From: Elizabeth Lewis
To: Kampmeyer, Christopher
Subject: [EXTERNAL] Re: GRN 001129: Sept. 6, 2023 Meeting Memo and Questions
Date: Tuesday, October 24, 2023 7:22:57 PM
Attachments: image001.png
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image018.png
Superbrewed Food_Clostridium_FDA Responses_10242023.pdf
FDA_Supplementary Info_Clostridium Protein_10242023.pdf

Dear Chris,

Many thanks for the email and for the helpful advice below. Please find attached supplementary information to address the questions raised by the FDA. A copy of the references can be provided if required. We have tried to provide comprehensive responses and remain at your disposal to address any further questions. Do not hesitate to come back to me if anything else is required.

Best regards,
Elizabeth

Elizabeth Lewis, PhD
Scientific & Regulatory Adviser
E: elizabeth.lewis@nutrasteward.com

NutraSteward
www.nutrasteward.com

On Tue, 10 Oct 2023 at 21:37, Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Hi Elizabeth,
>
> As an addition to my previous message—I would like to stress that if you consider previous GRNs in your response, please make sure to go through any amendments, which are considered part of the notice. These amendments usually contain our main line of questioning and how previous notifiers have tried to address them.

Best regards,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

From: Kampmeyer, Christopher
Sent: Tuesday, October 10, 2023 4:29 PM
To: Elizabeth Lewis <elizabeth.lewis@nutrasteward.com>
Subject: GRN 001129: Sept. 6, 2023 Meeting Memo and Questions

Dear Elizabeth,

I am writing to provide a memo copy for our meeting on September 6, 2023. Questions for the notifier are included within as an attachment.

We respectfully request a response to these questions within 10 business days. Please do not include any confidential information in your responses. Thank you in advance for your attention to our comments.

Thank you,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

Memorandum

Date: September 6, 2023, 1:30 pm – 2:30 pm ET

Location: ZoomGov

Participants:

NutraSteward Ltd.

Elizabeth Lewis	Scientific & Regulatory Advisor

Superbrewed Food, Inc.

Bryan Tracy	CEO and Co-founder
Carissa Wiedel	Research and Development

FDA/CFSAN/OFAS/DFI

Chris Kampmeyer	Regulatory Review Scientist
Shayla West-Barnette	Regulatory Review Team Lead
Mical Honigfort	Regulatory Review Branch Chief
Perry Wang	Chemistry Reviewer
Ying Li	Chemistry Reviewer
Hem Thapa	Chemistry Reviewer
Renata Kolanos	Chemistry Team Lead
Diana Doell	Chemistry Branch Chief
Anne Macgregor-Das	Toxicology Reviewer
Kotaro Kaneko	Toxicology Team Lead

Subject: Summary of teleconference to discuss GRAS Notice 001129

FDA (we) requested this teleconference with NutraSteward Ltd. (NutraSteward) and Superbrewed Food, Inc. (Superbrewed Food) to provide an overview of issues and questions we identified during our review of GRN 001129, describing the intended use of heat-killed *Clostridium tyrobutyricum* strain ASM#19. We discussed questions and concerns related to: whether the intended use includes foods with standards of identity; proposed specifications for the ingredient; mycotoxins for which the batches of the ingredient were tested; the sources of the enzymes intended for use as processing aids; estimates of dietary exposure for the U.S. population ages 2 years and older at the mean and 90th percentile; the allergenicity and tolerability of the ingredient; the bioinformatics analysis of the ingredient; and other deficiencies requiring clarification. To address these questions, we recommended that Superbrewed Food consider the GRAS conclusions of similar novel proteins for which we had no questions.

We noted that if Superbrewed Food is unable to address our questions within an appropriate

timeframe (10 business days), then we would recommend that Superbrewed Food request that we cease to evaluate GRN 001129. We noted that we would provide a list of deficiencies that were identified during the evaluation of the notice.

Christopher P. Kampmeyer -S

Digitally signed by Christopher P. Kampmeyer -S
Date: 2023.10.10 16:27:55 -04'00'

Christopher Kampmeyer
Regulatory Review Scientist

CC: GRN 001129

Attachments:

1) GRN 001129 Questions for the Notifier

GRN 001129 Questions for the Notifier

After reviewing the GRAS notice (GRN 001129) submitted by Superbrewed Food ("the notifier") regarding the intended use of heat-killed *Clostridium tyrobutyricum* strain ASM#19, we note the following questions for Superbrewed Food. We respectfully request a response to these questions within 10 business days.

Chemistry

1. Please confirm that the ingredient is not intended for use in foods where standards of identity preclude its use.
2. In Table 2.5 (page 20), the proposed specification limit for lead of ≤ 0.3 mg/kg is higher than levels of lead that we typically see in ingredients manufactured through controlled fermentation and in accordance with current good manufacturing practices. Furthermore, we note that in Table 2.6 (page 21), the levels of lead from the analyses of batches of the ingredient range from 0.023 mg/kg to 0.24 mg/kg. Please specify the source(s) of lead and discuss the observed variability of the lead levels in the final ingredient. We would also like to remind you about FDA's "Closer to Zero" initiative that focuses on reducing dietary exposure to heavy metals.
3. In Table 2.6, (page 21), we note that some of the results for heavy metals are reported as "<" [a value]. Please provide the limit of quantitation (LOQ) and limit of detection (LOD) for the analytical method(s) used to analyze the batches for heavy metals.
4. On page 26, you state, "with the exception of deoxynivalenol (DON) at 10 μg/kg in Lot DNII210401, no mycotoxins were identified above detection limits in any of the lots tested." Please specify other mycotoxins that the batches were tested for and provide the LOD for the analytical method(s) used to test for mycotoxins.
5. In Table 2.3 (page 16), alpha-amylase and glucoamylase are listed as processing aids. Please provide the source(s) of the enzymes and clarify whether the enzymes are present in the final ingredient.
6. In Tables 3.1 and 3.2 (pages 28-29), the estimates of dietary exposure are provided for several populations, including the total population. In addition to the provided estimates, please provide eaters-only estimates of dietary exposure for the US population aged 2 years and older at the mean and 90th percentile.

Toxicology

1. On p.39-40, you present the calculation of amino acid scores for *Clostridium* protein. In Table 6.3, the calculated amino acids scores for methionine + cysteine as well as tryptophan do not appear to be correct. Please confirm whether there is an error in either the *Clostridium* protein total amino acid content or the FAO/WHO recommended values. For example, it appears that the FAO/WHO Recommended value of 2 mg/g protein may be incorrect in this table. Likewise, in Table 6.4 it appears that the calculated amino acids score for tryptophan is incorrect. Please clarify this calculation.

2. You stated on p. 52 that the 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%. You also stated that the sequence

identity values noted for *C. tyrobutyricum* were much higher to other microbial proteins from sources not known to be allergenic than to established allergens. Please provide more information on the identified *C. tyrobutyricum* proteins with homologies to known allergens (such as names and/or list of the accession numbers and percent identity) with a short explanation as to why these identified proteins are not a safety concern. If it would help to further establish safety, please also include the functions of the identified microbial proteins.

3. We note that *Clostridium* protein is a novel microbial protein. You have provided a published bioinformatics evaluation of the potential allergenic risk of *Clostridium* protein; however, there appears to be a lack of human consumption data with this ingredient to support tolerability. Given that *Clostridium* protein will be used as a direct protein replacement, and at levels as high as 33g/p/d, please provide a narrative justifying why no human consumption data is needed to mitigate concerns pertaining to tolerability and allergenicity.

4. We note that historically, the specification for nucleic acids has been less than 2g/100g. In the safety narrative, you summarize an unpublished dose-range finding study in rats using a crude *Clostridium* protein preparation, where the nucleic acid content was analyzed and found to be 7.8g/100g. There were noted adverse findings, such as swelling of the hindlimbs with inflammation and edema noted upon microscopic evaluation, that were suggestive for gouty arthritis or synovitis, which were attributed to the high nucleic acid content. In the published 90-day dietary feeding study using a heat-treated *Clostridium* protein preparation containing 2.7g nucleic acids/100g, these adverse effects were not observed.

    a. Please provide additional narrative to support your conclusion that the nucleic acid specification of 4g/100g is adequate to ensure safety given your subchronic toxicological study where no adverse effects were noted was performed with a preparation containing just 2.7g nucleic acids/100g, substantially below your specified limitation.
    b. On p. 63 of the notice, you appear to reference the FAO/WHO/UNICEF Protein Advisory Group recommendation for a maximum daily intake of ≤ 2g/day of nucleic acids from single-cell protein foods, regardless of whether the biomass is bacterial or fungal-derived.[1,2,3] You also note that the 90th percentile intakes for nucleic acids for *Clostridium* protein containing the maximum amount of nucleic acids would be 1.33g/p/d. As it appears the Protein Advisory Group's recommendation on nucleic acids levels from single-cell proteins is a pivotal argument for the safety of your GRAS conclusion, please discuss the data used by

[1] Calloway, D. et al., 1969. Safety of single-cell proteins as evaluated by human feeding trials at the University of California, Berkeley. *Proceedings of the 16th meeting of the FAO/WHO/UNICEF Protein Advisory Group, Geneva Switzerland*; 8-11 September 1969; p.8-11.
[2] Coelho, M. et al., 2020. Short-Communication: Ingestion of a nucleotide-rich mixed meal increases serum uric acid concentrations but does not affect postprandial blood glucose or serum insulin responses in young adults. *Nutrients*; 12(4): 1115.
[3] Edozien, J. et al., 1970. Effects of high levels of yeast feeding on uric acid metabolism of young men. *Nature*; 228: 180.

the FAO/WHO/UNICEF Group to reach their conclusion on nucleic acid levels in the diet from single-cell proteins.

