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January 14, 2020

**Via Federal Express**

Office of Food Additive Safety
HFS-200
Center for Food Safety and Applied Nutrition
5001 Campus Drive
College Park, MD 20740-3835

Re: **New Generally Recognized as Safe Notice Submission for Fermented Microbial Protein; Our File No. 025299.00001**

Dear Sir or Madam:

On behalf of Sustainable Bioproducts, Inc., we hereby respectfully submit the enclosed Generally Recognized as Safe Notice (GRASN) for the use of Fermented Microbial Protein (FMP) in finished food to replace or supplement traditional sources of protein and other macronutrients in the human diet. This GRASN specifically concludes that FMP is GRAS under its intended conditions of use based on scientific procedures, as articulated in 21 C.F.R. § 170.30(a) and (b). FMP is not intended for use in infant formula or in meat or poultry products regulated by the United States Department of Agriculture (USDA). We are enclosing one CD copy of Form 3667, the GRASN and the relevant attachments.

We trust you will find that the information included in the enclosed GRASN is sufficient to demonstrate that FMP is GRAS under its intended conditions of use. Please contact us by telephone or e-mail if there are any questions regarding this FCN so that we can respond right away.

Sincerely yours,

[redacted]

Joan Sylvain Baughan

Enclosures

# Attachment 1

# GRAS Notice for FMP

**Generally Recognized as Safe (GRAS) Notification**
**Fermented Microbial Protein**

Prepared for:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, Maryland 20740

Submitted by:
Sustainable Bioproducts, Inc.
1452 East 53rd Street
Chicago, Illinois 60615

Preparer:
Steptoe & Johnson, LLP
1330 Connecticut Avenue NW
Washington, DC 20036

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**Summary**

Fermented microbial protein (FMP) is a new food product grown through surface fermentation of *Fusarium novum. yellowstonensis* and can be used as an ingredient or macronutrient. Fermented microbial protein (FMP) is the mycelial network of the fungal microorganism *Fusarium novum. yellowstonensis*,[1] discovered in Yellowstone National Park. Under appropriate cultivation conditions, FMP grows into a filamentous mat of mycelial biomass, termed biomat, with a texture profile similar to that of muscle fibers. The biomat is 20-30% solids and can be dried and ground to a flour of ~95% solids. It is intended for use in a wide variety of finished foods to replace or supplement traditional sources of protein and other macronutrients in the human diet.

FMP has an excellent nutritional profile as a macronutrient and protein source that provides all the essential amino acids and significant amounts of dietary fiber with no trans-unsaturated fatty acids. Based on its nutritional and textural profile, FMP can be used in a wide variety of finished foods to replace, as the primary source, or supplement traditional sources of protein and nutrients in the human diet.

Sustainable Bioproducts (SBP) has self-determined, using scientific procedures, that FMP is Generally Recognized as Safe (GRAS) for use as a food ingredient and macronutrient. A panel of experts, qualified by scientific training and experience to evaluate the safety of substances under the conditions of their intended use in food, also has reviewed the published and unpublished information and data on FMP and similar ingredients, and concluded that the product is GRAS for its intended use. FMP is not intended for use in infant formula or in meat or poultry products regulated by the U.S. Department of Agriculture and does not impart color to food. The report of the expert panel is included with this Notification. The safety assessment supporting this GRAS conclusion evaluated FMP's identity, nutritional composition, digestibility, potential allergenicity, and toxicology using publicly available information and data, and is supported by data generated both by the company and external accredited laboratories using accepted scientific procedures. In sum, these data provide ample basis to support a conclusion as to GRAS status for FMP as a food ingredient and protein replacement.

[1] ATCC PTA – 10698. Deposited March 2, 2010. At time of deposit the Identification Reference by Depositer was filed as *"Fusarium oxysporum:* MK7". Since 2010, further taxonomic characterization and genetic analysis has indicated the organism is a distinct species. The name for this distinct species is classified as *Fusarium novum. yellowstonensis.*

I. Signed Statements and Certification (Form 3667)

Introductory information about the submission, the Notifier, administrative information, and intended use information is included in Form 3667.

## II. Product Information

In accordance with 21 C.F.R. § 170.230, we provide here information on the identity, method of manufacture, specifications, and physical or technical effect of Fermented microbial protein (FMP).

### A. Identity

The subject of this GRAS Notice is Fermented microbial protein (FMP). FMP is the mycelial biomass grown through the surface fermentation of the fungal microorganism *Fusarium novum. yellowstonensis*. Mycelium is the vegetative part of a fungal organism and consists of a mass of thread-like filaments known as hyphae. *Fusarium novum. yellowstonensis* was discovered in Yellowstone National Park under a research permit, and under appropriate cultivation conditions grows into FMP. FMP can be used as a food ingredient, specifically as a source of macronutrients for food applications such as meat, dairy, and flour alternatives. FMP's primary component is protein although in contains fiber and other carbohydrates, minerals and other common food components.

*Fusarium novum yellowstonensis* is a distinct species of the Fusarium genus of fungi.[2] The wild type (i.e., unmodified) strain of *Fusarium novum. yellowstonensis* is being used for production of FMP.

**Kingdom**: Fungi
**Division:** Ascomycota
**Subdivision:** Pezizomycotina
**Class:** Sordariomycetes
**Order:** Hypocreales
**Family:** Nectriaceae
**Genus:** *Fusarium*
**Species:** *Fusarium novum. yellowstonensis*

Fermented microbial protein (FMP) refers to the biomat comprised of the mycelial network of the fungal microorganism *Fusarium novum. yellowstonensis.* This biomat is 20-30% solids, but also may be dried and ground to ~95% solids (e.g., Fusarium Mycelium flour). The only difference between the biomat and flour is the removal of water.

Typical composition of FMP on a dry weight basis is as follows:

- >45% Protein
- 5-15% Non-fiber carbohydrates
- 25-35% Total dietary fiber
- 5-10% Fat
- <5% Ash
- <0.5% Sugar

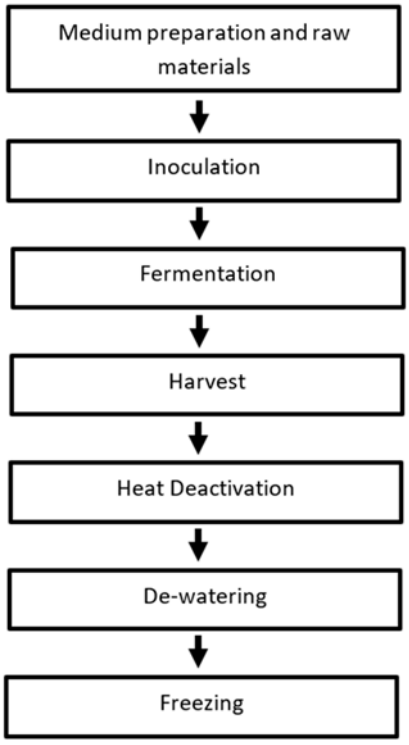
[2] Watanabe, M., and Yonezawa, T., et. al (2011). Molecular phylogeny of the higher and lower taxonomy of the Fusarium genus and differences in the evolutionary histories of multiple genes. BMC Evolutionary Biology, 11, 322nd ser. Retrieved from https://bmcevolbiol.biomedcentral.com/articles/10.1186/1471-2148-11-322#rightslink.

As FMP is the biomat of a *Fusarium sp.* fungal microorganism, secondary metabolites, such as mycotoxins, may be produced during cultivation. Extensive analysis has been conducted to determine if any mycotoxins are produced by *F. novum yellowstonensis* using both genetic sequencing and analytical testing of the finished product. This analysis has determined that only two mycotoxins, fumonisins and beauvericin, may be present in FMP at very low levels. This testing repeatedly demonstrated that these mycotoxins are not present in many cases and in all cases are below levels that would represent a safety concern based on safety levels established by regulatory authorities.

B. Manufacturing Process

FMP is grown through the surface fermentation of *Fusarium novum yellowstonensis* on a batch basis, using appropriate feedstock and cultivation conditions. The following flowchart provides an overview of the manufacturing steps:

**Figure 1: Overview of Manufacturing Process**




1. Raw Materials and Growth Medium Preparation

The raw materials used in the fermentation and growth of FMP are ingredients commonly used in the food, fermentation, and enzyme production industries. All the raw materials are food grade, or high-quality chemical or pharmaceutical grades (FCC, ACS, or USP grades) from approved suppliers. The fermentation medium is prepared in accordance with Good Manufacturing Practices (21 CFR Part 117) and specifications are set for the raw materials that include purity and heavy metals limits.

**Table 1: Manufacturing Process Medium Components**

| Medium Components | | |
|---|---|---|
| Food grade carbon source | Urea | Chloride source |
| Nitrate source | Yeast extract | Trace metals[3] |
| Phosphate source | Sulfate source | Water |

2. Inoculation

An inoculation starts by growing a pure frozen isolate culture into a master stock culture from which working culture stocks are prepared. The culture stock is grown in the liquid medium in the fermenter.

3. Fermentation and growth

FMP is grown by the fermentation of *Fusarium novum. yellowstonensis* in a cultivation chamber. The cultivation chamber is monitored for environmental conditions including, but not limited to, temperature and humidity. The growth process continues until the medium is consumed and the biomat is formed.

4. Harvest

Following completion of the growth stage, FMP is harvested by physically removing the biomat from the cultivation chamber in accordance with Good Manufacturing Practices. The harvest is conducted without the use of solvents or other chemicals.

5. Heat Deactivation

FMP is deactivated by exposure to a suitable heat treatment method resulting in cellular deactivation.

6. Dewatering

FMP is mechanically pressed to remove water resulting in a moisture content consistent with the specification in Table 3. The resulting product complies with the specifications summarized in Table 3 for use as a protein source and macronutrient in a variety of food applications. Further dewatering can be achieved using a suitable method such as dehydration and the dried product is ground to produce a flour.

7. Storage and Freezing

FMP, in both biomat and flour form, is sealed in food-grade packaging and frozen at or below -20$^{o}$C, at which point it can be held and will remain stable for up to 12 weeks. The frozen biomat or flour is

[3] Hutner et al., Proc. Am. Philos. Soc. 94: 152-170 (1950).

typically removed from -20°C frozen storage and used in its frozen state for processing into final product forms such as biomat in meat analogues or flour in fortified baked goods as described in Table 11: Intended Food Use Categories.

8. Stability

After manufacturing, FMP will be packaged and frozen for storage and shipping. To inform the shelf-life of FMP under these conditions, stability testing was conducted. Samples of FMP biomat were vacuum-sealed in food-grade plastic bags, frozen to -20°C and sampled at five time points: time zero and weeks three, six, nine, and twelve. The data from weeks zero, three, six, and twelve are present in the table below. The tested parameters are below and show that after twelve weeks of storage at frozen condition, FMP meets all the specifications set. Notably, no increase in mycotoxins or microorganisms was observed in any of the samples.

Based upon the results of the testing, FMP is stable under storage conditions of -20°C for at least twelve weeks.

**Table 2: Stability Testing Results Summary**

| | **Week 0** | **Week 3** | **Week 6** | **Week 12** |
|---|---|---|---|---|
| **Protein (%)** | 52.0% | 52.9% | 53.7% | 54.0% |
| **Ash (%)** | 2.6% | 1.42% | 3.34% | 2.4% |
| **Moisture (%)** | 79.6% | 79.2% | 78.4% | 80.0% |
| **Fat by acid hydrolysis (%)** | 8.8% | 8.7% | 8.8% | 10.0% |
| **Mycotoxin (Fumonisin B1+B2)** | <25.0 ppb | <25.0 ppb | <25.0 ppb | <25.0 ppb |
| **Aerobic plate count (CFU/g)** | 10 | NT | 10 | 35 |
| **Yeast (CFU/g)** | <10 | NT | <10 | <10 |
| **Mold (CFU/g)** | <10 | NT | <10 | <10 |
| **E. coli (CFU/g)** | <10 | NT | <10 | <10 |
| **Total coliforms (CFU/g)** | Negative<10 | NT | Negative | NT |
| **Salmonella (P or N)** | Negative | NT | Negative | NT |
| **Organoleptic Evaluation** | Tan solid<br>Clear vacuum sealed clear bag<br>Odorless | Tan solid<br>Clear vacuum sealed clear bag<br>Odorless | Tan solid<br>Clear vacuum sealed bag<br>Odorless | Tan solid<br>Clear vacuum sealed bag<br>Odorless |

C. Specifications

Product specifications are established for FMP based on its intended uses in food. Analyses of three non-consecutive production batches of FMP, conducted at accredited third-party laboratories, demonstrates compliance with the specifications as follows (results reported on dry weight basis, except for water content) (methods presented in Table 4 below):

