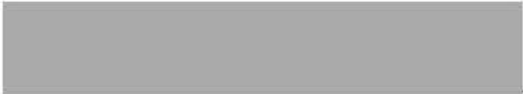
Following a critical evaluation of the data and information summarized above, it can be concluded that *Clostridium* protein manufactured by Superbrewed Food using suitable food-grade materials in accordance with cGMP and meeting appropriate food-grade specifications, is safe and suitable for the intended use as a source of protein in the range of specified conventional foods and beverages. It is further concluded that *Clostridium* protein is GRAS for the intended use in food based on scientific procedures.

CONCLUSIONS

We, the undersigned independent qualified members of the Expert Panel, have independently and collectively critically evaluated the data and information summarized above and conclude that *Clostridium* protein manufactured by Superbrewed Food in accordance with cGMP and meeting appropriate food-grade specifications as presented in the supporting dossier [Documentation to Support the Generally Recognized As Safe (GRAS) Status of *Clostridium* Protein for Use in Foods], is safe and suitable for use as an ingredient in specified food and beverage products.


We further unanimously conclude that the proposed use of *Clostridium* protein manufactured in accordance with cGMP and meeting food-grade specifications is GRAS based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.



Professor Eric Johnson
University of Wisconsin-Madison


5/15/2022
Date



Professor Kelly Swanson
University of Illinois, Urbana-Champaign

05/16/2022

Date



Dr. Ashley Roberts
AR Toxicology, Inc.

05/12/2022

Date

Superbrewed Food, Inc.
April, 2022

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DEPARTMENT OF HEALTH AND HUMAN SERVICES Food and Drug Administration GENERALLY RECOGNIZED AS SAFE (GRAS) NOTICE (Subpart E of Part 170)	Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022 (See last page for OMB Statement)	
	FDA USE ONLY	
	GRN NUMBER 001129	DATE OF RECEIPT Dec 14, 2022
	ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
	NAME FOR INTERNET	
KEYWORDS		

Transmit completed form and attachments electronically via the Electronic Submission Gateway (*see Instructions*); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission (<i>Check one</i>)	
<input checked="" type="checkbox"/> New	<input type="checkbox"/> Amendment to GRN No. _____
	<input type="checkbox"/> Supplement to GRN No. _____
2. <input checked="" type="checkbox"/> All electronic files included in this submission have been checked and found to be virus free. (<i>Check box to verify</i>)	
3. Most recent presubmission meeting (<i>if any</i>) with FDA on the subject substance (<i>yyyy/mm/dd</i>): <u>2022/06/29</u>	
4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? (<i>Check one</i>)	
<input type="checkbox"/> Yes	If yes, enter the date of communication (<i>yyyy/mm/dd</i>): _____
<input type="checkbox"/> No	

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Bryan P. Tracey	Position or Title CEO		
	Organization (<i>if applicable</i>) Superbrewed Food, Inc.			
	Mailing Address (<i>number and street</i>) 239 Lisa Drive			
City New Castle		State or Province Delaware	Zip Code/Postal Code 19720	Country United States of America
Telephone Number 1 (864) 921 5146		Fax Number	E-Mail Address btracy@superbrewedfood.com	
1b. Agent or Attorney (<i>if applicable</i>)	Name of Contact Person Elizabeth Lewis	Position or Title Scientific & Regulatory Advisor		
	Organization (<i>if applicable</i>) NutraSteward Ltd.			
	Mailing Address (<i>number and street</i>) Frederick House, Johnston			
City Haverfordwest		State or Province Pembrokeshire	Zip Code/Postal Code SA62 3AQ	Country United Kingdom
Telephone Number 44(0)7847301171		Fax Number	E-Mail Address elizabeth.lewis@nutrasteward.com	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Clostridium protein

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media _____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)*

☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))*

☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? *(see 21 CFR 170.225(c)(8) and 170.250(d) and (e))*

☐ Yes *(Proceed to Item 8)*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Clostridium protein is intended for use as a source of protein in meat and poultry analogs, dairy analogs, prepared meals, meal replacement products, milk and non-milk-based nutritional beverages, vegetable and fruit-based drinks, baked goods, breakfast cereals, soups, dressings and sauces at levels ranging from 1 to 40% by weight of the ready-to-eat (RTE) or the ready-to-drink (RTD) products. In protein powders for formulation into beverages using milk or water for consumption as a supplemental protein source in the diet, use levels will vary up to a maximum of 90% by weight in the powder.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes

☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes

☐ No, you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

- ☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- ☒ PART 3 of a GRAS notice: Dietary exposure (170.235).
- ☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- ☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- ☒ PART 6 of a GRAS notice: Narrative (170.250).
- ☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☒ Yes ☐ No

Did you include this other information in the list of attachments?

☒ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Dr. Bryan Tracey

(name of notifier)

has concluded that the intended use(s) of Clostridium protein

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Superbrewed Food, Inc. agrees to make the data and information that are the basis for the
(name of notifier) conclusion of GRAS status available to FDA if FDA asks to see them;
agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

239 Lisa Drive, New Castle, DE 19720

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official,
Agent, or Attorney

Elizabeth Lewis

Digitally signed by Elizabeth Lewis
Date: 2022.12.14 16:01:17 -04'00'

Printed Name and Title

Dr. Elizabeth Lewis, Scientific & Regulatory Advisor

Date (mm/dd/yyyy)

12/07/2022

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRAS Notice_Clostridium Protein_2022-1207.pdf	Submission
	RedactedbySubmitter_GRAS Notice_Clostridium Protein_2022-1207.pdf	Submission
	App A_Clostridium Protein_Consensus Statement_2022-1207.pdf	Submission
	RedactedbySubmitter_App A_Clostridium Protein_Consensus Statement_2022-1207.pdf	Submission

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, PRASStaff@fda.hhs.gov. (Please do NOT return the form to this address). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.