NutraSteward

Providing regulatory support for food and feed ingredients

October 24, 2023

Susan J. Carlson, Ph.D., Director
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety (HFS-200)
5100 Campus Drive
College Park, MD 20740

Dear Dr. Carlson,

Re: GRAS Notice 001129

In follow-up to the questions on the GRAS notice for heat-killed *Clostridium tyrobutyricum* strain ASM#19 received from the U.S. FDA on October 10, 2023, please find Superbrewed Food Inc.'s responses enclosed.

We trust that this meets with your immediate needs and remain at your disposal to provide any further information required.

Sincerely,

Elizabeth Lewis, PhD
Scientific & Regulatory Advisor
NutraSteward, Ltd.

NutraSteward, Ltd.
Registered office: Frederick House, Hayston View,
Johnston, Pembrokeshire SA62 3AQ
www.nutrasteward.com

Tel: 44(0)7847301171
Email: info@nutrasteward.com

Supplementary Information for GRAS Notice 001129

1. CHEMISTRY

Question 1

Please confirm that the ingredient is not intended for use in foods where standards of identity prelude its use.

Supplementary Information

In the notice, Superbrewed Food states its intention to use heat-killed *Clostridium tyrobutyricum* strain ASM#19 in several food categories, including foods for which standards of identity exist under Title 21 of the Code of Federal Regulations (21 CFR). Superbrewed Food confirms that the ingredient will only be added to foods if it is permitted by the applicable standard of identity.

Question 2

In Table 2.5 (page 20), the proposed specification limit for lead of ≤ 0.3 mg/kg is higher than levels of lead that we typically see in ingredients manufactured through controlled fermentation and in accordance with current good manufacturing practices. Furthermore, we note that in Table 2.6 (page 21), the levels of lead from the analyses of batches of the ingredient range from 0.023 mg/kg to 0.24 mg/kg. Please specify the source(s) of lead and discuss the observed variability of the lead levels in the final ingredient. We would also like to remind you about FDA's "Closer to Zero" initiative that focuses on reducing dietary exposure to heavy metals.

Supplementary Information

The data provided in the GRAS notice was on lots manufactured at pilot-scale using food-grade raw materials. Although food-grade corn was used as the substrate for fermentation, it has subsequently been shown that the levels of lead can vary between corn sources resulting in the variability in lead content displayed by heat-killed *C. tyrobutyricum* strain ASM#19.

For commercial production, Superbrewed Food will monitor the lead levels in food-grade corn as received and prior to use in the production process and establish an internal raw material specification that result in a lead content of heat-killed *C. tyrobutyricum* strain ASM #19 of less than 0.1 mg/kg will be used. A revised specification for lead in heat-killed *C. tyrobutyricum* strain ASM #19 of less than 0.1 mg/kg is proposed by Superbrewed Food consistent with the specifications set for fungal proteins previously notified for use as ingredients in food under similar conditions of use, i.e., *Fusarium venenatum* protein (GRN. No. 91; U.S. FDA, 2002), fungal protein from fermented *Fusarium* sp. mycelium (GRN No. 904; U.S. FDA, 2021) and fungal protein from *Fusarium* sp. mycelia (GRN No. 945; U.S. FDA, 2022). The levels of lead reported in 3 of the 5 analytical lots of heat-killed *C. tyrobutyricum* strain ASM#19 provided in Table 2.6 of the GRAS notice fall below 0.1 mg/kg confirming that the ingredient as manufactured can conform to the revised specification limit.

Question 3

In Table 2.6, (page 21), we note that some of the results for heavy metals are reported as "<" [a value]. Please provide the limit of quantitation (LOQ) and limit of detection (LOD) for the analytical method(s) used to analyze the batches for heavy metals.

Supporting Information

The results presented as "<" [a value] for heavy metals in Table 2.6 refers to values that were below the limit of quantification (LOQ). The LOQ for lead, cadmium, arsenic, and mercury was 0.010 mg/kg for the analytical method used to test these heavy metals in heat-killed *C. tyrobutyricum* strain ASM#19. The test method uses Inductively Coupled Plasma/Mass Spectrometry (ICP-MS) and the limits of detection (LOD) of the instrument for lead, cadmium, arsenic, and mercury were 0.3, 0.5, 0.5 and 1 μg/L.

Question 4

On page 26, you state, "with the exception of deoxynivalenol (DON) at 10 μg/kg in Lot DNII210401, no mycotoxins were identified above detection limits in any of the lots tested." Please specify other mycotoxins that the batches were tested for and provide the LOD for the analytical method(s) used to test for mycotoxins.

Supplementary Information

Aflatoxin B1, B2, G1 and G2, as well as the sum of aflatoxins B1, B2, G1 and G2 were tested using United States Pharmacopoeia (USD) method 561. The results were below the LOQ of 5.0 μg/kg for each parameter tested.

15-Acetyldeoxynivalenol (15-ADON), 3-acetyldeoxynivalenol (3-ADON), deoxynivalenol (DON or vomitoxin) and diacetoxyscirpenol (DAS) were tested using Romer Microsep[1] (Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) with a LOQ of <20, <20, <10 and <50 μg/kg, respectively. The method used by the external testing laboratory is accredited in accordance with ISO/IEC 17025:2017 and A2LA 2993.01.

Question 5

In Table 2.3 (page 16), alpha-amylase and glucoamylase are listed as processing aids. Please provide the source(s) of the enzymes and clarify whether the enzymes are present in the final ingredient.

Supplementary Information

The enzymes used in the manufacturing process to heat-killed *C. tyrobutyricum* strain ASM#19 are presented in Table S-1. The enzymes are notified as GRAS for the hydrolysis of starch or starch polysaccharides in food manufacture and the conditions of use in the hydrolysis of the corn starch for the production of heat-killed *C. tyrobutyricum* strain ASM#19 are equivalent to these traditional food processing applications.

[1] https://www.romerlabs.com/en/mycotoxin-cleanup-columns

Table S-1: Sources of the Enzymes used in the Final Ingredient	
Enzyme	**Regulatory Status**
Alpha-amylase **Description** Liquozyme® Standard 2X (Novozymes) – blended alpha-amylase enzyme preparation produced by submerged fermentation of a selected strain of genetically modified strains of *Bacillus licheniformis*	GRAS for use as a processing aid in starch applications in food (GRN 24 and 27; U.S. FDA, 2002). **Statutory Basis for GRAS** GRAS for the intended use through scientific procedures. **Specifications** Food-grade enzyme preparation manufactured according to cGMP and complying with FCC and FAO/WHO JECFA recommended purity specifications for food-grade enzymes **GRN 24** Use in the starch industry (for the liquefaction of starch in the production of syrups) and in the alcohol industry (for thinning of starch in distilling mashes) as a processing aid at the minimum levels necessary to accomplish the intended technical effect in accordance with cGMP. Identity and Technical Effect Alpha-amylase enzyme preparation from *Bacillus licheniformis* expressing a gene encoding a modified alpha amylase from *Bacillus stearothermophilus*. The enzyme catalyzes the hydrolysis of starch or starch polysaccharides in food. **GRN 79** Use as a processing aid for the liquefaction of starch in the production of syrups and for the thinning of starch in distilling mashes in the production of alcohol. The alpha-amylase is used at the minimum levels necessary to achieve the intended effects. The recommended use level for the alpha-amylase enzyme preparation is 0.35 kg/ton of starch. Identity and Technical Effect Alpha-amylase enzyme preparation from *B. licheniformis* carrying a gene constructed from a modified *B. licheniformis* alpha-amylase gene and a portion of *B. amyloliquefaciens* alpha-amylase gene. **Relevance of the GRAS Status for the Production of Heat-Killed *C. tyrobutyricum* ASM #19** Alpha-amylase is used to act on starch derived from corn for use in production of a sugar syrup or mash as a feedstock for the fermentation of *C. tyrobutyricum* ASM#19. Superbrewed Food uses the enzymes at the recommended levels as specified by the manufacturer and described in the GRAS notices.
Glucoamylase **Description** Amylase™ AG 300 L (Novozymes) – glucoamylase enzyme produced by submerged fermentation of a	GRAS for use as a processing aid in starch applications in food (GRN 89; U.S. FDA, 2002). **Identity and Technical Effect** Carbohydrates enzyme preparation from *Aspergillus niger* and containing glucoamylase. The enzyme catalyzes the hydrolysis of starch or starch polysaccharides in food.

Table S-1: Sources of the Enzymes used in the Final Ingredient	
Enzyme	**Regulatory Status**
selected strain of *Aspergillus niger*	**Statutory Basis for GRAS** GRAS for the intended use through experience based on common use in food before 1958. **Specifications** Food-grade enzyme preparation manufactured according to cGMP and complying with FCC and FAO/WHO JECFA recommended purity specifications for food-grade enzymes **Relevance of the GRAS Status for the Production of Heat-Killed *C. tyrobutyricum* ASM #19** Glucoamylase is used to act on starch derived from corn with only unavoidable levels in the final ingredient. Superbrewed Food uses the enzymes at the recommended levels as specified by the manufacturer and supported by the GRAS status.

At the end of the fermentation, the *C. tyrobutyricum* cell mass is separated from the fermentation broth by microfiltration. The enzymes will be removed in the fermentation broth and subsequent washing steps to ensure that the levels in the dried cells are reduced by at least 200 times. Thus, only unavoidable residues of the enzymes will present in heat-killed *C. tyrobutyricum* strain ASM#19. Any residual enzymes present in heat-killed *C. tyrobutyricum* strain ASM#19 will be inactivated under the conditions of the production process which includes heat-treatment and contribute a negligible amount to the total protein content. In this respect, the residual levels of inactivated enzymes present in the food ingredient will be metabolized by humans by the same pathways as other protein sources and not pose a safety concern to consumers.