**Table 3: Specifications and Batch Analysis Summary (dry weight)**

| Analyte | Specification | QCB 1091 | QCB 1099 | QCB 1112 |
|---|---|---|---|---|
| **Water content** | 70-80% | 76.0% | 76.5% | 77.2% |
| **Protein** | >45% | 47.9% | 50.0% | 51.3% |
| Total sugars | <0.5% | <0.35 | <0.35 | 0.49 |
| Glycerol | <4.0% | <1.0 | <1.0 | <1.0 |
| **Total dietary fiber** | 25-35% | 31.7% | 30.3% | 31.6% |
| Insoluble | None | 28.2% | 27.2% | 29.2% |
| Soluble | None | 3.5% | 3.0% | 2.4% |
| **Non-Fiber Carbohydrate** | 5-15% | 12.1% | 12.2% | 9.3% |
| **Total fat** | 4-10% | 5.0% | 4.5% | 4.6% |
| Saturated | 0-3% | 1.3% | 1.0% | 1.1% |
| Monounsaturated | 0-3% | 0.8% | 0.6% | 0.6% |
| Polyunsaturated | 2-6% | 3.0% | 2.9% | 2.9% |
| Trans | <1% | <0.02% | <0.02% | <0.02% |
| **Ash** | <5% | 3.0% | 2.9% | 3.0% |
| **RNA** | <2.0% | 0.02% | 0.00% | 0.01% |
| **Total Fumonisin** | <0.5 ppm | 0.06 ppm | <0.01 ppm | 0.05 ppm |
| **Total aerobic bacteria** | <10000 cfu/g | Pass | Pass | Pass |
| **Total yeast** | <100 cfu/g | Pass | Pass | Pass |
| **Total mold** | <100 cfu/g | Pass | Pass | Pass |
| ***E. coli*** | ND | ND | ND | ND |
| ***Salmonella*** | ND | ND | ND | ND |
| **Color** | Off white to light tan | Light tan | Light tan | Light tan |

**Table 4: Batch Analysis Analytical Methods**

| Analyte | Method |
|---|---|
| Water content | AOAC 925.09 |
| Protein | AOAC 990.03; AOAC 992.15 |
| Total sugars | AOAC 982.14, (Mod) |
| Glycerol | GC-MS |

| | |
|---|---|
| Total dietary fiber | AOAC 2009.01 & AOAC 2011.25 |
| Carbohydrate | Calculated |
| Total fat | AOAC 996.06 mod |
| Ash | AOAC 942.05 |
| RNA | Fluorometric RNA Assay |
| Fumonisin | LC-MS/MS |
| Total aerobic bacteria | U.S.P. Ch. 61 |
| Total yeast | U.S.P. Ch. 61 |
| Total mold | U.S.P. Ch. 61 |
| E. coli | U.S.P. Ch. 62 |
| Salmonella | AOAC 2003.09 |
| Color | Visual |

Specifications for the heavy metals lead, cadmium, arsenic, and mercury have been established at <0.1 ppm, similar to or below other protein replacement products (see, e.g., GRNs 91 (mycoprotein), 608 (pea protein), 609 (rice protein)). The 3-batch analysis demonstrated that FMP complied with the specifications for all the heavy metals.

D. Physical or Technical Effect

FMP is intended for use in a wide variety of finished foods to replace or supplement traditional sources of protein and other macronutrients in the human diet. Due to its texture and nutritional attributes, the FMP biomat (20-30% solids) can be used in food, for example as a meat analog. The biomat also can be dried and ground to a flour (~95% solids) and used as an ingredient providing a source of protein and other nutrients in the diet.

FMP is intended for use in products within the following categories: plant protein products, including meat and poultry analogs; dairy product analogs; milk products; beverages and beverage bases; breakfast cereals; fruit and vegetable juices; grain products and pastas; baked goods and baking mixes; soups and soup mixes; and fats and oils. FMP is not intended for use in infant formula or in meat or poultry products regulated by the U.S. Department of Agriculture and does not impart color to food. FMP is intended for use in applications similar to other meat and dairy alternative products, such as tofu, textured vegetable protein, rice protein, and mycoprotein.

FMP is suitable for use as a food because it provides a readily digestible source of protein with high levels of fiber and low levels of fats. It is particularly suited for use in protein-replacement applications because it supplies all the required amino acids, including the nine essential amino acids that cannot be made by the body and must be consumed in food.

1. Protein

FMP contains a high-quality protein as indicated by an average Protein Digestibility Adjusted Amino Acid Score (PDCAAS) of 0.91 out of 1.00.[4] The PDCAAS for FMP was measured using both *in vitro* and *in vivo* methods, resulting in scores of 0.92 and 0.91, respectively, (an average of 0.91). *In vitro* PDCAAS testing conducted on four batches of dried FMP reported scores of 0.92, 0.92, 0.93, and 0.91, for a mean score of 0.92. This PDCAAS is favorably comparable to common animal and non-animal-based protein sources listed in the table below.

**Table 5: PDCAAS Comparison**

| **Protein Source** | **PDCAAS Score[5]** |
|---|---|
| Casein | 1.00[a] |
| Whey protein isolate | 1.00[b] |
| Whey protein concentrate | 1.00[b] |
| Soy protein isolate | 1.00[b] |
| Egg | 1.00[b] |
| **FMP protein (*in vitro)*** | **0.92** |
| **FMP protein (*in vivo)*** | **0.91** |
| Beef | 0.92[a] |
| Pea protein concentrate | 0.89[b] |
| Pea | 0.67[a] |
| Cooked kidney beans | 0.65[b] |
| Cooked rice | 0.62[b] |
| Cooked peas | 0.60[b] |
| Roasted peanuts | 0.51[b] |
| Whole wheat | 0.45[a] |

[4] The nutritive value of a protein is determined by its capacity to provide nitrogen and amino acids in amounts sufficient to meet human needs. PDCAAS is a method of evaluating the quality of a protein based on the amino acid requirements of humans and their ability to digest it. It is the method recommended by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organisation (WHO) for evaluating protein quality. See FAO, *Protein Quality Evaluation, Report of Joint FAO/WHO Expert Consultation*, FAO Food and Nutrition Paper 51 (4-8 December 1989), https://apps.who.int/iris/bitstream/handle/10665/38133/9251030979_eng.pdf?sequence=1&isAllowed=y. The PDCAAS is required by FDA regulations (21 C.F.R. § 101.9(c)(7)) to support claims for the protein content of food.

[5] Scores annotated "a" were identified in van Vliet, S, Burd NA, and van Loon, LJ, *The Skeletal Muscle Anabolic Response to Plant-Versus Animal-Based Protein Consumption*, The Journal of Nutrition, 145(9): 1981-1991 (2015), while scores annotated "b" were identified in Rutherfurd, SM et al., *Protein Digestibility-Corrected Amino Acid Scores and Digestible Indispensable Amino Acid Scores Differentially Describe Protein Quality in Growing Male Rats*, Journal of Nutrition, 145(2): 372-379 (Feb. 2015).

2. Amino Acids

FMP is a complete protein because it is a source of all of the amino acids found in consumed proteins, including the nine essential amino acids that cannot be made by the human body and must be consumed in food.[6] The essential amino acids make up 20-24% of the total dry weight and the total amino acids make up 40-50% of the total dry weight. Branched chain amino acids, leucine, isoleucine, and valine make up 10-15% of the dry weight and 20-25% of the total protein content. The essential and non-essential amino acids combined comprise all the organic nitrogen content in the protein.

Based on analyses of three non-consecutive production batches of FMP, the average amino acid content is as tabulated below.[7] In addition, FMP compares favorably to other protein replacement products used as meat and dairy analogs.

**Table 6: Amino Acid Composition and Comparison to Alternative Protein Replacement Products**

| **Amino Acid** | **Content in FMP (% of total, dry weight basis)** | **Content in tofu (% of total, dry weight basis)[8]** | **Content in green peas (*Pisum sativum*) (% of total, dry weight basis)[9]** |
|---|---|---|---|
| Isoleucine | **2.32%** | 2.82% | 0.92% |
| Leucine | **3.83%** | 4.61% | 1.53% |
| Histidine | **1.17%** | 1.43% | 0.51% |
| Lysine | **4.04%** | 2.93% | 1.50% |
| Methionine | **0.88%** | 0.70% | 0.39% |
| Phenylalanine | **2.13%** | 2.77% | 0.95% |
| Threonine | **2.57%** | 2.60% | 0.96% |
| Tryptophan | **0.85%** | 0.78% | 0.18% |
| Valine | **5.12%** | 2.89% | 1.11% |
| Tyrosine | **1.64%** | 2.32% | 0.54% |
| Serine | **2.36%** | 3.36% | 0.86% |
| Proline | **2.22%** | 3.59% | 0.82% |
| Glycine | **2.30%** | 2.43% | 0.87% |
| Alanine | **2.99%** | 2.56% | 1.14% |
| Glutamic Acid | **5.51%** | 10.90% | 3.51% |
| Aspartic Acid | **4.67%** | 6.75% | 2.35% |

[6] The nine amino acids that must be consumed in food are: isoleucine, leucine, histidine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.
[7] Analyses conducted using the following methods: AOAC 982.30 mod, AOAC 994.12 mod, and AOAC 988.15.
[8] Calculated from data - USDA National Nutrient Database: Basic Report: 16427, Tofu, raw, Regular, prepared with calcium sulfate. https://ndb.nal.usda.gov/ndb/foods/show?ndbno=16427
[9] Calculated from data - USDA National Nutrient Database: Basic Report: 11304, Peas, green, raw. https://ndb.nal.usda.gov/ndb/foods/show/3052?manu=&fgcd=&ds=

| | | | |
|---|---|---|---|
| Arginine | **2.96%** | 4.54% | 2.02% |
| Cystine | **0.40%** | 0.19% | 0.15% |

3. Carbohydrate and Dietary Fiber

The total carbohydrates in FMP are composed of soluble fiber, insoluble fiber, glycogen, and polysaccharides.

Based on the analyses of three non-consecutive production batches of FMP, the average carbohydrate composition is as follows:

**Table 7: Carbohydrate and Dietary Fiber Composition**

| **Type** | **Content (% of FMP, dry weight basis)** |
|---|---|
| Total carbohydrates | 42.4% |
| Total fiber | 31.2% |
| Insoluble fiber | 28.2% |
| Soluble fiber | 3.0% |
| Glycogen | 2.5% |
| Polysaccharides | 8.7% |

4. Fats

The fat content is comprised of mostly polyunsaturated and monounsaturated fats, a small amount of saturated fat, and no trans-unsaturated fats.

Based on analyses of three non-consecutive production batches of FMP, the average fats composition is as follows:

**Table 8: Dietary Fat Composition**

| **Type** | **Content (% of FMP, dry weight basis)** |
|---|---|
| Total Fats | 4.7% |
| Total Saturated Fat | 1.1% |
| Total Monounsaturated Fat | 0.6% |
| Total Polyunsaturated Fat | 2.9% |
| Total Trans Fat | <0.02% |
| Cholesterol | <0.8% |

5. Essential Nutrients

Based on analyses of three non-consecutive production batches, the representative vitamin and mineral contents is as follows (values reported on a dry weight basis):

**Table 9: Essential Nutrients**

| Substance | Content | Analytical Method |
|---|---|---|
| Vitamin B1 (Thiamine) | 0.3 mg/100 g | AOAC 942.23 (Mod) |
| Vitamin B5 (Pantothenic acid) | 0.9 mg/100 g | AOAC 945.74 (Mod) |
| Vitamin B6 | 0.1 mg/100 g | JAOAC 88, 30-37 |
| Vitamin B12 | <0.440 µg/100 g | AOAC 952.20 (Mod) |
| Vitamin C | <0.440 µg/100 g | AOAC 967.22 (Mod) |
| Vitamin D | <4 IU/ 100g | LC-MS/MS |
| Vitamin E | <0.2 mg/100 g | AOAC 971.30 |
| Potassium | 5,000-6,500 ppm | AOAC 984.27 |
| Calcium | 1,000-2,000 ppm | AOAC 984.27, 927.02, 985.01, 965.17(Mod) |
| Sodium | 25-300 ppm | AOAC 984.27, 927.02, 985.01, 965.17 (Mod) |
| Chloride | <600 ppm | AOAC 969.10 (Mod) |
| Magnesium | 300-450 ppm | AOAC 984.27, 927.02, 985.01, 965.17 (Mod) |
| Phosphorus | 7,000-9,000 ppm | AOAC 984.27, 927.02, 985.01, 965.17 (Mod) |
| Iron | 15-40 ppm | AOAC 984.27, 927.02, 985.01, 965.17 (Mod) |

As FMP is intended for use as a food ingredient in a similar manner to other protein-replacement products such as tofu, pea protein, and mycoprotein, a comparison of the nutritional composition of these products is summarized below. This comparison illustrates that FMP provides a similar nutritional profile and does not result in a nutritional deficiency.

