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

[2]Calculation: 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 *Clostridium* Protein, Whey Protein, Casein and Pea Protein Isolate

Amino Acid	g/100 g of Product						Adult Requirement[3]
	Clostridium 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)
Histidine	***1.4***	***1.4***	***2.2***	***1.6***	***0.4***	***2.9***	***1.5***
Isoleucine	***6.3***	***3.8***	***3.0***	***2.3***	***0.6***	***4.9***	***3.0***
Leucine	***6.7***	***8.6***	***7.8***	***5.7***	***1.0***	***8.6***	***5.9***
Lysine	***9.0***	***7.1***	***5.9***	***4.7***	***0.9***	***7.1***	***4.5***
Methionine	***2.6***	***1.8***	***2.2***	***0.3***	***0.2***	***1.3***	***1.6***
Cysteine[4]	***0.7***	***0.8***	***0.1***	***0.2***	***-***	***0.3***	***0.6***
Phenylalanine	***3.8***	***2.5***	***3.1***	***3.7***	***0.5***	***6.9***	***3.8***
Tyrosine[4]	***3.2***	***2.4***	***3.4***	***2.6***	***0.2***	***3.2***	
Threonine	***4.2***	***5.4***	***3.5***	***2.5***	***0.6***	***12.3***	***2.3***
Tryptophan	***0.7***	***-***	***-***	***-***	***0.2***	***0.95***	***0.6***
Valine	***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 in **bold** are the essential amino acids (IOM, 2005);

[1]Mean 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;

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

[3]Based 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;

[4]Considered 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 *Clostridium* 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)];
[1]Calculated 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);
[2]Calculated 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:

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

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

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 *Clostridium* Protein (FAO, 1991)

Essential Amino Acids for Human Nutrition	*Clostridium* Protein Total Amino Acid Content (mg/g protein)[1]	FAO/WHO Recommended Values (mg/g protein; 1991)[2]	Calculated Amino Acid Scores for *Clostridium* 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 *Clostridium* Protein (FAO, 1991)			
Essential Amino Acids for Human Nutrition	***Clostridium* Protein Total Amino Acid Content (mg/g protein)[1]**	**FAO/WHO Recommended Values (mg/g protein; 1991)[2]**	**Calculated Amino Acid Scores for *Clostridium* 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;
[1]Amino acid profile of Lot DNII 210225 used in the *in vitro* digestibility study;
[2]FAO 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;
[3]Calculation: (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 *Clostridium* Protein (FAO, 2013)					
Essential Amino Acids for Human Nutrition	***Clostridium* 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 *Clostridium* Protein[3]**	**FAO/WHO Recommended Values (mg/g protein; 2013) for Older Children, Adolescents and Adults[2]**	**Calculated Amino Acid Scores for *Clostridium* 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;
[1]Amino acid profile of Lot DNII 210225 used in the *in vitro* digestibility study;

[2]FAO 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;
[3]Calculation: (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]	-
Clostridium protein	***72 (tryptophan)***	***101 (histidine)***

Abbreviations: FAO = Food and Agricultural Organization; WHO = World Health Organization;
[1]Based on reference pattern for 6 month old infants to 3 year-old children (used to score foods for older children, adolescents and adults);
[2]Taken from the Joint FAO/WHO Expert Consultation on Protein Quality Evaluation (1991);
[3]As 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 protein[3].

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).

Table 6.6: Examples of the RNA and DNA Content of Selected Foods (Taken from: Lassek and Montag, 1990; Jonas *et al.*, 2001)

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%.

[1]Calculation: 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% Clostridium 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;
[1]DM 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;
[2]Calculation: (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]
Meat substitute containing 40% Clostridium protein	*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.
[1]DM 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;
[2]Calculation: (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 acids[4] 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 protein[6]. 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% Clostridium 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 *Clostridium* Protein by Male Teenagers

Mineral	AIs or RDAs (mg/day)[1]	ULs (mg/day)	Mineral Intakes from *Clostridium* 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;

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

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

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

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

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

[6]The 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 source[7] 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% Clostridium 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 *Clostridium* Protein by Male Teenagers

Vitamin	RDAs[1]	Estimated Intake[2]	% Contribution to AI or RDA[3]	Estimated Intake[4]	% Contribution to AI or RDA[5]
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;
[1]RDAs as reported by the IOM (2011c);
[2]Calculated as: mean vitamin content (mg/100g or µg/100g; see Part 2) x (14.7 g/100 g);
[3]Calculated 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;
[4]Calculated as: mean vitamin content (mg/day or µg/day; see Part 2) x (33.2 g/100 g);
[5]Calculated 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 source[8] 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)
Clostridium 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 $1x10^{-7}$ 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 though 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 *Clostridium* Protein-Containing Treatment Diets

Crude *Clostridium* 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 *Clostridium* Protein-Containing Treatment Diets

Crude *Clostridium* 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 (FOB[9]; 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 count[10] was 0.52 x 10^9/L ± 0.22 (0.3 to 0.85; based on N=426). For monocyte counts, the historical values[11] were 0.072 x 10^9/L ± 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 x 10^9/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 x 10^9/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 x 10^9/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 x 10^9/L ± 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 x 10^9/L ± 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 µmol/L, respectively) remained within the reference range for this strain, sex, and age of rat (2.9 µmol/L ± 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 *Clostridium* Protein-Containing Treatment Diets

Crude *Clostridium* 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 *Clostridium* Protein			
Study	**Design**	**Concentrations**	**Findings**
Bacterial reverse mutation assay [GLP; OECD TG 471]	*Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and *Escherichia coli* tester strain WP$_2$*uvrA*-pKM101 Metabolic activation: +/- S9 Treat and wash modification	Expt 1: 0, 52, 164, 512, 800, 1,600, 5,000 µg/plate	Tester train WP$_2$*uvrA*-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	*Clostridium* protein was non-mutagenic under the conditions of the test
In vitro 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	*Clostridium* 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 µmol/L ± 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.