Question 6

In Tables 3.1 and 3.2 (pages 28-29), the estimates of dietary exposure are provided for several populations, including the total population. In addition to the provided estimates, please provide eaters-only estimates of dietary exposure for the US population aged 2 years and older at the mean and 90th percentile.

Supplementary Information

The consumer (eaters)-only estimates of dietary exposure for the U.S. population aged 2 years and older at the mean and 90th percentile are presented on absolute and body weight basis in Tables S-2 and S-3, respectively. On an absolute basis, mean and 90th percentile consumer-only intakes by the population 2-years of age and over were 10.8 and 26.1 g/day, respectively.

Table S-2: Summary of the Estimated Daily Intake of *Clostridium* Protein from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)					
Population Group	**Age Group (Years)**	**Consumer-Only Intake (g/day)**			
		%	**n**	**Mean**	**90th Percentile**
Population 2+ years	2 and up	83	5,720	10.8	26.1

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

On a body weight basis, mean and 90th percentile consumer-only intakes by the population 2-years of age and over were 0.20 and 0.50 g/kg body weight/day, respectively.

Table S-3: Summary of the Estimated Daily Per Kilogram Body Weight Intake of *Clostridium* Protein from Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)					
Population Group	**Age Group (Years)**	**Consumer-Only Intake (g/kg body weight/day)**			
		%	**n**	**Mean**	**90th Percentile**
Population 2+ years	2 and up	83	5,720	0.20	0.50

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

2. TOXICOLOGY

Question 1

On p.39-40, you present the calculation of amino acid scores for Clostridium protein. In Table 6.3, the calculated amino acids scores for methionine + cysteine as well as tryptophan do not appear to be correct. Please confirm whether there is an error in either the Clostridium protein total amino acid content or the FAO/WHO recommended values. For example, it appears that the FAO/WHO Recommended value of 2 mg/g protein may be incorrect in this table. Likewise, in Table 6.4 it appears that the calculated amino acids score for tryptophan is incorrect. Please clarify this calculation.

Supplementary Information

As noted by the U.S. FDA, the FAO/WHO recommended value for methionine +cysteine was incorrectly quoted as 2 mg/g protein rather than 25 mg/g protein. Additionally, the total amino acid content for tryptophan of heat-killed *C. tyrobutyricum* strain ASM#19 was listed as 82 mg/g protein rather than 8.2 mg/g protein. The only impact on the calculations is that the total amino acid score for heat-killed *C. tyrobutyricum* strain ASM#19 for methionine and cysteine should be 1.54. Table 6.3 has been updated to include the corrected values (adjustments highlighted in red).

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	80	66	1.22
Lysine	109	58	1.88
Methionine + cysteine	38	25	1.54
Phenylalanine + tyrosine	85	63	1.34
Threonine	50	34	1.48
Tryptophan	8.2	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).

Likewise, the value for total amino acid content for tryptophan of heat-killed *C. tyrobutyricum* strain ASM#19 was listed as 82 mg/g protein rather than 8.2 mg/g protein. This was a typographical error, and the value has been updated in Table 6.4. There is no impact on the calculated amino acid scores or overall conclusions.

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	8.2	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).

Question 2

You stated on p. 52 that the 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%. You also stated that the sequence identity values noted for C. tyrobutyricum were much higher to other microbial proteins from sources not known to be allergenic than to established allergens. Please provide more information on the identified C. tyrobutyricum proteins with homologies to known allergens (such as names and/or list of the accession numbers and percent identity) with a short explanation as to why these identified proteins are not a safety concern. If it would help to further establish safety, please also include the functions of the identified microbial proteins.

Supplementary Information

Further information on the evaluation of heat-killed *C. tyrobutyricum* strain ASM#19 protein sequences against the AllergenOne.org database is provided below.

The matches generated using BlastP comparison between amino acid sequences obtained from *C. tyrobutyricum* strain ASM#19 and the Allergen database are listed below. In considering the clinical relevance in each case, the strength of evidence for the allergen is considered briefly. The percentage and extent of match to the allergen is also considered: it can be inferred that there is minimal risk of

allergy where a *C. tyrobutyricum* protein matches housekeeping proteins, enzymes or chaperone proteins which are highly evolutionarily conserved in many species not known to be allergenic, to a greater extent than it matches the allergen.

Three *C. tyrobutyricum* proteins matched allergen: gi|27806257|gid|1565|Allergen collagen alpha-2(I) chain precursor [*Bos taurus*]. Two of these are identified as phage tail fiber protein, and the third as hypothetical protein formerly called flagellar hooklength control protein FliK.

The *Bos taurus* collagen protein match is considered an allergen due to basophil tests on human samples with claims of allergy to collagen in foods. However human collagen is approximately 65% identical to *Bos taurus* collagen over 1362 AA, compared with 54.4% identity of the *C. tyrobutyricum* sequence over only 114 AA, so this *Clostridium* protein represents no risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|94468818|gid|2708|Putative heat shock cognate 70 [*Aedes aegypti].* The *C. tyrobutyricum* protein was identified as chaperone protein DnaK – a housekeeping protein involved in protein folding. The mosquito heat shock protein 70 is a putative allergen based on 2 D immunoblot with 9 of 15 showing IgE binding, but not bioactivity. There are over 1,000 alignments of 77% identity or longer, full- length to this protein in public databases so the approximately 51% match over 630 amino acids to the heat-killed *C. tyrobutyricum* strain ASM#19 is unlikely to represent any risk of allergy.

Two C. *tyrobutyricum* proteins matched allergen: gi|171853012|gid|2371|Putative seed maturation-like protein precursor [*Sesamum indicum*]. Both *C. tyrobutyricum* proteins were identified as belonging to the oxidoreductase, short chain dehydrogenase/reductase family. The *Clostridium* protein has 49% identity to a putative sesame allergen, seed maturation like protein that was identified by 2D IgE binding and proteomics with limited sequence data and few patients. Based on public protein database, there are long alignments of the sesame protein to proteins from a wide variety of common foods at >70% identity. Heat-killed *C. tyrobutyricum* strain ASM#19 has 49 % match over 286 amino acids to the allergen, indicating it is not a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|328900101|gid|2846|Putative triosephosphate isomerase [*Procambarus clarkii*]. The *C. tyrobutyricum* protein was identified as triosephosphate isomerase (EC 5.3.1.1). The *Procambarus clarkii* protein was identified as an allergen for crawfish allergic people in China based on relatively weak IgE binding data. Triosephosphate isomerase sequences are also shared by other taxa, including a broad spectrum of non-allergenic species including primates and regularly consumed organisms. Based on public databases, there are over 100 alignments of this protein to a wide variety of proteins at 70% identity. The modest match of heat-killed *C. tyrobutyricum* strain ASM#19 at 47% over 247 amino acids is unlikely to represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|253783729|gid|2301|Putative glyceraldehyde-3-phosphate dehydrogenase (GADPH) [*Triticum aestivum*]. Heat-killed *C. tyrobutyricum* strain ASM#19 was identified as NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). This wheat enzyme was identified as a putative allergen in baker's asthma. The wheat GAPDH has high identity matches above 88% to GAPDH of many commonly consumed plants. The low match to the heat-killed *C.*

tyrobutyricum strain ASM#19 of 45.7% over 337 amino acids suggests this is unlikely to represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|385145180|gid|1959|Allergen enolase [*Salmo salar*]. The *C. tyrobutyricum* protein was identified as enolase (EC 4.2.1.11). The salmon enolase allergen is 98% identical to over 100 proteins from a wide variety of sources. Heat-killed *C. tyrobutyricum* strain ASM#19 is an enolase which has only a 50.9 % match over 430 amino acids to the salmon protein, but greater than 80% matching to many bacterial protein sequences. The weak similarity to the allergen suggests it is unlikely to be a risk for allergy.

One *C. tyrobutyricum* protein matched allergen: gi|225810599|gid|1206|Allergen Sal k 3 pollen allergen [*Salsola kali*]. The *C. tytobutyricum* protein was identified as 5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase (EC 2.1.1.14). The Sal k 3 pollen allergen was identified as binding IgE from 19 of 30 pollen allergic subjects when used as a purified protein in ELISA (757 AA long protein), and with strong skin prick test reactivity in weed pollen allergic subjects. However, the heat-killed *C. tyrobutyricum* strain ASM#19 only has a 44.4 % match over 780 amino acids, while the Sal k 3 protein has over 100 matches with > 88% identity to proteins of many plants. The weak similarity to the antigen suggests this protein is unlikely to represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|4138173|gid|651| Putative allergen [*Malassezia sympodialis*]. The *C. tyrobutyricum* protein was identified as Peptidyl-prolyl cistrans isomerase (EC 5.2.1.8). The *Malassezia* cyclophilin protein was identified as an IgE binding protein based on phage display and serum IgE binding, while its high level of conservation in many eukaryotic protein sources, including human cyclophilin, was noted. This *Malassezia* cyclophilin has over 100 matches with greater than 75% identity to proteins of many species. Heat-killed *C. tyrobutyricum* strain ASM#19 has 41.3 % match to the putative allergen over 150 amino acids. This represents no risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|1098871171|gid|2576| Putative enamine/imine deaminase [*Dermatophagoides farinae*]. Heat-killed *C. tyrobutyricum* strain ASM#19 was identified as UK114 superfamily protein. The house dust mite protein Der f 34, was identified as a putative allergen based on limited IgE binding ELISA data without biological activity. UK114 superfamily members are conserved across all domains of life. The dust mite protein matches 100 bacterial and mite species down to 48% identity, while heat-killed *C. tyrobutyricum* strain ASM#19 has only 40 % identity over 125 amino acids. This heat-killed *C. tyrobutyricum* strain ASM#19 match does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|91680609|gid|876|Putative thioredoxin [*Aspergillus fumigatus*]. The *C. tyrobutyricum* protein was identified as thioredoxin. The thioredoxin from *Aspergillus* was identified as a putative allergen based on limited IgE binding from patients allergic or with bronchopulmonary aspergillosis. Thioredoxin of *Aspergillus* has more than 100 matches to thioredoxins of many fungi with >55% identity, while the *C. tyrobutyricum* protein has only 37.7 % identity over 106 amino acids. This heat-killed *C. tyrobutyricum* strain ASM#19 match does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|94468818|gid|2708|Putative heat shock cognate 70 [*Aedes aegypti*]. The *C. tyrobutyricum* protein was identified as chaperone protein HscC. *Aedes aegypti*