**Table 10: Nutritional Comparison**

| Per 100 g (wet weight) | | | | |
|---|---|---|---|---|
| **Nutrient** | **FMP** | **Mycoprotein[10]** | **Tofu, raw[11]** | **Peas, green, raw (*Pisum sativum*)** |
| Water % | **75** | 75 | 85 | 79 |
| Energy (kcal) | **97[12]** | 85 | 76 | 81 |
| Protein (g) | **12** | 12 | 8.1 | 5.4 |
| Total fat (g) | **1.1** | 3 | 4.8 | 0.4 |
| Saturated fatty acids (g) | **0.3** | 0.5 | 0.7 | 0.1 |
| Monounsaturated fatty acids (g) | **0.2** | 0.4 | 1.1 | 0 |
| Polyunsaturated fatty acids (g) | **0.7** | 1.4 | 2.7 | 0.2 |
| Total Carbohydrates (g) | **2.4** | 3.0 | 1.9 | 14.5 |
| Sugars (g) | **<0.5** | 0.5 | 0.6 | 5.7 |
| Dietary fiber (g) | **7.4** | 6.0 | 0.3 | 5.7 |
| Vitamin B12 (ug) | **0.5** | 0 | 0.0 | 0 |
| Sodium (mg) | **2.2** | 5.0 | 7.0 | 5.0 |
| Cholesterol (g) | **0** | 0 | 0 | 0 |
| Iron (mg) | **0.7** | 0.5 | 5.4 | 1.5 |
| Zinc (mg) | **1.9** | 9.0 | 0.8 | 1.2 |
| Selenium (ug) | **0** | 20.0 | 8.9 | 1.8 |

[10] Miller SA and JT Dwyer. Evaluating the Safety and Nutritional Value of Mycoprotein. Food Technology 55:7, 42-46 (July 2001).

[11] USDA National Nutrient Database: Basic Report: 16427, Tofu, raw, Regular, prepared with calcium sulfate. https://ndb.nal.usda.gov/ndb/foods/show?ndbno=16427

[12] Calories calculated based on United States Department of Agriculture: "How many calories are in one gram of fat, carbohydrate, or protein?" https://www.nal.usda.gov/fnic/how-many-calories-are-one-gram-fat-carbohydrate-or-protein.

## III. Estimate of Dietary Exposure

In accordance with 21 C.F.R. § 170.235, this section provides an estimate of the dietary exposure to FMP and a discussion of the data and information used to support the estimate. In addition, estimates of dietary exposure have been calculated for two categories of substances that may be present in FMP: (1) mycotoxins; and (2) nitrates. Mycotoxins are secondary metabolites that may naturally be generated by *F. novum yellowstonensis* during biomat growth, while nitrates may be carried through the fermentation process from raw material inputs.

Given that this is the first and only known use of FMP, the estimated daily intake (EDI) of FMP was conservatively calculated based on the maximum intended incorporation rate of FMP in representative foods drawn from the intended food categories, in combination with food consumption data provided in the National Health and Nutrition Examination Survey (NHANES) for 2011-2012.[13]

Mycotoxins are known to be present in a variety of food crops, such as cereal grains (e.g., corn and wheat), peanuts and tree nuts, apple juice, and coffee. Nitrates and nitrites are currently present in the food supply due in part to current FDA clearances for use in food under a variety of FDA regulations, as well as the natural presence of these substances in food. Where possible, the dietary exposure estimate incorporates information and data on current exposures to calculate a cumulative estimated dietary intake (CEDI).

### A. Estimated Daily Intake of Fermented microbial protein (FMP)

FMP is intended for use as a food ingredient and macronutrient in the following general food categories: plant protein products, including meat and poultry analogs; dairy product analogs; milk products; beverages and beverage bases; breakfast cereals; fruit juices and vegetable juices; grain products and pastas; baked goods and baking mixes; soups and soup mixes; and fats and oils.

The range of intended incorporation rates of FMP in various foods is presented in Table 11 below. As FMP may be used as a food ingredient in two forms, a wet biomat and dry flour, the intended use levels are presented below on a dry weight basis. Anticipated minimum and maximum use levels are provided.

[13] The NHANES is a program of studies conducted by the National Center for Health Statistics (NCHS) within the Centers for Disease Control and Prevention (CDC) and is designed to assess the health and nutritional status of adults and children in the United States. One component of the NHANES is a 2-day dietary survey of the study participants. The data from this survey is made publicly available and can be used to conduct intake estimates, see https://www.cdc.gov/nchs/nhanes/index.htm.

**Table 11: Intended Food Use Categories**

| **Food Category** | **Example Foods** | **Minimum Use Level** (% dry wt) | **Maximum Use Level** (% dry wt) |
|---|---|---|---|
| Plant protein products, including meat/poultry analogs | Meatless chicken nuggets, veggie burgers, lunch meat, meal replacement protein bars and instant breakfasts | 10.0% | 23.3% |
| Dairy analogs - non-beverage | Non-dairy yogurt | 3.8% | 17.5% |
| Dairy analogs – beverage | Non-dairy milk, creamer, cheese, milk-based meal replacements | 3.8% | 12.5% |
| Fruit and vegetable juices | High protein juices | 3.8% | 15% |
| Prepared meals and soups | Frozen or shelf stable prepared meals and soups | 3.8% | 15% |
| Pasta and grain products | Dried pasta, noodles | 2.5% | 10% |
| Baked goods and baking mixes | Breads and muffins, cakes, brownies, cookies, doughnuts, biscuits, pie, pancakes, waffles, tortillas, breakfast/granola bars, crackers, doughs | 2.5% | 10% |
| Fats and oils | Salad dressings, margarine, non-dairy cheese sauce, condiments, mayonnaise, gravies | 3.8% | 7.5% |

The EDI of FMP was calculated based on the maximum intended incorporation rate of representative foods, on a dry weight basis, in combination with food consumption data provided in the NHANES for 2011-2012. The data from the NHANES dietary surveys from these years has been previously evaluated for purposes of deriving an EDI for rice protein concentrate, another protein replacement, as summarized in Appendix I of GRN No. 609 (Rice protein concentrate).

For purposes of GRN 609, the notifier used data from the 2011-2012 surveys on the basis that they provided data from two 24-hour dietary recall surveys administered on 2 non-consecutive days, which would be considered more robust and representative of likely intakes.[14]

As the intended food categories for FMP are similar to GRN 609 as a protein replacement product, calculations for the mean all-person intake for the total population were performed for each proposed

[14] NHANES 2011-2012 survey data were collected from individuals and households via 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout the year. Day 1 data were collected in-person, and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S., of which 15 PSUs are visited per year. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. For NHANES 2011-2012, 13,431 individuals were selected for the sample, 9,756 were interviewed (72.6%) and 9,338 were sampled (69.5%).

use based on the food consumption estimate provided in GRN 609, adjusted for the different use levels of FMP in the food product as compared to GRN 609. For example, the intake of FMP from consumption of health bars is calculated as follows: 0.6 g/day rice protein intake ÷ 20% max rice protein use level × 10% max FMP use level = 0.3 g/day FMP intake.

**Table 12: Estimated Daily Intake of FMP (dry weight)**

| **Food Category** | **Rice Protein All-Person Mean Intake g/day** | **Rice Protein Max. Use Level** | **FMP Max. Use Level** | **FMP Intake g/day** |
|---|---|---|---|---|
| Meat analogues | | | | |
| Meat patty with soy protein | <0.1 | 34.3% | **23.3%** | **0.1** |
| Prepared soups and chili, with meat alternative[15] | 0.2 | 0.96% | **15%** | **3.1** |
| Dairy analogues - non-beverage / Non-dairy frozen desserts | | | | |
| Yogurt (regular and frozen) | 0.2 | 2% | **17.5%** | **1.8** |
| Prepared Soups, dairy-based | 0.1 | 0.96% | **15%** | **1.6** |
| Dairy analogues - beverage | | | | |
| Soy/imitation milks | 0.1 | 1.04% | **12.5%** | **1.2** |
| Flavored milk drinks | 0.3 | 1.04% | **12.5%** | **3.6** |
| Milk-based meal replacements | 0.1 | 1.04% | **12.5%** | **1.2** |
| Pasta and grain products | | | | |
| Ready-to-eat breakfast cereals | 1.1 | 16% | **10%** | **0.7** |
| Health bars and grain-based bars containing fruit and vegetable | 0.6 | 20% | **10%** | **0.3** |
| Prepared soups, noodle-based | 0.1 | 0.96% | **15%** | **1.6** |
| Fruit and vegetable juices | | | | |
| Fruit smoothies | 1.3 | 20% | **15%** | **1.0** |
| Vegetable juices (including smoothies) | 0.7 | 20% | **15%** | **0.5** |
| Baked goods and baking mixes | | | | |
| Breads | 1.5 | 4.8% | **10%** | **3.1** |
| Rolls | 0.8 | 4.8% | **10%** | **1.7** |
| Bagels | 0.2 | 4.8% | **10%** | **0.5** |
| English muffins | 0.1 | 4.4% | **10%** | **0.2** |
| Sauces and dressings | | | | |
| Margarine | 0.6 | 17.12% | **7.5%** | **0.3** |
| Salad dressings | 0.7 | 8% | **7.5%** | **0.7** |
| Meal replacement | | | | |
| Non-milk based meal replacements | <0.1 | 1.04% | **12.5%** | **1.2** |
| **Sum** | | | | **24.4** |

[15] The prepared soups category identified in GRN 609 has been divided to more properly reflect the intended uses of FMP as a replacement for meat- and dairy-based prepared soups.

Calculation of the 90th percentile all-person intakes were calculated following the FDA's "Guidance for Industry: Estimating Dietary Intake of Substances in Food"[16] based on a "pseudo" 90th percentile that is twice the mean EDI. As summarized above, the mean intake of FMP is calculated to be 24.4 g/day. This results in a pseudo-90th percentile intake of 48.8 g/day (dry weight).[17]

To put such an intake into a lay perspective, we can consider FMP as a major ingredient perhaps 25% of a given food based on the highest use level in Table 12. If this example food contained 50g of dry weight FMP it would have a total of 200g of ingredients by dry weight and the wet version of the food containing two-thirds to three quarters water by weight would be between 600g and 800 g of food eaten each day. In comparison, the same 50g of FMP used at a maximum of 7.5% by weight in food would require a dry food weight of 665g. This dry weight of food would have a wet version of between 1995g and 2660g. In these terms it is easy to see that to achieve the highest consumption levels of FMP estimated an individual would need to consume very large quantities of food containing FMP as an ingredient at the highest levels to achieve our 90th percentile intakes. Thus, we believe that these intakes must be conservative.

B. Estimate of Dietary Exposure to Mycotoxins

As discussed above, Fusarium species have the potential to produce mycotoxins as secondary metabolites. SBP has conducted extensive analysis to determine if any mycotoxins are produced by *F. novum yellowstonensis* using both genetic sequencing and analytical testing of the finished product. Genome sequencing and analysis indicated the *potential* for formation of the mycotoxins fumonisins, beauvericin, fusarin C, and fusaric acid. Analytical testing using accepted methods was then used to determine whether *F. novum yellowstonensis* produces these mycotoxins under the specific manufacturing conditions used for FMP. This testing has repeatedly demonstrated that these mycotoxins either are not present or are below levels that would present a safety concern when consuming FMP.

The results of analytical testing on three-batches of FMP for the potential mycotoxins are summarized in the following table:

[16] FDA, *Guidance for Industry: Estimating Dietary Intake of Substances in Food*, August 2006, https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-estimating-dietary-intake-substances-food.

[17] We note that although GRN 609 used the "all-user" values for its EDI, the calculated "all-person" and "all-user" values are almost identical (10.1 g/day vs. 10.3 g/day for all-person as compared to all-user). Moreover, the 90th percentile intake for both all-person and all-user intakes are less than double the mean values (e.g., the mean all-person intake is 10.1 g/day, while the 90th percentile all-person intake is 17.2 g/day), indicating the conservatism of our use of the "pseudo" 90th percentile approach.

**Table 13: Summary of 3-Batch Mycotoxin Analysis (Dry weight basis)**

| Analyte | Limit of Quantitation | QCB 1091 | QCB 1099 | QCB 1112 |
|---|---|---|---|---|
| **Fumonisins** | 0.01 ppm | 0.06 ppm | <0.01 ppm | 0.05 ppm |
| **Beauvericin**[18] | 0.01 ppm | 0.05 ppm | 0.12 ppm | 0.06 ppm |
| **Fusarin C** | 0.10 ppm | ND | ND | ND |
| **Fusaric Acid** | --[19] | ND | ND | ND |

1. Fusarin C and Fusaric Acid

Although genomic analysis indicated the potential for formation of fusarin C and fusaric acid, neither substance is identified in analytical testing.[20] [21] Therefore, no intake of fusarin C and fusaric acid is indicated from consumption of FMP and an intake value is not calculated.