HS protein 70 Aed a 8 is a putative allergen which is more than 89% identical to heat shock proteins from more than 100 insects, but has a low level match of 36.9% over 545 amino acids to the heat-killed *C. tyrobutyricum* strain ASM#19, which therefore presents no risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|442565872|gid|2075| Putative triose-phosphate Isomerase [*Dermatophagoides farinae*]. The *C. tyrobutyricum* protein was identified as triosephosphate isomerase (EC 5.3.1.1). The *Dermatophagoides farinae* triosphosphate isomerase was identified as a putative antigen with sera from 9 subjects based on immunoblotting without any biological activity. Triosephosphate isomerase of *Dermatophagoides farinae* is over 70% identical to homologous protein of many insects and arachnids but has a low-level match of 36.5% over 249 amino acids to the heat-killed *C. tyrobutyricum* strain ASM#19, which therefore presents no risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|62147665|gid|115|Allergen hyaluronidase b [*Vespula vulgaris*]. The *C. tyrobutyricum* protein was identified as beta-glucoside bgl operon antiterminator, BglG family. The *Vespula vulgaris* hyaluronidase b was identified as a venom allergen with biological activity and is considered a clear allergen with cross-reactivity to venom proteins of many wasps, yellow jackets and other stinging insects with more than 45% identity over their full length. The *Clostridium* protein had 26% identity to the *Vespula* protein, which could be maximised at 36.2 over 80 amino acids using a sliding 80mer search. The low matching indicates heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen:gi|3243234|gid|234| Allergen isoflavone reductase related protein [*Pyrus communis*]. The *C. tyrobutyricum* protein was identified as indole-3-glycerol phosphate synthase (EC 4.1.1.48). The isoflavone reductase of pear has been shown to bind IgE from a number of subjects and be responsible for 20% of basophil histamine release compared to whole pear protein, so is considered an allergen. Heat-killed *C. tyrobutyricum* strain ASM#19 matched over 100 proteins from plants with more than 79% identity over full length but had only 31% sequence identity to the pear protein, which could be maximized to 36.2% identity over 80 amino acids with a sliding 80mer search. The low matching indicates heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|114841683|gid|424|Putative pollen allergen [*Chamaecyparis obtusa*]. The *C. tyrobutyricum* protein was identified as xanthine dehydrogenase iron-sulfur subunit (EC 1.17.1.4). The *Chamaecyparis* pollen putative allergen was shown to bind IgE and has high identity to Japanese cypress pollen allergen and potential biological activity in basophils. However, the heat-killed *C. tyrobutyricum* strain ASM#19 has identity matches of over 45% identity over full length proteins to more than 100 proteins from diverse plant species, while the overall sequence identity was only 35.7% over 80 AA to *Chamaecyparis obtusa* polygalacturonase. The low matching indicates heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|2677826|gid|239| Allergen major allergen protein homolog [*Prunus armeniaca*]. The *C. tyrobutyricum* protein was identified as peptidoglycan D,D transpeptidase MrdA (EC 3.4.16.4). The apricot and cherry proteins are nearly identical and have been shown to bind IgE to subjects allergic to these fruits in various European countries. Basophil activity was shown to the apricot protein, which is considered an allergen. However, heat-killed *C. tyrobutyricum*

strain ASM#19 has only a modest match of 35.6% identity over 90 amino acids to the *Prunus armeniaca* protein. The low matching indicates heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|2182106|gid|116|Putative group 2 allergen [*Tyrophagus putrescentiae*]. The *C. tyrobutyricum* protein was identified as probable transcriptional regulatory protein YebC. The *Tyrophagus* protein Tyr p 2 was shown to have IgE binding to people with allergies to the food storage mite. Heat-killed *C. tyrobutyricum* strain ASM#19 is only 35.3% identical to the *Tryrophagus* allergen Tyr p 2 over 85 AA. The low matching indicates heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|288860106|gid|160| Allergen high molecular weight glutenin subunit 5 [*Triticum aestivum*]. The *C. tyrobutyricum* protein was identified only as a hypothetical protein. This wheat gluten has been shown to bind IgE and cause biological activity in a number of subjects. There are many proteins of high identity over part or much of the protein lengths in many wheat species. The hypothetical *Clostridium* protein has only one protein match using BLASTP and that is to *Rhizopus microsporus* over 130 AA, with 73% identity. Its match to the wheat glutenin subunit is only 35.2 % over 108 amino acids. The low matching indicates this hypothetical heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

One *C. tyrobutyricum* protein matched allergen: gi|308193268|gid|1776| Putative thioredoxin [*Plodia interpunctella*]. The *C. tyrobutyricum* protein was identified as thioredoxin. The *Plodia sp.* thioredoxin is a putative allergen, but without bioactivity. It had 35.2% ID to the *Clostridium* thioredoxin over 90 amino acids. The *Clostridium* thioredoxin only has a high identity match only other *Clostridium* thioredoxins. The low matching to a weak antigen suggests heat-killed *C. tyrobutyricum* strain ASM#19 does not represent a risk of allergy.

Taken together, it is concluded that heat-killed *C. tyrobutyricum* strain ASM#19 is of low allergenicity concern.

Question 3

We note that Clostridium protein is a novel microbial protein. You have provided a published bioinformatics evaluation of the potential allergenic risk of Clostridium protein; however, there appears to be a lack of human consumption data with this ingredient to support tolerability. Given that Clostridium protein will be used as a direct protein replacement, and at levels as high as 33g/p/d, please provide a narrative justifying why no human consumption data is needed to mitigate concerns pertaining to tolerability and allergenicity.

Supplemental Information

Heat-killed *C. tyrobutyricum* strain ASM#19 represents the first bacterial protein ingredient intended for market in the U.S. The pivotal evidence of safety is provided by the results of published toxicity testing and a bioinformatics evaluation of the allergenic potential of the ingredient. Corroborating evidence of the tolerability to humans of heat-killed *C. tyrobutyricum* strain ASM#19 is provided by the findings of a study in which 20 g was consumed in one sitting by male subjects who were then monitored over a 3-

hour period. The study was designed to assess efficacy rather than safety but parameters relevant to tolerability were included such as bloating and adverse event reporting.

A randomized, double-blinded, within subjects, acute study compared the effects of Superbrewed Food's heat-killed *C. tyrobutyricum* strain ASM#19 and whey protein concentrate when ingested alone or in combination with maltodextrin, on amino acid absorption kinetics, as well as the glucose and insulin response over a 3-hour postprandial period (Broughton *et al*., 2023 – Unpublished). All subjects in the study were healthy males (aged 18 to 25 years old, with a BMI of <30 kg/m^2 and body fat percentage <25%), which were classed as recreationally active (exercising 2 or more days a week). In the study, 17 subjects received in a randomized order, beverage formulations at amounts providing 0.3 g/kg lean body mass (LBM) heat-killed *C. tyrobutyricum* strain ASM#19, 0.3 g/kg LBM whey protein concentrate, 0.3 g/kg heat-killed *C. tyrobutyricum* strain ASM#19 + 75 g maltodextrin, 0.3 g/kg LBM whey protein concentrate + 75 g maltodextrin or 0.3 g/kg LBM maltodextrin. Although the LBM will have varied per individual, exposure to heat-treated *C. tyrobutyricum* strain ASM#19 was approximately 20 g/day. The amount of maltodextrin included in the study is the same level used in the OGTT to identify diabetes.

Prior to study start all participants were provided a 24-hour diet to adhere to and were instructed to record all food consumed in the 24 hours prior to each treatment. Subjects were also requested to refrain from any strenuous activity (heavy resistance training or running) during the 48 to 24 hours prior to testing, and not to perform any exercise at all in the 24 hours prior to study start. Subjects were assessed at screening (pre-treatment) by means of an oral glucose tolerance test (OGTT), to ensure that participants had resting blood glucose levels of <100 mg/dL prior and values of up to 200 mg/dL after 1 hour and 140 mg/dL after 2 hours during the test. Blood samples were taken 15 minutes prior to intervention and then at 0 (baseline), 15-, 30-, 45-, 60-, 90-, 120-, and 180-minutes following ingestion for total amino acids, essential amino acid, branched chain amino acid, leucine, glucose, and plasma levels of insulin. Subjective assessments were taken including bloating and adverse effects.

There were no reports of adverse effects reported for any of the interventions. When protein samples were ingested with the dextrose, participants indicated that the combination was not as palatable when consumed alone. Two (2) individuals receiving 0.3 g/kg LBM heat-killed *C. tyrobutyricum* strain ASM#19 + 75 g maltodextrin felt nausea at termination of blood sampling, however this was not observed within the first 3 hours after ingestion and the investigators postulated that these effects were due to a hypoglycemic response to the dextrose ingestion. One individual fainted at the sight of a catheter needle and was not included in the study. Another single participant did not complete the study due to a lack of time to lack of adherence, however this individual had only completed the OGTT at screening and all participants that started the protein ingestion phase completed the study.

Heat-killed *C. tyrobutyricum* strain ASM#19 when ingested alone, elevated blood glucose levels from 92 to 100 mg/dL and maintained plasma glucose at about 95 mg/dL for 3 hours; these values were higher than levels observed with whey protein which induced mild induced hypoglycemia (levels as low as 87 mg/dL) for about two hours before returning to normal levels. The insulin response was virtually identical in individuals ingesting heat-killed *C. tyrobutyricum* strain ASM#19 and whey protein when consumed on their own.

When heat-killed *C. tyrobutyricum* strain ASM#19 and whey protein was ingested with dextrose, the insulin response was the same over the first 30 minutes. However, after 30 minutes heat-killed *C. tyrobutyricum* strain ASM#19 attenuated the insulin response, reducing it by approximately 17% after one hour and to normal levels at 90 minutes. After 2 hours a rebound hypoglycemia induced by dextrose was similar to the response when dextrose was consumed alone. In comparison individuals receiving whey protein + dextrose observed an increase in insulin, which remained >50% elevated at 90 minutes, returning to normal between 2 to 2.5 hours. These changes in blood glucose are anticipated after significant sugar consumption and the findings using heat-killed *C. tyrobutyricum* strain ASM#19 and whey protein were considered by the investigators to be acceptable.

Overall, when ingested alone both heat-killed *C. tyrobutyricum* strain ASM#19 and whey protein were equally effective in maintaining blood levels of aromatic acids, branched chain amino acids (BCAA; isoleucine and valine) and short chain amino acids (i.e., alanine, glycine, serine, and proline). The BCAA, leucine had higher levels in individuals receiving whey protein than *heat-killed C. tyrobutyricum strain ASM#19* and this reflected the higher leucine content in whey protein. When both protein sources were consumed with dextrose, there was an increased rate of amino acid clearance from the blood, which was likely attributable to an increased insulin response stimulated by the BCAAs, primarily leucine. Short-chain amino acids were increased when heat-killed *C. tyrobutyricum* strain ASM#19 was consumed with dextrose, and this may reflect conversion of amino acids or precursors derived from glucose to the short-chain amino acids.

In conclusion, heat-killed *C. tyrobutyricum* strain ASM#19 was well-tolerated in healthy male individuals with no reports of bloating or adverse effects within 3-hours of consuming approximately 17 kg of ingredient in a beverage formulation. There were also no adverse effects on insulin response or the uptake of amino acids among subjects relative to a whey protein reference.

It is also noteworthy that *Clostridium butyricum* was the subject of a novel food assessment in the European Commission and a daily intake of 1.35×10^8 CFU/g is authorized for use in food (dietary) supplement products (EC, 2023). The safety of the live microorganism was supported by an assessment conducted by the United Kingdon (UK) Advisory committee for Novel Foods and Processes (ACNFP, 2013). Although these use levels are relatively low compared to heat-killed *C. tyrobutyricum* strain ASM#19, they provide further evidence of the tolerability of repeated and long-term exposure to *Clostridium* species as a component of the diet.

Further evidence for the tolerability of humans to heat-killed *C. tyrobutyricum* strain ASM#19 is provided by published data on the natural presence of the species in milk and cheese (Herman *et al*., 1995; Herman *et al*., 1997; Burtscher *et al*., 2020). Although the presence of *C. tyrobutyricum* can be detrimental to the cheese-making process under conditions favorable fermentation, it carries through from the milking process into milk and dairy products.

Importantly, *C. tyrobutyricum* is a commensal microorganism which has been isolated from human adults and infants, as well as cattle, dogs, and other animals (Ivy and Wiedmann, 2014; Hrncirova *et al*., 2018; Luo et al., 2021). On the basis that it is found naturally in the gut of humans, it is reasonable to assume that the species is well-tolerated by humans.

Question 4

We note that historically, the specification for nucleic acids has been less than 2g/100g. In the safety narrative, you summarize an unpublished dose-range finding study in rats using a crude Clostridium protein preparation, where the nucleic acid content was analyzed and found to be 7.8g/100g. There were noted adverse findings, such as swelling of the hindlimbs with inflammation and edema noted upon microscopic evaluation, that were suggestive for gouty arthritis or synovitis, which were attributed to the high nucleic acid content. In the published 90-day dietary feeding study using a heat-treated Clostridium protein preparation containing 2.7g nucleic acids/100g, these adverse effects were not observed.

a. *Please provide additional narrative to support your conclusion that the nucleic acid specification of 4g/100g is adequate to ensure safety given your subchronic toxicological study where no adverse effects were noted was performed with a preparation containing just 2.7g nucleic acids/100g, substantially below your specified limitation.*
b. *On p. 63 of the notice, you appear to reference the FAO/WHO/UNICEF Protein Advisory Group recommendation for a maximum daily intake of ≤ 2g/day of nucleic acids from single-cell protein foods, regardless of whether the biomass is bacterial or fungal-derived.1,2,3 You also note that the 90th percentile intakes for nucleic acids for Clostridium protein containing the maximum amount of nucleic acids would be 1.33g/p/d. As it appears the Protein Advisory Group's recommendation on nucleic acids levels from single-cell proteins is a pivotal argument for the safety of your GRAS conclusion, please discuss the data used by the FAO/WHO/UNICEF Group to reach their conclusion on nucleic acid levels in the diet from single-cell proteins.*

Supplementary Information

Part a

Superbrewed Food proposes a revised maximum limit on nucleic acids in heat-killed *C. tyrobutyricum* strain ASM#19 of 3 g/100 g. The revised specification limit better reflects the nucleic acid content of the test article used in the toxicity studies (2.7 g/100 g). The nucleic acids content of 5 representative lots of heat-killed *C. tyrobutyricum* strain ASM#19 analyzed as part of the GRAS notice (Table 2.6), demonstrated that the levels varied from 1.6 to 2.9 g/100 g. Thus, the nucleic acid content of these representative lots conforms with the revised specification limit of maximum 3 g/100 g and also supports that the test article used in the 90-day dietary feeding study in rats contained nucleic acids towards the upper end of the article of commerce.

The NOAEL allocated from the 90-day dietary feeding study in rats using heat-killed *C. tyrobutyricum* strain ASM#19 was equivalent to 149 and 179 mg nucleic acids/kg body weight/day in males and females, respectively. Under the conditions of intended use, infants and young children were determined to have the highest mean and 90th percentile consumer-only intakes of heat-killed *C. tyrobutyricum* strain ASM#19 of 600 and 1,370 mg/kg body weight/day, respectively, equating to 18 mg and 41.1 mg nucleic acids/kg body weight/day for an ingredient containing the maximum content of nucleic acids of 3 g/100 g (revised specifications). Thus, exposure by female rats to nucleic acids from the dietary administration of heat-killed *C. tyrobutyricum* strain ASM#19 fed to female rats in the 90-day

study was 3.6-fold higher than the 90th percentile intakes estimated from the proposed food uses of the ingredient.

Fungal proteins such as mycoprotein (Quorn™) contain a maximum of 2 g/100 g of nucleic acids, as noted by the U.S. FDA. However, unlike heat-killed *C. tyrobutyricum* strain ASM#19, which will be a component of a formulated product, and provide for example 40 g/serving when used in meat substitute products (e.g., vegetable-based burger), mycoprotein may represent the primary protein source in a meal (e.g., Quorn™ based bolognaise) with a serving size in the region of 110 g. As such, a meal containing 110 g of Quorn™ may provide up to 2.1 g nucleic acids/serving. By comparison, a 40 g serving of heat-killed *C. tyrobutyricum* strain ASM#19 based on the revised specification limit, will provide up to 1.2 g nucleic acids/serving. In this respect, heat-killed *C. tyrobutyricum* strain ASM#19 will not lead to greater exposure to nucleic acids from microbial protein source than from this well-established fungal protein.

Furthermore, as mentioned with the GRAS notice, nucleic acids are present naturally in vegetable- and animal-derived foods that form part of the normal diet of the U.S. population. Liver for example, 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 of DNA, equating to a total (RNA + DNA) of 4.0 to 4.7 g/100 g dry matter. Other relatively high sources of nucleic acids include chestnut mushrooms and oyster fungi with levels of 2.2 to 2.4 g/100 g dry matter RNA and 0.14 g/100 g of DNA, equating to approximately 2.3 to 2.6 g/100 g dry matter in total (RNA + DNA). Although on a per serving basis, the exposure by the human population may be lower than from a single serving of heat-killed *C. tyrobutyricum* strain ASM#19, when considering the ubiquitous nature of these and similar foods the diet, the contribution of the bacterial protein is not expected to be significantly different than that obtained by an offal- and vegetable-rich meal.

Taken together, the revised specification of a maximum of 3 g/100 g of nucleic acids in heat-killed *C. tyrobutyricum* strain ASM#19 provides further support that under the conditions of intended use, the nucleic acid content does not pose a safety concern.

Part b

As noted by the U.S. FDA, where a microbial protein (or single cell protein) is intended for use as a primary protein source for humans, the Protein Advisory Group (PAG) of the WHO/FAO/UNICEF guideline no. 12 (PAG, 1983) indicated that nucleic acid intake should not exceed 2 g/100 g for adults. This maximum has been documented by others and is based on the findings of two studies in humans (Waslien *et al*., 1968; Edozien *et al*., 1970; Maul *et al*., 1970; Bratosin *et al*., 2021). In the study by Edozien *et al*. (1970), 4 healthy males were fed diets containing varying levels of food yeast contributing 2.9, 5.8 or 8.7 g/day nucleic acids and the serum uric acid levels measured. When 2.9 g of yeast nucleic acid was fed to the healthy male subjects, uric acid excretion in the urine increased to more than 1,000 mg/day.