2. Fumonisins

Low levels of fumonisins may be present in *F. novum yellowstonensis* as cultivated. FMP is subject to a specification for total fumonisins of <0.5 ppm, on a dry weight basis, based on the publicly available safety data. As evidenced in the three-batch analysis, fumonisin levels typically are well below the specification, with one batch below the detection limit of 0.01 ppm and two batches averaging 0.055 ppm. The results from the 3-batch analysis are consistent with fumonisin analyses of more than 250 batches of FMP that have been produced.

We have calculated, therefore, EDI values based on both the established specification and the average levels from the 3-batch analysis.

The EDI based on consumption at the specification is calculated as follows for the 90th percentile consumer (mean value would be half):

$$\frac{0.5\ mg}{kg\ TP} \times \frac{48.8\ g}{day} \times \frac{1\ kg}{1000\ g} = 0.024\ mg/day$$

A more representative exposure to fumonisins is determined conservatively using the results only from those batches in which fumonisins were detected, as follows (90th percentile consumer):

[18] Results corrected for recovery.
[19] No analytical standard is available for fusaric acid, thus limits of quantitation or detection cannot be determined.
[20] Fusarin C has not been identified in *F. novum yellowstonensis* through analysis using liquid chromatography-mass spectrometry (LC-MS/MS) and High-Resolution MS (LC-QTOFMS). Specifically, no peaks characteristic of fusarin C were identified in the mass spectrometry chromatograms.
[21] Sulyok M., Krska R., Schuhmacher R. A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. Analytical and Bioanalytical Chemistry (2007) 289:1505-1523.

$$\frac{0.055\ mg}{kg\ TP} \times \frac{48.8\ g}{day} \times \frac{1\ kg}{1000\ g} = 0.0027\ mg/day$$

The intake of fumonisins from FMP may be summarized as follows:

**Table 14: Dietary Intake of Fumonisins from FMP**

| Impurity | Specification mg/kg | Measured mg/kg | Mean EDI mg/day | 90th %tile EDI mg/day |
|---|---|---|---|---|
| Total Fumonisins (spec.) | 0.5 mg/kg | -- | 0.012 | 0.024 |
| Total Fumonisins (measured) | -- | 0.055 mg/kg | 0.0013 | 0.0027 |

As discussed in more detail in Section VI.D.1 below, fumonisins have been the subject of an evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

As part of its evaluation, JECFA provided information on current estimated daily intakes of fumonisins in a variety of countries, including the United States. As reported by JECFA, in the US, the estimated mean chronic dietary exposures to total fumonisins for the general population are 0.068-0.28 µg/kg bw/day, while the estimated 90th percentile exposures are 0.14-0.57 µg/kg bw/day for total fumonisins. To calculate the cumulative estimated daily intake (CEDI), we first have calculated the EDI for fumonisins from FMP on a body weight basis, using FDA's default assumption that a person weighs 60 kg. For example, the measured intake of fumonisins (90th percentile consumer) would be calculated as follows: 0.0027 mg/day ÷ 60 kg/person = 0.045 µg/kg bw/day.

When calculated for both the mean and 90th percentile consumers, using both the specification and the measured fumonisin levels in FMP, the EDIs are summarized as follows:

**Table 15: Estimated Daily Intake of Fumonisins**

| Impurity | Mean EDI mg/day | 90th %tile EDI mg/day | Mean EDI µg/kg bw/day | 90th %tile EDI µg/kg bw/day |
|---|---|---|---|---|
| Total Fumonisins (spec. <0.5ppm) | 0.012 | 0.024 | 0.2 | 0.4 |
| Total Fumonisins (measured, 0.055 ppm) | 0.0013 | 0.0027 | 0.022 | 0.045 |

When the calculated dietary exposure to fumonisins from consumption of FMP is added to the existing US dietary exposure indicated in the JECFA evaluation, the mean and 90th percentile upper-bound (UB) CEDIs are as follows:

**Table 16: Summary of Fumonisin EDIs and CEDIs**

| | Mean EDI µg/kg bw/day | 90th %tile EDI µg/kg bw/day | Mean UB CEDI µg/kg bw/day | 90%tile UB CEDI µg/kg bw/day |
|---|---|---|---|---|
| Total Fumonisins (spec. <0.55 ppm) | 0.2 | 0.4 | 0.48 | 0.97 |
| Total Fumonisins (measured) | 0.022 | 0.045 | 0.3 | 0.62 |

3. Beauvericin

Beauvericin has been detected in analytical testing at low levels. The three-batch analysis of FMP indicates an average beauvericin content of <0.1 ppm, and these results are consistent with other testing for beauvericin conducted on FMP. The EDI is calculated based on the average results from the three-batch analysis, with a mean of 0.08 ppm, or 0.08 mg/kg, as follows (90th percentile consumer):

$$\frac{0.08\ mg}{kg} \times \frac{48.8\ g}{day} \times \frac{1\ kg}{1000\ g} = 0.004\ mg/day$$

As discussed in more detail below, beauvericin has been evaluated by the European Food Safety Authority's (EFSA) Panel on Contaminants in the Food Chain (CONTAM).

One component of the CONTAM review determined the existing intake of beauvericin from food across 17 European countries based on the submission of occurrence data.[22] We have summarized the calculated mean and 95th percentile chronic exposures in the table below.

**Table 17: Existing Dietary Intake of Beauvericin (EU)**

| | EU Intake Mean Consumer µg/kg bw/day | EU Intake 95th %tile µg/kg bw/day |
|---|---|---|
| **Beauvericin** | 0.003-0.05 | 0.006-0.93 |

We have identified no intake estimate of beauvericin for US consumers, and given the relative similarity of the European and US diets, we have considered it reasonable to utilize the EU data regarding current intakes of beauvericin to be representative of the US population. Indeed, as the EU data calculated a 95th percentile intake (rather than 90th percentile), use of this data is conservative. We have, therefore, calculated the CEDI to beauvericin on the basis of the EU intake data in combination with the calculated intakes from FMP. We again first calculated the beauvericin EDIs (mean and 90th percentile consumer) on a body weight basis assuming an individual body weight of 60 kg:

[22] Although the CONTAM panel calculated existing upper- and lower-bound values for both chronic and acute dietary exposures, we have focused on the chronic data as being most relevant.

**Table 18: Beauvericin EDI**

| Impurity | Mean EDI mg/day | 90th %tile EDI mg/day | Mean EDI µg/kg bw/day | 90th %tile EDI µg/kg bw/day |
|---|---|---|---|---|
| Beauvericin | 0.002 | 0.004 | 0.033 | 0.067 |

These intakes were added to the upper-bound intake values for the mean and 95% percentile consumers from the CONTAM evaluation to calculate the CEDIs. Thus, the CEDIs are summarized below.

**Table 19: Summary of Beauvericin EDI and CEDI**

| | FMP Mean EDI µg/kg bw/day | 90 %tile EDI µg/kg bw/day | CONTAM EDI UB Mean µg/kg bw/day | CONTAM EDI 95%tile µg/kg bw/day | CEDI Mean UB µg/kg bw/day | CEDI 95%tile UB µg/kg bw/day |
|---|---|---|---|---|---|---|
| **Beauvericin** | 0.033 | 0.067 | 0.05 | 0.93 | 0.083 | 1.0 |

*Mycotoxin summary*

As fumonisin and beauvericin have been identified and quantified in the developmental production of FMP, a rigorous analytical testing program by LC-MS/MS has been established by SBP for these mycotoxins to ensure that exposures remain consistently within safe levels.

**Table 20: Summary of Mycotoxin EDIs and CEDIs**

| Impurity | Specification mg/kg | Measured mg/kg | Mean EDI mg/day | 90th %tile EDI mg/day | Mean CEDI µg/kg bw/day | 90th% CEDI µg/kg bw/day |
|---|---|---|---|---|---|---|
| Total Fumonisins (spec.) | 0.5 | -- | 0.012 | 0.024 | 0.48 | 0.97 |
| Total Fumonisins (measured) | -- | 0.055 | 0.0013 | 0.0027 | 0.3 | 0.62 |
| Beauvericin | -- | 0.08 | 0.002 | 0.004 | 0.083 | 1.0 |

4. Nitrates

Only one potential residual input, nitrate, has been identified in FMP as it is carried through from the manufacturing process. Nitrates are present in the fermentation medium as the constituent nitrogen is an essential building block for the natural synthesis of amino acids and proteins.

The three-batch analysis indicates that nitrates are typically present at a level of 25 ppm (25 mg/kg), on a dry weight basis. Based on FMP daily intake values of 24.4 g/day and 48.8 g/day for the mean and 90$^{th}$ percentile, respectively, this equates to nitrate EDIs of 0.61 mg/person/day and 1.2 mg/person/day, respectively,[23] or 0.01 mg/kg bw/day and 0.02 mg/kg bw/day, assuming a 60 kg individual.

Given the many potential sources of intake to nitrates in the food of common US and EU diets and the fact that the EDIs above are a tiny fraction of the typical intake of nitrates in the diet, we have not calculated a CEDI.

[23] E.g. (25 mg NO3/kg FMP) × (1 kg/1000 g) × (24.47 g FMP/p/day) = 0.61 mg/p/day.

## IV. Self-Limiting Levels of Use

No self-limiting levels of use have been identified.

V. Experience based on common use in food before 1958

This section is not applicable.

## VI. Basis for Conclusion of GRAS Status

Extensive information relating to product identity, nutritional composition, digestibility, potential contaminants and by-products, allergenicity, and toxicology has been assessed to determine Fermented microbial protein's (FMP) safety and the basis for its status as GRAS. Safety studies, including animal toxicology studies commissioned by the company and referenced in the publicly available literature on similar Fusarium-derived products, have indicated that there are no acute or chronic exposure concerns from consumption of FMP. Additionally, the available literature provides evidence that compositionally similar Fusarium-derived products support normal growth and development in animals and humans.[24] Through assessment by an independent expert institution, *F. novum yellowstonensis* has been determined to represent no significant additional risk for allergenicity to foods containing FMP. There are no impurities or secondary metabolites that present a safety concern from consumption of FMP, even at conservatively high intake levels and considering other potential sources of intake. The culmination of these data supports the conclusion that Fermented microbial protein(FMP) is GRAS for uses in food including those described here.

The safety of FMP is discussed below for each of these components.

### A. Available Safety Data

Based on toxicity testing conducted on FMP using published and accepted methods and information available in the public literature on other mycoprotein food ingredient products, there is a robust set of data available to support the safety of FMP for its intended uses in food. An acute toxicity test was completed on FMP, along with two genotoxicity tests. In addition, the publicly available literature of repeated dose toxicity studies on related mycoprotein products were evaluated and support the safety of FMP. Based on the acute *in vivo* test and genotoxicity testing on FMP, and the available repeated dose toxicity testing of similar mycoprotein food ingredients, FMP may be considered safe for its intended use.

#### 1. Acute Oral Toxicity Test

A short-term oral range-finding study for FMP was conducted in Sprague-Dawley rats (5/sex/dose) over 14-days at doses of 0, 5%, 10%, and 15% of the diet. The animals were observed at least once daily for viability, signs of gross toxicity, and behavioral changes, and weekly for a battery of detailed physical observations.[25] There were no abnormal physical observations resulting from FMP. All animals were subject to gross necropsy and no macroscopic observations were identified. No significant differences were noted in bodyweight gain, feed consumption, or feed efficiency over the course of the study as

[24] [GRN 91: Mycoprotein]

[25] The physical observations looked for included: 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), changes in gait, posture, and response to handling, the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling), or bizarre behavior (e.g., self-mutilation, walking backwards).

compared to the control groups, and no clinical observations were noted in the groups fed FMP, regardless of dose rate.

2. Genetic Toxicity

As part of the evaluation common for a new food ingredient, *F. novum yellowstonensis* [26]was assessed for potential genotoxic effects using two *in vitro* methods, the Ames and micronucleus tests. These tests, in addition to a review of the genotoxicity evaluation completed by Marlow Foods for their Fusarium-derived product, provided no evidence of genotoxic effects in *F. novum yellowstonensis* or in similar Fusarium spp.

a) Bacterial Reverse Mutation Test (Ames)

The bacterial reverse mutation test (Ames) test is a standard biological assay that uses bacteria to test the mutagenic potential of a food ingredient. *F. novum yellowstonensis* has been tested, following GLP guidelines, using the following bacterial strains: *Salmonella typhimurium* TA 98, TA 100, TA 1535, and TA 1537 and *E. coli* WP2 uvrA in both the absence and presence of S9 mix, at levels up to 5000 µg/plate. The S9 mix is a mammalian liver fraction used to mimic the mammalian metabolic conditions that are lacking in the *S. typhimurium* and *E.coli* cells. No increases in the number of revertant colonies were observed with any strain, in both the presence and absence of S9 mix. On these bases, *F. novum yellowstonensis* did not elicit evidence of bacterial mutagenicity in the Ames assay.

b) *In vitro* Micronucleus Test in Human Lymphocytes

The *in vitro* micronucleus test is used to determine the genotoxicity of a new food ingredient by evaluating the presence of micronuclei that contain chromosome fragments produced from DNA breakage or whole chromosomes produced by the disruption of mitosis. *F. novum yellowstonensis* has been tested, following GLP guidelines, using human peripheral blood lymphocytes both with and without S9 mix. *F. novum yellowstonensis* produced no cytotoxic effects up to the highest concentrations tested of 50ug/ml, in both the presence and absence of S9 mix. The evaluation of micronuclei frequencies did not show a biologically relevant increase compared to the controls.

c) Publicly Available Genotoxicity Literature

We have reviewed the publicly available literature for similar food products. Most relevant is the available toxicological data for a Fusarium-derived product subject to a GRAS Notice submitted by Marlow Foods, which is a food product produced from a *Fusarium sp*. organism, in this case *Fusarium venenatum*. As discussed in GRN 91, Marlow Foods conducted *in vitro* genetic toxicity testing on its mycoprotein product using the bacterial reverse mutation test (Ames) in *S. typhi* (strains not identified).