Waslien *et al*. (1968) also provided healthy males with 0, 2, 4 or 8 g yeast RNA for 5 consecutive days in the form of 75 g egg protein in the diet, split over 4 equal meals and evaluated serum uric acid levels. Urinary uric acid excretion values were reported to be 373, 667, 939 or 1,393 mg/day which were lower

than the values observed by Edozien *et al*. (1970). The general population consuming normal diets were considered to excrete less than 600 mg/day.

From the available information, it was concluded that 3 g nucleic acids/day can result in relatively high and undesirable levels of uric acid in urine. A dietary intake of 2 g/day was considered to involve little risk of stone formulation. Slightly higher levels were also suggested to probably pose no hazards as long as urine volume and pH were not abnormally low. No gastrointestinal disturbances were reported from exposure to up to 135 g yeast, corresponding to 8.7 g nucleic acids/day.

The human data on which the 2 g/day limit was based is relatively old and is derived from only a few subjects. On this basis, it was not given significant prominence in the GRAS notice, although reference to the 2 g/day was made for completeness.

Under the proposed conditions of use of heat-killed *C. tyrobutyricum* strain ASM#19, 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 0.44 and 1.0 g/person/day, respectively for heat-killed *C. tyrobutyricum* strain ASM#19 containing the maximum content of nucleic acids of 3 g/100 g. These intake levels, based on the maximum and mean nucleic acid levels in heat-killed *C. tyrobutyricum* strain ASM#19, fall below the 2 g nucleic acids/day limit considered acceptable for microbial proteins used as primary protein sources for humans. Thus, the higher nucleic acid content in *Clostridium* protein compared to *Fusarium* protein products currently on the market is not considered to be nutritionally disadvantageous under the intended conditions of use.

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From: Elizabeth Lewis
To: Kampmeyer, Christopher
Subject: Re: [EXTERNAL] Re: GRN 001129: Sept. 6, 2023 Meeting Memo and Questions
Date: Thursday, December 7, 2023 3:40:27 PM
Attachments: image001.png
image002.png
image003.png
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Superbrewed Food_Clostridium_FDA Responses_12072023.pdf
FDA Supplementary Info_12072023.pdf

Dear Chris,

Many thanks again for the email below and follow-up questions from the reviewers. Please find attached supplementary information to address the points below. If you have any further questions or require copies of the references cited therein, let me know.

Best regards,
Elizabeth

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www.nutrasteward.com

On Tue, 28 Nov 2023 at 16:22, Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Dear Elizabeth,
>
> Our review team identified a few follow-up questions during our review of your responses:

1. For the administrative record, please state whether any raw materials or processing aids used in the manufacturing process are derived from any of the major nine allergens and are expected to be present in the final ingredient.

2. In your amendment dated October 24, 2023, you provided further information on the matches between *C. tyrobutyricum* protein sequences and the Allergen Online database. We note that on p. 8, you discuss two *C. tyrobutyricum* protein sequences that match a putative sesame allergen[1]. In your conclusion, you indicate these proteins likely don't pose a risk of allergy as there are a wide variety of common foods with higher identity scores. Your basis for concluding that these homology matches do not indicate significant allergenicity risk is based on your evaluation that "there are long alignments of the sesame protein to proteins from a wide variety of common foods at >70% identity." It is not clear from your statement whether you are implying that a wide variety of common foods contain allergic proteins or that the bioinformatic analyses are not adequately sufficient to assess risk for allergenicity. Please provide further details and context of your statement on these common foods that you identified with higher identity scores as well as additional information to support your conclusion that these two proteins would not pose a risk of allergy in consumers.

3. In the same amendment, you conclude that *C. tyrobutyricum* is a commensal microorganism, and cite three publications to support this point (p. 13). We note that the Ivy and Wiedmann (2014) book chapter does state that *C. tyrobutyricum* has been isolated from human adults and infants, but it does not offer a reference for this conclusion. The Luo et al. (2021) paper does not appear to state that *C. tyrobutyricum* is a gut microbe, but rather, it references the Hrncirova et al. (2019) paper. The Hrncirova et al. (2019) paper does isolate *C. tyrobutyricum* from three healthy adult males. Based on these publications, there appears to be very little published evidence that *C. tyrobutyricum* is part of the human gut microbiome. If available, please provide any further support to your conclusion that *C. tyrobutyricum* protein is a commensal microorganism.

[1] In 2021, the Food Allergy Safety, Treatment, Education, and Research (FASTER) Act was signed into law, adding sesame as the 9th major food allergen in the US.

Your responses to these are respectfully requested within 10 business days.

Thank you,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

NutraSteward

Providing regulatory support for food and feed ingredients

December 7, 2023

Susan J. Carlson, Ph.D., Director
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety (HFS-200)
5100 Campus Drive
College Park, MD 20740

Dear Dr. Carlson,

Re: GRAS Notice 001129

In follow-up to the questions on the GRAS notice for heat-killed *Clostridium tyrobutyricum* strain ASM#19 received from the U.S. FDA on November 28, 2023, please find Superbrewed Food Inc.'s responses enclosed.

We trust that this meets with your immediate needs and remain at your disposal to provide any further information required.

Sincerely,

Elizabeth Lewis, PhD
Scientific & Regulatory Advisor
NutraSteward, Ltd.

NutraSteward, Ltd.
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Supplementary Information for GRAS Notice 001129

FOLLOW-UP QUESTIONS

Question 1

For the administrative record, please state whether any raw materials or processing aids used in the manufacturing process are derived from any of the major nine allergens and are expected to be present in the final ingredient.

Supplementary Information

Superbrewed Food confirms that none of the raw materials or processing aids used in the manufacturing process are derived from any of the major nine allergens[1] and none of these allergens are expected to be present in the final ingredient.

Question 2

In your amendment dated October 24, 2023, you provided further information on the matches between C. tyrobutyricum protein sequences and the Allergen Online database. We note that on p. 8, you discuss two C. tyrobutyricum protein sequences that match a putative sesame allergen. In your conclusion, you indicate these proteins likely don't pose a risk of allergy as there are a wide variety of common foods with higher identity scores. Your basis for concluding that these homology matches do not indicate significant allergenicity risk is based on your evaluation that "there are long alignments of the sesame protein to proteins from a wide variety of common foods at >70% identity." It is not clear from your statement whether you are implying that a wide variety of common foods contain allergic proteins or that the bioinformatic analyses are not adequately sufficient to assess risk for allergenicity. Please provide further details and context of your statement on these common foods that you identified with higher identity scores as well as additional information to support your conclusion that these two proteins would not pose a risk of allergy in consumers.

Supplementary Information

The putative seed maturation-like protein precursor of sesame indicum was identified only as a potential minor allergen and not published. The NCBI entry is provided in Figure 1. It is a member of the oxidoreductase, short chain dehydrogenase/reductase family which is highly conserved among all organisms.

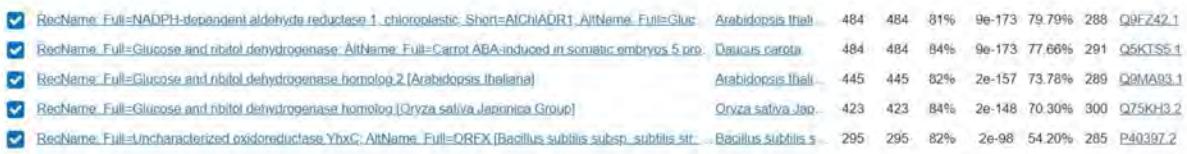
[1] U.S. FDA 9 major food allergens: soybeans, sesame, milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, and wheat. Further information available at: https://www.fda.gov/food/food-labeling-nutrition/food-allergies

Figure 1: Entry for Putative Sesame Antigen in NCBI

```
LOCUS       ACB55491                345 aa            linear   PLN 30-DEC-2014
DEFINITION  seed maturation-like protein precursor [Sesamum indicum].
ACCESSION   ACB55491
VERSION     ACB55491.1
DBSOURCE    accession EU410074.1
KEYWORDS    .
SOURCE      Sesamum indicum (sesame)
  ORGANISM  Sesamum indicum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliopsida; eudicotyledons; Gunneridae;
            Pentapetalae; asterids; lamiids; Lamiales; Pedaliaceae; Sesamum.
REFERENCE   1  (residues 1 to 345)
  AUTHORS   Grishina,G., Beyer,K., Bardina,L. and Sampson,H.
  TITLE     Isolation of a seed maturation-like protein from sesame seed, a new
            potential minor allergen
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 345)
  AUTHORS   Grishina,G., Beyer,K., Bardina,L. and Sampson,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JAN-2008) Pediatrics, Icahn School of Medicine at
            Mount Sinai, 1425 Madison Ave., Box 1089, New York, NY 10029, USA
COMMENT     Method: conceptual translation supplied by author.
```

A BLAST P search of the UniprotKB/Swiss-Prot database using the full-length amino acid sequence of the putative sesame antigen from the NCBI database identified the highest homology to members of this protein family from wild carrot (*Daucus carota*), rice (*Oryza sativa*) and thale cress (*Arabidopsis thaliana*) with similarities between 70 and 80% (Figure 2). These plants are all edible so the high match of the sesame protein to proteins in these plants suggests it is unlikely to be an allergen. The statement was not meant to imply that common foods contain allergenic proteins, but rather that the match to proteins in common foods that are not known allergens implies that this protein is not a clinically relevant antigen.