[26] As autoclaved culture.

The mycoprotein product was determined to be non-mutagenic in both the presence and absence of S9 mix metabolic activation.[27]

3. Repeated Oral Dose Studies of Marlow Foods' Fusarium-Derived Product

For repeated dose oral toxicity, there are a number of relevant studies that support the safe consumption of food products from *Fusarium sp*. Most relevantly, animal studies conducted on the compositionally similar Marlow Foods' Fusarium-derived product were considered. There is a robust database of repeated dose oral feeding studies, including four subchronic studies in rats, a 13-week study in baboons, a two-year carcinogenicity study (with an in-utero phase), a one-year dog study, a two-generation reproductive and developmental toxicity study (including a teratology phase), and studies in humans to assess tolerance to Marlow Food's Fusarium-derived product. These studies are summarized in the following table.

**Table 21: Summary of Relevant Repeated Dose Toxicity Studies**

| **Study Type** | **Species** | **Dosing (% of diet)** | **Results** | **Publication Status** |
|---|---|---|---|---|
| 22-week oral feeding study | Rats | 26-52% | With exception of caecal enlargement attributed to the high fiber content of the food, no significant findings were observed. | Miller and Dwyer (2001). |
| 13-week oral feeding study with dried, cooked mycoprotein | Rats | 13%, 35%[28] | With the exception of caecal enlargement, no significant differences were found in growth, blood parameters, organ weights, and histopathology. | Miller and Dwyer (2001). |
| 13-week oral feeding study with undried mycoprotein | Rats | 13%, 35% | No significant differences were found in all parameters studied, including availability and balance of calcium, phosphorus, magnesium, iron, copper, and zinc. | Miller and Dwyer (2001). |
| 90-day oral feeding study, with in utero exposure and 90- | Rats | 20%, 40% | All diets demonstrated normal growth and development. At weaning, however, both male and female pups fed the high | Miller and Dwyer (2001). |

[27] Miller SA and JT Dwyer. Evaluating the Safety and Nutritional Value of Mycoprotein. Food Technology 55:7, 42-46 (July 2001).

[28] We note that there is a discrepancy between the reported dosing levels between the Miller and Dwyer article, which identifies the dosages as 13% and 35%, as compared to GRN 91, which states the dosages as 17.5% and 35%.

| | | | | |
|---|---|---|---|---|
| day studies in offspring | | | mycoprotein diet had lower body weights at weaning than the casein controls. While the proportional differences diminished during the 90-day feeding period, statistical differences remained. In addition, plasma cholesterol and triglycerides were lower in the rats fed mycoprotein. Liver weights were also lower. All of these effects could be attributed to the reduction in food intake observed in these animals. To test this hypothesis, the physical form of the diet was changed to improve its palatability. The differences in food intake and weight gain were abolished, confirming the hypothesis. | |
| 13-week oral feeding study | Baboons | 26%, 51.5% | All baboons survived the study in good condition and there were no effects on growth, food and water intake, or hematology. Serum alkaline phosphatase activity was increased in the groups fed mycoprotein and serum alanine transaminase and aspartate transaminase activities were increased from normal levels in the casein control group. There were no changes in electrocardiograms. The test groups showed marginally higher liver weights than the casein control and colon and rectum weights were higher, but this reflected the increased contents. No histopathological changes were seen. It was concluded that mycoprotein was without adverse effect, but the | Unpublished[29] |

[29] As summarized in GRN 91.

| | | | | |
|---|---|---|---|---|
| | | | casein diet produced a minimal degree of liver damage. | |
| Two-year Carcinogenicity study (with in-utero phase) | Rats | 21%, 41% (at 41%, all dietary protein was derived from mycoprotein) | Although minor intergroup variations were observed in some parameters, no significant adverse effects were noted in either phase of the study on growth, survival, incidence or onset of tumors or in hematological, urinary or histopathological examinations, indicating that mycoprotein is not toxic or carcinogenic. | Miller and Dwyer (2001). |
| One-year oral feeding study | Beagle dogs | 20%, 40% | The study examined growth, clinical condition, urine, hematology, and histopathology. All diets supported good growth and no clinical signs that were diet-related were reported. As in the rat, dogs fed diets containing mycoprotein showed lower plasma cholesterol and triglycerides; females showed marginally greater thyroid weight. No gross or microscopic pathological findings were made that were diet related. | Miller and Dwyer (2001). |
| Two-generation reproductive and developmental toxicity study | Rats | 12.5%<br>25%<br>50%<br><br>50% casein as control. | The mycoprotein diets supported good growth and maturation in both parental generations and offspring, and fertility was within normal limits. There were no changes in reproductive function that were attributable to mycoprotein, and there were no microscopic or pathological findings | Miller and Dwyer (2001). |
| Teratogenicity studies (7) | Rats and rabbits | | No evidence of teratogenic or embryo-toxic effects associated with mycoprotein-containing diets was observed. | Miller and Dwyer (2001). |

Additionally, the ingredient from *F. venenatum* has a 30+ year history of consumption as a food ingredient, being consumed in 17 countries and an estimated 5 billion servings have been consumed worldwide with low levels of reported adverse effects.[30] Although substances used as new food ingredients, such as FMP and the Marlow Foods Fusarium-derived product generally cannot be evaluated for safety in the same manner as traditional food additives because they are intended to be replacements for conventional macronutrients for use at relatively high levels in food, in this case this robust dataset strongly supports a conclusion that there will be no acute or chronic adverse effects from the consumption of FMP.[31]

B. Allergenicity

An evaluation of the potential for a new food protein to cause allergic reactions in consumers is a key component of a safety assessment. *F. novum yellowstonensis* has been evaluated by the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln to assess risks of allergy from consumption of FMP, using: 1) analysis and comparison of genetic sequence information, 2) a literature review, and 3) two *in vitro* digestibility studies. The genetic analysis portion extensively utilized the National Center for Biotechnology Information (NCBI) Protein[32] and FARRP's Allergen Online[33] databases. Additionally, in the literature review, particular attention was given to allergenicity concerns raised by exposure to Marlow Foods' Fusarium-derived product and potential cross-reactions for those with existing allergies. Based on these three components of work, FARRP concluded that food produced from *F. novum yellowstonensis* is unlikely to represent any additional risk of food allergy beyond what is currently available in the human food supply.

While there currently are no methods to accurately predict the probability of *de novo* sensitization to a new food, there are methods, such as those developed by *Codex Alimentarius*, for identifying proteins that have the potential for allergenicity. FARRP therefore assessed evidence of allergic reactions to the source material and genetic similarity of specific proteins known to cause allergies. Their evaluation followed the basic steps described by Codex Alimentarius for the evaluation of allergy risks for genetically engineered (GE) Crops.[34] Though Fermented microbial protein (FMP) is not a GE product (as noted above, the wild strain is being used in the manufacture of the product subject to this GRAS notice) the evaluation procedure was considered appropriate and relevant for a new food ingredient.

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[30] https://www.quorn.us/whats-new

[31] Specifically, it may not be feasible to establish a margin of exposure using standard toxicological safety factors, that is, as a fraction of the highest oral dose that has no adverse effects in animals. FDA, *Draft Guidance, Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food,* Redbook II, Chapter VII, *Emerging Issues in Safety Assessment of Food Additives and Color Additives Used in Food* (August 1993), https://www.fda.gov/media/72348/download.

[32] https://www.ncbi.nlm.nih.gov/protein.

[33] www.AllergenOnline.org.

[34] Codex Alimentarius, *Foods derived from modern biotechnology*, *Principles for risk analysis and guidelines for the conduct of food safety assessment of foods derived from recombinant-DNA plants, micro-organisms and animals*. 2nd Ed., WHO/FAO 2009.

1. Genetic Sequencing and Allergenic Protein Identification

FARRP analyzed the genetic sequence of *F. novum yellowstonensis* to identify coding regions for potential allergenic proteins. FARRP determined that *F. novum yellowstonensis* proteins have high homology to proteins that are in the mycoprotein product and other evolutionarily close *Fusarium sp.* Additionally, FARRP determined there was no homology to high potency allergenic proteins that are common in the food supply chain such as the eight major food allergens that are identified by the FDA.[35] In summary, FARRP concluded that *F. novum yellowstonensis* is unlikely to represent any additional risk of food allergy beyond what is currently available in the human food supply.

*F. novum yellowstonensis* has been the subject of genetic sequencing and annotation. Based on the highest genetic identity matches, *F. novum yellowstonensis* is predicted to be most closely related to members of the *Fusarium fujikuroi* species complex. FARRP analyzed the annotated genome and identified 14,239 potential proteins from sequence predicted proteins based on the gene sequence of *F. novum yellowstonensis*. FARRP evaluated the protein sequences[36] and characterized the similarity of sequences identified for *F. novum yellowstonensis* to the Allergen Online database.[37] FARRP then determined[38] the highest matched sequences to *Fusarium sp*. proteins as well as those in other species.[39]

A number of allergens in the Allergen Online database were identified as matching predicted *F. novum yellowstonensis* proteins using FASTA35 alignments, with identities above the conservative Codex criteria of >35% identity over 80 amino acids. Further searches were performed to understand the potential risks associated with the sequence matched proteins, including comparison of the published predicted proteins from *Fusarium venenatum* (i.e., mycoprotein). While FARRP concluded that nearly all of the proteins in *F. novum yellowstonensis* were close homologues of the mycoprotein product proteins, more detailed studies showed that they generally are homologues of fungi that are evolutionarily close to *Fusarium sp*.

As discussed, FARRP additionally concluded that there were no high identity matches to other common and potent allergenic proteins. There were no matches to 2S albumins of peanut or tree nuts, and no matches to lipid transfer proteins from peach or related fruits. There were no significant matches to vicilins or tropomyosins that are major sources of severe reactions from peanut, tree nut, shrimp, cockroach, or house dust mites. Of the sequence identity matches to allergenic proteins in the Allergen Online database, all were to moderate allergenic proteins only that do not show evidence of clinical reactivity.

[35] https://www.fda.gov/food/buy-store-serve-safe-food/what-you-need-know-about-food-allergies
[36] https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=select&pgm=fa.
[37] Version 18B.
[38] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins.
[39] Goodman, R.E., *New Fusarium species fermentation product proteins: Bioinformatics to evaluate potential allergenicity*, Study No. REG 2019 Sustainable 3 (24 March 2019). Dr. Goodman used the FASTA35 program for the analysis.

On the basis of the genetic sequencing and protein identification, FARRP concluded that food produced from *F. novum yellowstonensis* is unlikely to represent any additional risk of food allergy beyond what is currently available in the human food supply.

2. Summary of FARRP Literature Review[40]

To complement the genome analysis, FARRP conducted a literature review to qualify the risk of the allergenic proteins in *Fusarium sp.*, identifying and conducting a safety evaluation of four proteins recognized for their potential reactivity below. In addition, due to identified similarities between *F. novum yellowstonensis* and Marlow Food's Fusarium-derived product, which is produced with *Fusarium venenatum*, FARRP included an allergenicity review of the Marlow Food's product. Based on the literature review, FARRP concluded that allergic reactions from consumption of foods containing proteins from various *Fusarium sp.* are rare in published studies.

Specifically, FARRP used the WHO/IUIS Allergen Nomenclature database[41] and the Allergen Online database to identify allergenic proteins from the *Fusarium fujikuroi* species complex, which includes *Fusarium proliferatum* and *Fusarium mangiferae*. FARRP then identified published scientific reports of allergy in the literature, using the PubMed database and general internet searches with relevant keywords. Four proteins identified in *Fusarium sp.* (Fus c 1 (Ribosomal P2), Fus c 2 (Thioredoxin), Fus p 4 (Enolase) and Fus p 9 (transaldolase) have been accepted as allergens in the WHO/IUIS Allergen Nomenclature database and are listed in the AllergenOnline.org database. An additional potential allergen from *Fusarium culmorum* (Cytrochrome C, which has not been recognized by the WHO/IUIS Allergen Nomenclature committee) was also included in the literature review. References identified for the five allergens were evaluated in the context of known food allergies and reviewed for evidence of possible cross-reactivity.

Additionally, FARRP reviewed self-reported cases of allergenicity to the Marlow Foods Fusarium-derived product that were made available by Marlow Foods[42] and Center for Science in the Public Interest (CSPI), an unrelated non-governmental entity.[43] The CSPI data involved alleged allergenic reactions from 312 reports received over the course of twelve years (2002-2014), as reported by Jacobson and DePorter. Of these reports, only two included medical records that permitted a more thorough review by the authors. Although additional post-2014 reports are included where skin-prick tests indicated allergenic responses for two individuals, *Fusarium sp.* proteins were not identified as the source of the allergenic response and it may have been due to other ingredients included in Marlow Foods' final product

[40] Goodman, R.E., *Fusarium mycoprotein fermentation product literature survey*, Study No. REG 2019 Sustainable 1 (24 March, 2019). The BLASTP program and NCBI Protein database were used for the analysis.
[41] http://www.allergen.org/.
[42] Finnegan T, et al. *Mycoprotein: The Future of Nutritious Nonmeat Protein, a Symposium Review,* Current Developments in Nutrition, 3:nzz021 (2019). Marlow foods has tracked worldwide consumer complaints since 1985. In the last 15 years, the frequency of self-reported illnesses per packages sold was 1 per 683,665 or 1 per 1.85 million servings. The frequency of IgE mediated reactions was 1 per 8.99 million packages or 1 per 24.3 million servings.
[43] Jacobson MF and DePorter J. *Self-reported adverse reactions associated with mycoprotein (Quorn-brand) containing foods*, Ann. Allergy Asthma Immunol., 120: 626-630 (2018).

formulation. FARRP concluded that "the described prevalence and severity of reactions is not remarkable based on the source and amount of food that is consumed in a meal…and may be due to consumption of high concentrations of fiber" in the mycoprotein product.[44]

In summary, FARRP concluded that allergic reactions from consumption of foods containing proteins from various *Fusarium sp*. are rare in published studies and that *F. novum yellowstonensis* presents minimal risks of food allergy to consumers.

3. FARRP Digestibility Studies and Allergenicity

For the third component of the allergenicity analysis, as discussed above FARRP conducted *in vitro* digestibility studies on *F. novum yellowstonensis*. The studies give an approximation of the digestibility of proteins. Longer time to digest and incomplete digestion can indicate a potential for an allergic response that would need to be investigated further. The studies showed that most of the proteins in *F. novum yellowstonensis* are quickly and readily digestible by the enzymes and conditions common for human digestion and that there is relatively low risk for dietary allergenicity.

FARRP's *in vitro* digestibility study measured the protein concentrations in *F. novum yellowstonensis* and tested the stability of the proteins in a standard pepsin digestion assay. The assay used a simulated gastric fluid (SGF) model with porcine pepsin to digest the material at pH 2, followed by removal of timed samples from time 0 minutes to 60 minutes. The samples were run in SDS-PAGE reducing gels followed by staining with Coomassie Brilliant Blue to evaluate the disappearance of detectable protein bands. The undigested sample bands ranged from nearly 125 kDa to approximately 5 kDa in size. At 30 seconds of digestion the higher molecular weight (MW) bands were no longer visible. Bands up to 30 kDa diminished over time with the smallest bands barely detectable at 13 kDa after 60 minutes of digestion.

Stability in simulated intestinal fluid (SIF) was assessed using porcine pancreatin at pH 7.5 in a timed assay up to 120 minutes of digestion. A sample of spinach leaf Rubisco was used as a digestion control protein in parallel with the *F. novum yellowstonensis* sample. Samples were removed at specific times and the reactions quenched. All samples were evaluated in an SDS-PAGE reducing gel after staining with Coomassie Brilliant Blue. As in the SGF assay, the undigested samples showed protein bands from 125 kDa down to 5 kDa. Those bands disappeared rapidly during digestion, without visible bands after 2 minutes.

The results of these assays demonstrated that the proteins in *F. novum yellowstonensis* samples digested moderately well in SGF, leaving only 20% residual stained bands based on densitometry by five minutes of digestion. The samples digested more rapidly in SIF with less than 10% residual stained bands based on densitometry that were visible by 30 seconds of digestion. While these assays do not predict the specific behavior of proteins in the human digestive tract, due to large variations in digestive responses between humans and under diverse circumstances, they do show that most of the proteins in

[44] Goodman, supra note 39.

this species are digestible and labile by the enzymes and conditions common for human digestion. FARRP concluded, therefore, that the ready digestibility suggests a low risk for dietary allergenicity.

4. Major Food Allergens and Sensitivities

None of the major food allergens required to be labeled pursuant to the Food Allergen Labeling and Consumer Protection Act (FALCPA) are present in the raw materials or manufacturing process of fermented microbial protein (FMP). Even though there is no anticipated concern, allergen testing was conducted at an analytical food contract laboratory for the three-batch analysis. No allergenic proteins for the following foods/food groups were identified in the testing: milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans. In addition to these allergens, FMP was tested also for the presence of sulfites and none were detected at a detection limit of 10 ppm. The testing performed and technical literature establish that there is no safety concern, therefore, for the presence of the major food allergens nor the presence of common additives that can cause sensitivities in FMP.

C. Nutrition and Digestibility

As a food ingredient, FMP provides a high-quality, readily digestible source of protein and other essential macronutrients. It has high levels of fiber, low levels of fat, and supplies all the required amino acids, including the nine essential amino acids that cannot be made by the human body and must be consumed in food. As discussed below, the publicly available literature indicates that products of this type (e.g., mycoprotein) offer potential cholesterol, blood sugar, and satiety benefits, with a very low risk of adverse gastrointestinal or physiological effects at usual intakes. The analytical testing on FMP, supported by the public literature, demonstrates that FMP presents a beneficial nutritional profile.

*In vitro* PDCAAS testing conducted on four batches of dried FMP demonstrated a mean score of 0.92, while in vivo tests reported an average score of 0.91. The Fusarium-derived product from GRN 91 reports a PDCAAS of 0.91, showing consistency between the two *Fusarium sp*. The PDCAAS scores for FMP compare favorably with other protein sources and products, such as beef and pea protein concentrate.[45]

As part of the battery of allergenicity testing, the digestibility of FMP was evaluated through two digestion assays using simulated gastric fluids and simulated intestinal fluids.[46] These tests showed that most FMP proteins are digested to smaller protein molecular weights by pepsin, and more efficiently by pancreatin. One protein of ~ 30 kDa was visible through 30 minutes of digestion in pepsin, but gone at 60 minutes. Pancreatin digested all proteins to below 15 kDa. Semi-stable bands were visible above 10

[45] See van Vliet, S, Burd NA, and van Loon, LJ, *The Skeletal Muscle Anabolic Response to Plant-Versus Animal-Based Protein Consumption*, The Journal of Nutrition, 145(9): 1981-1991 (2015) and Rutherfurd, SM et al., *Protein Digestibility-Corrected Amino Acid Scores and Digestible Indispensable Amino Acid Scores Differentially Describe Protein Quality in Growing Male Rats*, Journal of Nutrition, 145(2): 372-379 (Feb. 2015).

[46] Goodman R. and S. Ramadan, *Fusarium mycoprotein fermentation product proteins in vitro digestibility study in a simulated gastric fluid (pepsin) assay (pH 2.0) and in a simulated intestinal fluid assay (pH 7.5)*, 23 March 2019.

kDa, but as smeared stainable bands, and mostly gone by 60 minutes. The results established that the proteins in FMP are readily digestible.

Food products similar to FMP have been the subject of interest and research to determine their potential role in lowering blood cholesterol concentrations, reducing energy intakes and controlling blood sugar levels.[47] Published studies have investigated the cholesterol-lowering effects of mycoprotein and, overall, have reported statistically significant reductions in total cholesterol amongst hypercholesterolaemic subjects (in the order of 4–14%).[48] A number of studies have investigated the effects of mycoprotein in comparison with other protein sources on satiety and have indicated a potential for greater effects on satiety as compared to an equivalent amount of chicken.[49]

Reports of adverse gastrointestinal effects from consumption of the mycoprotein product,[50] such as bloating, gas, constipation, and/or diarrhea, may be linked to the fiber content of the product. FMP includes dietary fiber at levels of 25-35%, on a dry weight basis. The mean EDI calculated in Section III.A is 24.4 grams/person/day with a 90$^{th}$ percentile EDI of 48.8 grams/person/day. Based on a 2,000-calorie diet, the FDA's adult Daily Value for fiber is 28 g per day.[51] To consume enough FMP to meet the Daily Value of 28 g per day, an adult human would need to consume 80 -112 grams of FMP on a dry basis, which equates to 320 - 448 grams of FMP biomat (~25% solids, with the biomat expected to be the more commonly consumed version of the product). It is, therefore, very unlikely that usual intakes of FMP would result in either adverse gastrointestinal or physiological effects.

D. Mycotoxins

As discussed in Section II.A, *F. novum yellowstonensis* has the potential to produce certain mycotoxins as secondary metabolites. SBP has conducted extensive analysis to determine if any mycotoxins are produced by *F. novum yellowstonensis* using both genetic sequencing and analytical testing of the finished product. Genome sequencing and analysis indicated the *potential* for formation of the mycotoxins fumonisins, beauvericin, fusarin C, and fusaric acid. Analytical testing was then used to determine whether *F. novum yellowstonensis* produces these mycotoxins under the specific manufacturing conditions used for FMP. This testing has repeatedly demonstrated that these mycotoxins are not present at levels that would represent a safety concern.

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[47] Denny, A., Aisbitt, B., and J. Lunn. *Mycoprotein and health*, Nutrition Bulletin 33(4):298-310 (November 2008); Dunlop, et al. *Mycoprotein represents a bioavailable and insulinotropic non-animal-derived dietary protein source: a dose–response study*, British Journal of Nutrition, 118(9): 673-685 (November 2017); Derbyshire, E. and KT Ayoob, *Mycoprotein Nutritional and Health Properties*, Nutrition Today, 54(1): 7-15 (January-February 2019); Finnegan, T. et al., *Mycoprotein: The Future of Nutritious Nonmeat Protein, a Symposium Review, Current Developments in Nutrition*, 3(6) (June 2019).

[48] Denny (2008) (supra note 46).

[49] Id.

[50] Jacobson MF and DePorter J. *Self-reported adverse reactions associated with mycoprotein (Quorn-brand) containing foods*, Ann. Allergy Asthma Immunol., 120: 626-630 (2018).

[51] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=101.9

As discussion in Section III.B above, the only mycotoxins for which there is the potential for dietary exposure are fumonisins and beauvericin. A summary of the potential dietary intakes are provided below:

**Table 22: Summary of Mycotoxin EDI and CEDI**

| Impurity | Specification mg/kg | Measured mg/kg | Mean EDI mg/day (µg/kg bw/day) | 90th %tile EDI mg/day (µg/kg bw/day) | Mean CEDI µg/kg bw/day | 90th% CEDI µg/kg bw/day |
|---|---|---|---|---|---|---|
| Total Fumonisins (spec.) | 0.5 | -- | 0.012 (0.2) | 0.024 (0.4) | 0.48 | 0.97 |
| Total Fumonisins (measured) | -- | 0.055 | 0.0013 (0.02) | 0.0027 (0.045) | 0.3 | 0.62 |
| Beauvericin | -- | 0.08 | 0.002 | 0.004 | 0.083 | 1.0 |

Although genomic analysis indicated the potential for formation of fusarin C and fusaric acid, neither substance is detected in analytical testing. Fusarin C (CAS Reg. No. 79748-81-5) is a member of a family of unstable compounds, which includes fusarins A, B, C, D, E and F, none of which are the subject of established levels of concern for safety by regulatory or expert authorities. Quantitative measurement of fusarin C has not been possible due to the lack of an analytical standard for the substance. However, qualitative analysis using LC-MS/MS and High-Resolution MS (LC-QTOFMS) was conducted to determine the presence or absence of fusarin C in *F. novum yellowstonensis* samples using m/z 454, in the retention time between fumonisin B3 and B2.[52] No peaks of interest were observed in this time window, leading to the conclusion that although genetic sequencing indicated the possibility for FMP to produce fusarin C, the substance in fact is not produced during the specific cultivation conditions for the production of FMP. [53]

Fusaric acid is a mycotoxin that typically comes from *Fusarium* species. Fusaric acid has not been the subject of an established level of concern for safety by regulatory or expert authorities. The 3-batch analytical testing conducted on FMP using LC-MS/MS and High-Resolution MS (LC-QTOFMS) did not detect fusaric acid at a level of quantitation of 100 ppb or a detection limit of 25 ppb. None of the additional testing conducted on FMP has detected fusaric acid at these levels, again leading to the conclusion that although genetic sequencing indicated the possibility for FMP to produce fusarin C, the

[52] Han, et al., *Screening survey of co-production of fusaric acid, fusarin C, and fumonisins B1, B2 and B3 by Fusarium strains grown in maize grains*, Mycotoxin Research 30:4, 231-240 (Nov. 2014).