Figure 2: Top five matches in the SwissProtKB/UniProt database for the putative sesame antigen



RecName: Full=NADPH-dependent aldehyde reductase 1, chloroplastic; Short=AtChlADR1; AltName: Full=Gluc	Arabidopsis thali	484	484	81%	9e-173	79.79%	288	Q9FZ42.1
RecName: Full=Glucose and ribitol dehydrogenase; AltName: Full=Carrot ABA-induced in somatic embryos 5 pro	Daucus carota	484	484	84%	9e-173	77.66%	291	Q5KTS5.1
RecName: Full=Glucose and ribitol dehydrogenase homolog 2 (Arabidopsis thaliana)	Arabidopsis thali	445	445	82%	2e-157	73.78%	289	Q9MA93.1
RecName: Full=Glucose and ribitol dehydrogenase homolog [Oryza sativa Japonica Group]	Oryza sativa Jap	423	423	84%	2e-148	70.30%	300	Q75KH3.2
RecName: Full=Uncharacterized oxidoreductase YhxC; AltName: Full=ORFX [Bacillus subtilis subsp. subtilis str	Bacillus subtilis s	295	295	82%	2e-98	54.20%	285	P40397.2

It is now understood that bioinformatic analysis alone is not sufficient to assess risk for allergenicity. Abdelmoteleb *et al.* (2021) found that matches to many proteins that are highly conserved through evolution do not predict the risk of allergy due to cross-reactivity. Furthermore, Abdelmoteleb *et al.* (2021) identified several of the same allergens found in our search, in an investigation of two species of algae and a fungus with potential for use as new food ingredients, and specifically eliminated all of them from further consideration as relevant allergens based on matches to a panel of 23 diverse and non-

allergenic genomes. The allergens eliminated in their study and also identified in this report, include the putative sesame antigen (Abdelmoteleb *et al.*, 2021; Table 4, p.7). The authors found that a protein in the algae *Chlorella variabilis* had 44.5% similarity over 330 amino acids to the same putative sesame allergen which Superbrewed Food identified as having a 49% match to the *C. tyrobutyricum* proteins, but higher matches to 15 of 23 species commonly consumed as food. [It is worth noting that the same argument can be applies to heat shock protein 70 cognates, triosephosphate isomerase, GAPDH and peptidyl- prolyl cis-trans isomerase: proteins also found in both Abdelmoteleb *et al.* 2021 and Superbrewed Food's analysis of *C. tyrobutyricum*]. Overall, the authors concluded that the species they investigated "do not represent a significant risk of food allergy to the general population as matches to similar proteins from many diverse species are very common." (Abdelmoteleb *et al.*, 2021; p.11). Based on the analysis, the investigators suggested the CODEX cut-off as being very conservative.

It can be inferred from this related analysis that there is minimal risk of allergy where a *C. tyrobutyricum* protein matches housekeeping proteins, enzymes or chaperone proteins which are highly evolutionarily conserved in many species (often across kingdoms) due to their essential functions, and which are not known to be allergenic.

It is of note that the predicted *C. tyrobutyricum* proteins also have their highest matches to the same proteins from thale cress, rice and wild carrot, and with a similarity in the same range, as that of the sesame protein (45-50%, as shown in Figure 3), further supporting that the similarities are due to conservation of these proteins.

Figure 3: Top 10 matches in the UniProtKB/Swiss-Prot database for the *C. tyrobutyricum* protein which was similar to a putative sesame antigen

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc Len	Accession
RecName: Full=Uncharacterized oxidoreductase YhxC; AltName: Full=ORFX [Bacillus subtilis subsp. subtilis str...	Bacillus subtilis s...	350	350	97%	7e-121	60.79%	285	P40397.2
RecName: Full=Uncharacterized oxidoreductase YhdF [Bacillus subtilis subsp. subtilis str. 168]	Bacillus subtilis s...	334	334	96%	1e-114	58.12%	289	O07572.1
RecName: Full=General stress protein 39; Short=GSP39 [Bacillus subtilis subsp. subtilis str. 168]	Bacillus subtilis s...	324	324	100%	1e-110	55.24%	286	P80873.3
RecName: Full=Glucose and ribitol dehydrogenase; AltName: Full=Cannot ABA-arrested in somatic embryos 5 pr...	Daucus carota	282	282	95%	6e-94	50.36%	291	Q5KTS5.1
RecName: Full=Glucose and ribitol dehydrogenase homolog 2 [Arabidopsis thaliana]	Arabidopsis thali...	275	275	95%	3e-91	49.47%	289	Q8VA93.1
RecName: Full=NADPH-dependent aldehyde reductase 1, chloroplastic; Short=AtChlADR1; AltName: Full=Gluc...	Arabidopsis thali...	272	272	98%	4e-90	47.22%	288	Q9FZ42.1
RecName: Full=Glucose and ribitol dehydrogenase homolog; [Oryza sativa Japonica Group]	Oryza sativa Jap...	259	259	95%	8e-85	48.28%	300	Q75KH3.2
RecName: Full=Uncharacterized oxidoreductase YhdF; AltName: Full=ORFY [Bacillus subtilis subsp. subtilis str...	Bacillus subtilis s...	243	243	93%	8e-79	49.08%	299	P40398.2
RecName: Full=Uncharacterized oxidoreductase YghA [Escherichia coli K-12]	Escherichia coli...	242	242	95%	3e-78	45.29%	294	P0AC84.1
RecName: Full=Uncharacterized oxidoreductase C4H3.06 [Schizosaccharomyces pombe 972h-]	Schizosaccharo...	217	217	97%	9e-69	40.28%	286	Q10216.1

Question 3

In the same amendment, you conclude that C. tyrobutyricum is a commensal microorganism, and cite three publications to support this point (p. 13). We note that the Ivy and Wiedmann (2014) book chapter does state that C. tyrobutyricum has been isolated from human adults and infants, but it does not offer a reference for this conclusion. The Luo et al. (2021) paper does not appear to state that C. tyrobutyricum is a gut microbe, but rather, it references the Hrncirova et al. (2019) paper. The Hrncirova et al. (2019) paper does isolate C. tyrobutyricum from three healthy adult males. Based on these publications, there appears to be very little published evidence that C. tyrobutyricum is part of the human gut microbiome. If

available, please provide any further support to your conclusion that C. tyrobutyricum protein is a commensal microorganism.

Supporting Information

C. tyrobutyricum is found naturally in dairy products and so must be frequently ingested by the general population. Recent studies of the human microbiome have identified *C. tyrobutyricum* in at least one sample. Examples include a study by the Ichan School of Medicine at Mount Sinai (2020) in which bacterial strains were isolated from donors who provided samples of fecal transplantation to several patients with different diseases. Four sequences from different strains of *C. tyrobutyricum* were deposited during this project:

- Genome assembly *C. tyrobutyricum* ASM1567006v1: https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_015670065.1/
- Genome assembly *C. tyrobutyricum* ASM1567112v1 https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_015671125.1/
- Genome assembly *C. tyrobutyricum* ASM1556045v1 https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_015560455.1/
- Genome assembly *C. tyrobutyricum* ASM1555695v1 https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_015556955.1/

The Unified Human Gastrointestinal Genome catalogue (2019) is a project intended to collect a comprehensive set of reference data for accurate taxonomic and functional characterization of the human gut microbiome. The collection includes an isolated *C. tyrobutyricum* strain: https://www.ncbi.nlm.nih.gov/bioproject/559126

Likewise, more recent studies of the human microbiome have identified *C. tyrobutyricum* in at least one sample as evidenced in supplemental reporting spreadsheets:

Expanded reference map of the human gut (Leviatan *et al*., 2022) identified *C. tyrobutyricum* at Line 239508 of this supplementary information file: https://static-content.springer.com/esm/art%3A10.1038%2Fs41467-022-31502-1/MediaObjects/41467_2022_31502_MOESM4_ESM.xlsx

Unified catalogue of reference genomes from human gut microbiome (Almeida *et al*., 2021) identified *C. tyrobutyricum* at Line 128 of this supplementary information file: https://static-content.springer.com/esm/art%3A10.1038%2Fs41587-020-0603-3/MediaObjects/41587_2020_603_MOESM3_ESM.xlsx

Reconstruction of genomes from fecal metagenomics from geographically and phenotypically diverse humans (Nayfach *et al*., 2019) identified *C. tyrobutyricum* at Line 23283: https://static-content.springer.com/esm/art%3A10.1038%2Fs41586-019-1058-x/MediaObjects/41586_2019_1058_MOESM4_ESM.xlsx

Furthermore, Podrzaj *et al*. (2022) carried out a phylogenetic analysis of 28 *C. tyrobutyricum* strains from different sources, including 5 strains of human origin. Four of the five clustered in one clade, with mud and silage isolates, while the fifth clustered with isolates from milk and cheese.

Supporting evidence of safety is provided by studies evaluating the role of live *C. tyrobutyricum* on gut health of humans and animals. The ability of *C. tyrobutyricum* to ferment oligosaccharides and other fermentable fibers to produce short-chain fatty acids, particularly butyrate, are postulated to have potential benefits for human health (Liu *et al*., 2020). Available published data include studies in mice on spores as well as the species. Liang *et al*. (2020) reported that *C. tyrobutyricum* spores helped maintain intestinal homeostasis. Xiao *et al*. (2021, 2021a and 2021b) found that *C. tyrobutyricum* improves intestinal barrier function in mice, and protects from lipopolysaccharide (LPS) induced inflammation. Similarly, C*. tyrobutyricum* pre-treatment attenuated oxidative stress, inflammatory reaction and injury severity, as well as positively impacting the balance of the intestinal microbiota (Liu *et al*., 2017).

The information above, together with that provided previously within the GRAS notice and supplementary information, support that *C. tyrobutyricum* is consumed naturally from foods, and is routinely identified as part of the gut microbiome of humans where it is not associated with any toxigenicity or pathogenicity, or other effects on health.

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Liang, Q., Liu, J., Wei, J., Jia, J., Shen, H., Chen, W., Liang, W., Gao, B., Xu, Z. and Zhang, L., 2020. The effect of Clostridium tyrobutyricum Spo0A overexpression in the intestine of mice. *Beneficial Microbes*, *11*(6), pp.573-589.

Liu, T., Zhu, L., Zhu, Z. and Jiang, L., 2020. Genome sequence analysis of Clostridium tyrobutyricum, a promising microbial host for human health and industrial applications. *Current Microbiology*, *77*, pp.3685-3694.

Nayfach, S., Shi, Z.J., Seshadri, R., Pollard, K.S. and Kyrpides, N.C., 2019. New insights from uncultivated genomes of the global human gut microbiome. *Nature*, *568*(7753), pp.505-510.

Podrzaj, L., Burtscher, J. and Domig, K.J., 2022. Comparative genomics provides insights into genetic diversity of Clostridium tyrobutyricum and potential implications for late blowing defects in cheese. *Frontiers in Microbiology*, *13*, p.889551.

Unified Human Gastrointestinal Genome Catalogue, 2019. A unified sequence catalogue of the human gut microbiome. Registration date 7-Aug-2019. Available at: https://www.ncbi.nlm.nih.gov/bioproject/559126

Xiao, Z., Liu, L., Jin, Y., Pei, X., Sun, W. and Wang, M., 2021. Clostridium Tyrobutyricum protects against LPS-induced colonic inflammation via IL-22 signaling in mice. *Nutrients*, *13*(1), p.215.

Xiao, Z., Liu, L., Jin, Y., Pei, X., Sun, W. and Wang, M., 2021a. A Potential Prophylactic Probiotic for Inflammatory Bowel Disease: The Overall Investigation of Clostridium tyrobutyricum ATCC25755 Attenuates LPS-Induced Inflammation via Regulating Intestinal Immune Cells. *Molecular Nutrition & Food Research*, *65*(14), p.2001213.

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From: Elizabeth Lewis
To: Kampmeyer, Christopher
Subject: [EXTERNAL] Re: GRN 001129 clarification
Date: Wednesday, February 14, 2024 4:17:32 PM
Attachments: image013.png
image014.png
image015.png
image016.png
image017.png
image018.png
FDA_Supplementary Info_02142024.pdf
Superbrewed Food_Clostridium_FDA Responses_02142024.pdf

Dear Christopher,

Many thanks for the email and please find attached a response to the questions below. If there is any further information required, please do not hesitate to contact me and we will respond as quickly as possible.

Best regards,
Elizabeth

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On Tue, 13 Feb 2024 at 13:08, Kampmeyer, Christopher <Christopher.Kampmeyer@fda.hhs.gov> wrote:

> Dear Elizabeth,
>
> I am writing to request clarification on the following points. As we're approaching the end of our evaluation period, we would appreciate responses as soon as possible.
>
> 1. According to the flowchart on page 18, microfiltration is performed at step 12, which is after the cell concentration (step 9), heat treatment (step 10) and washing (step 11). However, the description on page 19 did not seem to match the order of the steps in the flowchart because it states that the microfiltration occurs prior to the cell concentration and heat treatment. Please clarify the discrepancy and state where

exactly in the manufacturing process the microfiltration step is performed.
2. Could you please clarify the month/year at which the conducted literature search ended?

Thank you,

Chris

Chris Kampmeyer, M.S.

Regulatory Review Scientist

Office of Food Additive Safety

Center for Food Safety and Applied Nutrition

U.S. Food and Drug Administration

christopher.kampmeyer@fda.hhs.gov

NutraSteward

Providing regulatory support for food and feed ingredients

February 14, 2024

Susan J. Carlson, Ph.D., Director
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety (HFS-200)
5100 Campus Drive
College Park, MD 20740

Dear Dr. Carlson,

Re: GRAS Notice 001129

In follow-up to the questions on the GRAS notice for heat-killed *Clostridium tyrobutyricum* strain ASM#19 received from the U.S. FDA on February 13, 2024 please find Superbrewed Food Inc.'s responses enclosed.

We trust that this meets with your immediate needs and remain at your disposal to provide any further information required.

Sincerely,

Elizabeth Lewis, PhD
Scientific & Regulatory Advisor
NutraSteward, Ltd.

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Supplementary Information for GRAS Notice 001129

FOLLOW-UP QUESTIONS

Question 1

According to the flowchart on page 18, microfiltration is performed at step 12, which is after the cell concentration (step 9), heat treatment (step 10) and washing (step 11). However, the description on page 19 did not seem to match the order of the steps in the flowchart because it states that the microfiltration occurs prior to the cell concentration and heat treatment. Please clarify the discrepancy and state where exactly in the manufacturing process the microfiltration step is performed.

Supplementary Information

The description provided on p. 19 of the GRAS notice is correct. Separation (Stage 8) and cell concentration (Stage 9) identified on the flow-chart can be conducted by microfiltration or centrifugation. Microfiltration allows for a more complete recovery of the cells and is preferred by Superbrewed Food, Inc. Washing (Stage 11) of the cells is also conducted using microfiltration with a final microfiltration step (Stage 12) conducted post-washing and before passing the material to the drum dryer. In summary:

At the end of the fermentation, the C. tyrobutyricum cell mass is separated from the fermentation broth by microfiltration. The cell mass is concentrated to 15% total solids by microfiltration and heated to 70°C for 20 minutes in order to reduce the nucleic acid levels. The cells are washed by using a counter current to microfiltration which is performed over 2 stages. A final microfiltration step is performed before the cells are dried using a dual-drum dryer at 120°C.

Question 2

Could you please clarify the month/year at which the conducted literature search ended?

Supplementary Information

The systematic and comprehensive literature search was conducted up to March 2022 in order to identify all publicly available information considered pertinent to the safety assessment of heat-killed *Clostridium tyrobutyricum*. After this time, updated searches were conducted periodically including in November 2022 (at the point of submission of the GRAS notice) in order to identify the availability of any new data pertinent to the original safety assessment. Since submission of the GRAS notice, periodic brief searches of the published literature have also been performed.

From the most recent literature searches, it is pertinent that *Clostridium tyrobutyricum* has been recommended for inclusion in the Qualified Presumption of Safety (QPS) list established by the European Food Safety Authority (EFSA) (EFSA, 2024). The conclusions of the EFSA Panel were as follows:

"*No safety concerns were reported related to C. tyrobutyricum. No cytogenic strains of C. tyrobutyricum were found (Schallehn & Wolff, 1998). This was confirmed by a more recent study (Podzaj et al., 2022) on C. tyrobutyricum genomes indicating that so far this bacterium does not possess genes coding for*

toxins and virulence factors……C. tyrobutyricum is recommended for the QPS list with the qualification "absence of genetic determinants for toxin production."

The rationale for the assessment of *C. tyrobutyricum* under the QPS update in 2024 was the submission by Superbrewed Food, Inc. of a novel food application for heat-killed *Clostridium tyrobutyricum* to the European Union (EU). Superbrewed Food, Inc. confirms that the data submitted to the EU is the same as that submitted as part of the GRAS notice.

References cited by EFSA in QPS assessment:

Bao, T., Feng, J., Jiang, W., Fu, H., Wang, J. and Yang, S.T., 2020. Recent advances in n-butanol and butyrate production using engineered Clostridium tyrobutyricum. *World Journal of Microbiology and Biotechnology*, *36*, pp.1-14.

Liang, Q., Liu, J., Wei, J., Jia, J., Shen, H., Chen, W., Liang, W., Gao, B., Xu, Z. and Zhang, L., 2020. The effect of Clostridium tyrobutyricum Spo0A overexpression in the intestine of mice. *Beneficial Microbes*, *11*(6), pp.573-589.

Mosconi, M., Fontana, A., Daza, M.V.B., Bassi, D. and Gallo, A., 2023. *Clostridium tyrobutyricum* occurrence in silages and cattle feed: Use of molecular and simulation data to optimize predictive models. *Frontiers in Microbiology*, *14*, p.1118646.

Podrzaj, L., Burtscher, J., Küller, F. and Domig, K.J., 2020. Strain-dependent cheese spoilage potential of *Clostridium tyrobutyricum*. Microorganisms 8: 1836.

Podrzaj, L., Burtscher, J. and Domig, K.J., 2022. Comparative genomics provides insights into genetic diversity of *Clostridium tyrobutyricum* and potential implications for late blowing defects in cheese. *Frontiers in Microbiology*, *13*, p.889551.

Schallehn, G. and Wolff, M.H., 1988. Morphologische Veränderungen humaner embryonaler Lungenfibroblasten durch cytotoxine verschiedener clostridium-spezies. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. Series A: Medical Microbiology, Infectious Diseases, Virology, Parasitology*, *267*(3), pp.367-378.

Xiao, Z., Liu, L., Jin, Y., Pei, X., Sun, W. and Wang, M., 2021. Clostridium Tyrobutyricum protects against LPS-induced colonic inflammation via IL-22 signaling in mice. *Nutrients*, *13*(1), p.215.

Yang, Z., Amal, F.E., Yang, L., Liu, Y., Zhu, L., Zhu, Z. and Jiang, L., 2022. Functional characterization of Clostridium tyrobutyricum L319: a promising next-generation probiotic for short-chain fatty acid production. *Frontiers in Microbiology*, *13*, p.926710.

EFSA QPS Reference:

EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Hilbert, F., Lindqvist, R. and Nauta, M., 2024. Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 19: Suitability of taxonomic units notified to EFSA until September 2023. *EFSA Journal*, *22*(1), p.e8517.