[53] Fusarin C has been evaluated by the International Agency for Research on Cancer (IARC), IARC Monograph v. 56, https://monographs.iarc.fr/wp-content/uploads/2018/06/mono56-16.pdf. Based on the genotoxicity data available, IARC concluded that Fusarin C demonstrated some evidence of genotoxicity, but only in the presence of an exogenous metabolic system, and there is limited evidence in experimental animals for the carcinogenicity of fusarin C.

substance in fact is not generated under the specific cultivation conditions for Fermented microbial protein (FMP). In the absence of potential dietary intake of either fusarin C or fusaric acid, there is a reasonable certainty of no harm due to these mycotoxins from consumption of FMP.[54] The remainder of the safety assessment will focus, therefore, on fumonisins and beauvericin.

1. Fumonisins

Fumonisins are a group of mycotoxins produced by *Fusarium* species. A number of different types of fumonisin are known, but fumonisins B1 (FB1, CAS Reg. No. 116355-83-0), B2 (CAS Reg. No. 116355-64/84-1), B3 (CAS Reg. No. 136379-59-4) and B4 (CASRN 136379-60-7) are the major forms found in food, with FB1 considered to be ubiquitous in nature and the most prevalent, especially in corn/maize. Fumonisins have been evaluated for safety by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), as well as multiple national authorities, and based upon the acceptable intakes established by those reviews the potential exposure to fumonisins from consumption of FMP does not present a safety concern.

Specifically, fumonisins have been the subject of evaluations by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), as well as FDA,[55] USDA,[56] and others.[57] In its most recent evaluation, published in 2018, based on a thorough and updated review of the available toxicity data on fumonisins, JECFA retained its previously established provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg bw/day for fumonisins B1, B2 and B3, alone or in combination.[58] As part of its evaluation, JECFA

[54] Although there is a sizable body of pharmaceutical-related literature on fusaric acid, there are limited toxicology data available relevant to oral feeding exposures and we have not identified any authoritative reviews. The publicly available literature that we have identified indicate that, even if there were some dietary intake at levels below 25 ppb, the resulting exposure from consumption of [FMP] would not approach the therapeutic dose of fusaric acid (10-30 mg/kg), and would be well below the level at which in vitro assays indicate fusaric acid is cytotoxic or that there would be a concern for repeated dose toxicity. See, e.g., Stack, B., et al. (2014), *Determination of Oral Bioavailability of Fusaric Acid in Male Sprague-Dawley Rats*, Drugs R D, 14(2):139-45; Vesonder, R., Gasdorf, H., Peterson, R. (1993), *Comparison of the cytotoxicities of Fusarium metabolites and Alternaria metabolite AAL-toxin to cultured mammalian cell lines*, Arch. Environ. Contam. Toxicol., 24(4):473-477; Reddy, R.V., et al. (1996), *Developmental Toxic Effects of Fusaric Acid in CD1 Mice*, Bulletin of Environmental Contamination and Toxicology, 57(3): 354-360; Voss, K.A. et al. (1999), *Fusaric Acid and Modification of the Subchronic Toxicity to Rats of Fumonisins in* F. Moniliforme *Culture Material*, Food Chem. Toxic., 37: 853-861; Mamur, S. et al. (2018), *Evaluation of the cytotoxic and genotoxic effects of mycotoxin fusaric acid*, Drug Chem. Toxicol., 11: 1-9.

[55] https://www.fda.gov/RegulatoryInformation/Guidances/ucm109231.htm.

[56] Norred, et al. (1996), *Fumonisin toxicity and metabolism studies at the USDA. Fumonisin toxicity and metabolism*, Adv. Exp. Med. Biol. 392: 225-36.

[57] See, e.g., EFSA Panel on Contaminants in the Food Chain (CONTAM) (January 2018), Appropriateness to set a group health-based guidance value for fumonisins and their modified forms, https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5172; Voss, K.A. et al. (2001). *An overview of rodent toxicities: liver and kidney effects of fumonisins and* Fusarium moniliforme*.* Environ. Health Perspect. 109 (Supp 2) 259-266.

[58] JECFA (2018) *Safety Evaluation of Certain Mycotoxins in Food (WHO Food Additives Series No. 74), 83rd Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)*, Geneva, International Programme on Chemical Safety, World Health Organization. Available at https://apps.who.int/iris/bitstream/handle/10665/276868/9789241660747-eng.pdf?ua=1

determined current US intakes of fumonisins for the general population to be 0.068-0.28 μg/kg bw/day, and for the 90th percentile consumer to be 0.14-0.57 μg/kg bw/day.

As described in Section III.B.2, very low levels of fumonisins are detected in batches of FMP, resulting in conservatively calculated mean and 90th percentile EDIs of 0.0013 mg/day and 0.0027 mg/day, respectively. When the calculated dietary exposure to fumonisins from consumption of FMP is added to the existing US dietary exposure indicated in the JECFA evaluation, the upper bound 90th percentile CEDI (measured) is 0.62 μg/kg bw/day. This is only a very slight increase over the existing levels of fumonisin intake at 0.57 μg/kg bw/day and represents less than half of the JECFA PMTDI. The 90th-percentile CEDI calculated conservatively using the specification of <0.5ppm total fumonisin for FM is 0.97 μg/kg bw/day. This highly conservative value represents less than half of the JECFA PMTDI of 2 μg/kg bw/day.

As the CEDI for total fumonisins is well below the JECFA PMTDI, there is a reasonable certainty of no harm regarding fumonisins ingested as a component of FMP.

2. Beauvericin (BEA, CAS Reg. No. 26048-05-5)

Beauvericin is a common mycotoxin produced by many *Fusarium spp*. and occurs naturally on corn and corn-based foods and feeds. No regulatory or expert authority has established a specific threshold of safety concern for beauvericin. Nevertheless, an assessment has been conducted to ensure the safety of potential exposures to this substance. The genetic sequencing described above indicated the potential for *F. novum yellowstonensis* to produce beauvericin, and analytical testing has detected beauvericin at very low levels in FMP.[59] Based on information in the public literature regarding existing dietary intakes of beauvericin from commonly consumed foods, the calculated dietary exposure resulting from the presence of beauvericin in FMP does not increase the existing exposure. As there is only a slight change to the existing exposures of beauvericin, there is a very low additional risk of harm from exposure to beauvericin as a result of eating FMP.

As noted in Section III.B.3 above, beauvericin has been the subject of a comprehensive toxicological review by the EFSA CONTAM panel. Based on the CONTAM panel's conclusions, additional toxicity studies were undertaken by French and Italian national health and safety authorities (ANSES and ISS, respectively).[60,61] At the time of the CONTAM panel's 2014 report, the available toxicity data for

[59] Enniatins are reported in the publicly available literature as being structurally related to beauvericin and as potentially being present on food products were beauvericin is detected. However, the genetic sequencing of *F. novum yellowstonensis* indicated that enniatins were not predicted to be produced by *F. novum yellowstonensis* and analytical testing has not detected the presence of enniatins. Thus, enniatins are not reasonably expected to be present in FMP.

[60] EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2014. *Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed*. EFSA Journal 2014;12(8):3802, https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2014.3802.

[61] Istituto Superiore di Sanità (ISS), Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) and French Agency for Food, Environmental and Occupational Health & Safety (ANSES), 2018. *In vivo toxicity and genotoxicity of beauvericin and enniatins. Combined approach to study in vivo*

beauvericin were very limited,[62] so the panel, compared estimated chronic exposure levels with the doses reported to cause adverse effects upon therapeutic use of the drug fusafungine, which is a mixture of the structurally-related mycotoxins, enniatins. The panel compared the calculated exposure to the lowest observed adverse effect level (LOAEL) for fusafungine, which ranged from 0.09 to 0.17 mg/kg bw/day (90-170 µg/kg bw/day), and while noting the limitations and uncertainties of the approach, evaluated the margins of safety, which ranged from about 57,000 to 1,800 for the mean dietary exposure, and from about 17,000 to 1,000 for the 95$^{th}$ percentile dietary exposure.

The CONTAM panel's review has not yet been updated to address the additional studies reported by ISS/ANSES. *In vivo* micronucleus and Comet assays reported no cytotoxicity of beauvericin at doses up to 200 mg/kg bw, additionally *in vitro* micronucleus tests (OECD TG474) using human lymphoblast thymidine kinase heterozygote cell line (TK6) and human undifferentiated hepatic (HepaRG) cells were considered to be negative. These studies add weight to a conclusion that beauvericin is not genotoxic.

Additionally, the two *in vitro* Ames and micronucleus studies conducted on FMP, which were both negative, provide further support for the absence of genotoxicity.

A combined repeated dose oral toxicity study (OECD TG 407) and Reproductive/ Developmental Toxicity Screening Test (OECD TG 422) was conducted on BEA, finding NOAELs of 0.1 mg/kg bw/day and above.[63,64]

As described in Section III.B.3, very low levels of beauvericin are detected in FMP, resulting in calculated mean and 90$^{th}$ percentile EDIs of 0.002 mg/day and 0.004 mg/day, respectively. When the calculated dietary exposure to beauvericin from consumption of FMP is added to the existing dietary exposure indicated in the CONTAM evaluation, the upper bound 90$^{th}$ percentile CEDI is 1.0 µg/kg bw/day, a value

*toxicity and genotoxicity of mycotoxins beauvericin (BEA) and enniatin B (ENNB)*. EFSA supporting publication 2018:EN-1406, https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2018.EN-1406.

[62] No subchronic, chronic, reproduction and developmental toxicity, neurotoxicity or carcinogenicity studies. One acute toxicity study was available, with a reported LD50 of 100 mg/kg bw. The panel indicated that limited in vitro genotoxicity data were equivocal although some studies suggested a potential genotoxic effect. Specifically, beauvericin was negative in an Ames test in S.typhi strains TA97, TA98, TA100, TA102 and TA1535, with and without S9, but showed some potential evidence of cytotoxicity in chromosome aberration, sister-chromatid exchange and micronucleus assays in human lymphocytes. A micronucleus test and Comet assay in porcine kidney PK15 cells and a Comet assay in human lymphocytes showed cytotoxicity at some time- and dose- thresholds but not others.

[63] The study used doses of 0.1, 1, and 10 mg/kg bw/day and reported No Observed Adverse Effect Levels (NOAELs) as follows: female mice of 1 mg/kg bw/day; male mice of 0.1 mg/kg bw/day (reduced colloid and altered T4 serum levels); for reproductive effects, a maternal NOAEL of 0.1 mg/kg bw/day (increased thymus weight; and a developmental NOAEL of 10 mg/kg bw/day.

[64] Within the repeated dose toxicity study, genotoxicity was comprehensively assessed for gene mutation, DNA breakage, and chromosomal damage by employing the Pig-a gene mutation assay in peripheral blood, the Comet assay in different target organs (liver, duodenum, blood, kidney and testis/ovary cells), and the MN test in peripheral blood. Immunotoxicity and immunological functions upon repeated dose exposure were studied by ex vivo/in vitro assays on treated and control mice. All the genotoxicity endpoints analyzed in the repeated dose study yielded negative results, with the exception of BEA in the Comet assay in the duodenum and kidneys of male mice treated with the intermediate dose.

that represents only a very slight increase over the existing exposure of 0.93 µg/kg bw/day to BEA and does not substantially affect the existing margins of safety as compared to current intakes. In light of the very small addition to existing dietary exposures that the consumption of beauvericin from the intended use of FMP represent, we conclude there is a reasonable certainty of no harm for the ingestion of FMP regarding any expected residue of beauvericin.

3. Commonly Occurring Mycotoxins

FDA has established action or advisory levels for various commonly occurring mycotoxins, such as aflatoxins. With the exception of fumonisins, none of these mycotoxins were predicted to be present in FMP based on genetic sequencing and analytical testing. These mycotoxins were tested in the 3-batch analysis and have not been detected at the detection limits presented below. Additional testing was conducted for other mycotoxins for which FDA has not established action levels, as indicated below.

**Table 23: Summary of Commonly Occurring Mycotoxins Not Detected in FMP**

| **Analyte** | **Limit of Detection in FMP testing** | **Lowest FDA Action Level** |
|---|---|---|
| Aflatoxin B1 | ND @ 1.3 ppb | 20 ppb[65] |
| Aflatoxin B2 | ND @ 1.2 ppb | 20 ppb |
| Aflatoxin G1 | ND @ 1.1 ppb | 20 ppb |
| Aflatoxin G2 | ND @ 1.6 ppb | 20 ppb |
| Ochratoxin A | ND @ 1.1 ppb | 20 ppb[66] |
| Deoxynivalenol | ND @ 0.6 ppm | 1 ppm in finished wheat products[67] |
| Acetyldeoxynivalenol | ND @ 0.8 ppm | None |
| Fusarenon X | ND @ 0.4 ppm | None |
| Nivalenol | ND @ 0.6 ppm | None |
| T-2 Toxin | ND @ 0.2 ppm | None |
| HT-2 Toxin | ND @ 0.2 ppm | None |
| Neosolaniol | ND @ 0.4 ppm | None |
| Diacetoxyscirpenol | ND @ 0.4 ppm | None |
| Zearalenone | ND @ 51.7 ppb | None |

[65] 20 ppb is the action level for foods generally. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-action-levels-poisonous-or-deleterious-substances-human-food-and-animal-feed#afla.

[66] This is not a true action level, but FDA Compliance Guidance Program Manual 7307.001 indicates that levels below 20 ppb identified in tested samples upon import need no further action. http://wayback.archive-it.org/7993/20170404002445/https://www.fda.gov/downloads/Food/ComplianceEnforcement/UCM073294.pdf.

[67] https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-and-fda-advisory-levels-deoxynivalenol-don-finished-wheat-products-human.

In light of the repeated analyses indicating that these substances are non-detectable and the genetic analysis indicating that these mycotoxins are not expected to be produced by *F. novum yellowstonensis*, there is reasonable certainty of no harm from these mycotoxins.

E. Nitrates

Only one potential residual input, nitrate, has been identified in FMP as it is carried through from the manufacturing process. Nitrates are present in the fermentation medium as the constituent nitrogen is an essential building block for the natural synthesis of amino acids and proteins. As calculated above, the 90th percentile exposure would be 1.1 mg/person/day (equivalent to 0.02 mg/kg bw/day, assuming a 60 kg individual), well below the JECFA upper bound ADI of 3.7 mg/kg bw/day, giving a reasonable certainty of no harm from nitrates by consuming FMP.

Nitrates, in the forms of sodium nitrate and potassium nitrate, are currently permitted for use in food under a variety of FDA regulations permitting their direct addition to food.[68] Concerns about the safety of nitrate exposure has resulted in reviews by a number of authoritative bodies, including JECFA and FDA, several of which have established safety levels compared to which exposures from FMP are not anticipated to present a safety risk.

JECFA has evaluated the safety of nitrates in coordination with the safety of nitrites and nitrosamines on the basis that, although nitrate can generally be considered to be of relatively low toxicity, nitrate can be metabolized in the human body to form nitrite and N-nitroso compounds can also be formed from nitrite and N-nitrosatable compounds under certain conditions.[69] In its most recent evaluation in 2002, JECFA retained the acceptable daily intake (ADI) for nitrate of 0-3.7 mg nitrate ion/kg bw/day established in 1995.[70] This ADI specifically addressed concerns about the potential conversion of nitrates to nitrates. As calculated in Section III.B.4, the exposure to nitrates from consumption of FMP is estimated to be 0.01 mg/kg bw/day (mean) and 0.02 mg/kg bw/day (90$^{th}$ percentile). This exposure is more than 185-fold below the upper-bound ADI established by JECFA. A reevaluation conducted by the EFSA in 2017 reiterated the ADI for nitrate at 3.7 mg/kg bw/day.[71]

In addition to the JECFA review, FDA and the US Environmental Protection Agency (EPA) have both established safety thresholds for the presence of nitrates in drinking water (an application for which the expected consumption is much higher than that expected for FMP). The drinking water standard is

[68] See, e.g., 21 C.F.R. §§ 172.160, 172.170, 181.33.
[69] JECFA Toxicological Monograph, *WHO Food Additive Series 35, Nitrate*, http://www.inchem.org/documents/jecfa/jecmono/v35je14.htm; Joint FAO/WHO Expert Committee on Food Additives Evaluation of Certain Food Additives, *WHO Food Additive Series: 50, Nitrate (and potential endogenous formation of N-nitroso compounds)*, 1995, http://www.inchem.org/documents/jecfa/jecmono/v50je06.htm.
[70] Joint FAO/WHO Expert Committee on Food Additives Evaluation of Certain Food Additives: 59$^{th}$ Report, WHO Technical Report Series 913, 2002, https://apps.who.int/iris/bitstream/handle/10665/42601/WHO_TRS_913.pdf;jsessionid=E66F435DD346ABB2346A4E371433F875?sequence=1.
[71] https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/nitrates-nitrites-170614.pdf.

10 mg/L, measured as nitrogen.[72] The National Academies of Sciences, Engineering, and Medicine has determined that an adequate daily fluid intake is about 15.5 cups (3.7 liters) of fluids for men and about 11.5 cups (2.7 liters) of fluids a day for women, of which about 20% is absorbed from food.[73] Assuming a fluid intake entirely from tap or bottled plain water, the daily consumption of nitrate-nitrogen for men would be 29.6 mg/person/day (equivalent to 130 mg nitrate ion/p/day, as 1 mg nitrate-nitrogen = 4.4 mg nitrate).[74] The consumption of nitrates from FMP calculated above is well below this level.

EPA also has established an oral reference dose (RfD) for chronic exposures to nitrate-nitrogen of 1.6 mg/kg bw/day, based on critical effects seen in infants (equivalent to about 7.0 mg nitrate ion/kg bw/day).[75] The calculated exposure of 0.02 mg/kg bw/day for FMP is 350-fold below this threshold.

On these bases, there is a reasonable certainty of no harm from the calculated exposure to nitrates from consumption of FMP.

F. Ribonucleic Acid (RNA)

FMP is subject to a specification of 2% RNA. This is the same specification as provided for Marlow Foods' mycoprotein product in GRN 91. RNA is regularly consumed as a significant component of the human diet, with relatively high concentrations are present in edible offals, animal muscle tissues, vegetables, and fungi.[76] There are no safety issues inherent in the consumption of RNA, and there are no specific concerns that have been identified through genetic sequencing for *F. novum. yellowstonensis.* Additionally, as presented in Section II.C (Specifications), the results from the 3-batch analysis showed an average RNA level of 0.1%, much below the 2% specification. Fermented microbial protein (FMP) is produced using the wild strain of the organism, so there are no genetic modifications that need to be addressed. The level of RNA in FMP is similar to that of the foods it is intended to replace (i.e., animal muscle tissue) and other commonly consumed fungi (e.g., mushroom), as illustrated below, and thus presents no safety concerns.[77]

[72] https://safewater.zendesk.com/hc/en-us/articles/211401718-4-What-are-EPA-s-drinking-water-regulations-for-nitrate-.; 21 C.F.R. § 165.110.
[73] http://www.nationalacademies.org/hmd/Reports/2004/Dietary-Reference-Intakes-Water-Potassium-Sodium-Chloride-and-Sulfate.aspx.
[74] (10 mg/L) × (3.7 L × 0.8) = 29.6 mg nitrogen.
[75] https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0076_summary.pdf. The RfD also was based in part on the Walton study referenced in the ATSDR review.
[76] Jonas, D.A., et al., Safety Considerations of DNA in Food, Annals of Nutrition & Metabolism, 45: 235-254 (2001), https://www.karger.com/Article/PDF/46734.
[77] Adapted from Jonas, et al.

**Table 24: Comparison of RNA Content**

| Food | RNA (g/kg dry matter) |
|---|---|
| FMP | 20 g/kg |
| Beef | |
| Liver | 21.4–22.8 |
| Heart | 6.1 |
| Pig | |
| Liver | 31.2–35.5 |
| Kidney | 15.5 |
| Heart | 9.4 |
| Broccoli | 20.6 |
| Yeast, Baking | 66.2 |
| Oyster fungi | 24.1 |
| Boletus (yellow) | 23.1 |
| Chestnut mushrooms | 21.1 |

G. Pesticides

As FMP is produced using highly purified plant-based raw materials and is manufactured using a surface fermentation method, there is no reasonable expectation that any pesticide residues would be present. Nevertheless, testing was conducted as part of the three-batch analysis using the QuEChERS method in combination with GC and LC mass spectrometry. No pesticide residues were detected.

H. Conclusion

The extensive and thorough information and data described above support the conclusion that Fermented microbial protein (FMP) is generally recognized as safe for use as a food ingredient and source of macronutrients intended for use as meat, dairy, and flour alternatives. The safety profile of FMP has first been considered in light of the identity of the source organism, *F. novum yellowstonensis*, and the cultivation process of that organism to produce the FMP biomat. The biomat is a vegetative mass that is comprised of protein, carbohydrates, and fats. Surface fermented F. *novum yellowstonensis* biomat is a novel food ingredient containing a high-quality, complete protein, comparable to existing sources of dietary protein, such as egg and beef. It contains significant levels of dietary fiber, is low in fats and sugars, and is nutritionally comparable to other protein replacement products.

The available published and unpublished toxicity data support the safety of FMP at the calculated dietary intake levels. Acute oral toxicity and genetic toxicity testing conducted using OECD methods on FMP showed no evidence of adverse effects; in addition, while the robust dataset publicly available for other mycoprotein food ingredients demonstrate no safety concern in multiple genotoxicity and repeated dose studies at high intake levels.

A multi-pronged assessment for potential allergenicity of *F. novum yellowstonensis* has been conducted by the nationally renowned Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln using analysis and comparison of genetic sequence information using scientific principles, *in vitro* digestibility studies, and an evaluation of published literature. Based upon its extensive analysis, FARRP concluded that food produced from *F. novum yellowstonensis* is unlikely to represent any additional risk of food allergy beyond what is currently available in the human food supply.

Finally, FMP contains no components or impurities that present a safety concern, even when the product is consumed at 90$^{th}$ percentile levels. *Fusarium* species are known for their potential production of mycotoxins; however, genetic sequencing and rigorous analytical testing using accepted scientific principles, and comparison to published established provisional maximum tolerable daily intake, have demonstrated that only two mycotoxins are present in FMP, at levels that represent no safety concern, as established by comparison to safety thresholds determined by authoritative risk assessment bodies. Moreover, there are no impurities resulting from the manufacturing process that present a safety risk.

On the basis of the substantial scientific data supporting the safety of the potential dietary intakes, Sustainable Bioproducts and an independent panel of expert reviewers have determined that FMP is GRAS for its intended use.

## VII. List of Supporting Data and Information

### A. References


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B. Attachments

*Fusarium* Mycoprotein fermentation product literature survey 21 March 2019.

*Fusarium* Mycoprotein fermentation product proteins *in vitro* digestibility study in a simulated gastric fluid (pepsin) assay (pH 2.0) and in simulated intestinal fluid assay 23 March 2019.

New *Fusarium* species fermentation product proteins: Bioinformatics to evaluate potential allergenicity 24 March 2019.

*Fusarium* Mycoprotein fermentation product overall summary of allergenic risk 23 March 2019.

## VIII. Report of the Expert Panel

Please see below for signed statements from the Expert Panel.

The undersigned agrees that the Report of the GRAS Panel (copied below) and the revised GRAS Notice regarding the proposed uses of Fermented microbial protein (FMP) properly reflect the consensus of the Panel.

The GRAS Assessment included above was reviewed by a panel of Qualified Experts including Dr. Andrew Bartholomeus, Dr. Mitchell Cheeseman and Dr. James Hoadley. The panel notes that the assessment itself includes reports by qualified experts on allergenicity and digestibility and related issues for the proposed ingredient. The panel agreed that the Assessment provides an adequate basis for the GRAS status of Fusarium novum yellowstonensis mycelium, referred to as fermented microbial protein (FMP) for the intended uses in food described above. The above assessment was provided to the panel as well as underlying data, information and references. After review of the assessment and supporting documentation, the panel met and discussed the adequacy of the assessment. The panel made suggestions regarding limited additional discussions to complete the assessment but raised no substantive issues regarding the safety of FMP for the intended uses described above or about the general recognition of the safety of such intended uses. A signed consensus document from each panel member is attached to the GRAS Notice and the CVs for each panel member will be made available to FDA if the Agency so requests.[1]

Mitchell A. Cheeseman, Ph.D.

[1] During discussion of the GRAS assessment the food substance was identified by several potential names (Fusarium mycelium, Fusarium mycoprotein, and fermented microbial protein). Each of these names refers to the same food substance identified in this GRAS notice as Fermented microbial protein (FMP) produced from the fermentation of *F. novum yellowstonensis*.

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Andrew Bartholomeus, Ph.D.

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James Hoadley, Ph.D.

[1] During discussion of the GRAS assessment the food substance was identified by several potential names (Fusarium mycelium, Fusarium mycoprotein, and fermented microbial protein). Each of these names refers to the same food substance identified in this GRAS notice as Fermented microbial protein (FMP) produced from the fermentation of *F. novum yellowstonensis*.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

# GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
*(See last page for OMB Statement)*

**FDA USE ONLY**

| GRN NUMBER | DATE OF RECEIPT |
|---|---|
| 000904 | Jan 20, 2020 |
| ESTIMATED DAILY INTAKE | INTENDED USE FOR INTERNET |
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Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

## SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2019/05/02

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

## SECTION B – INFORMATION ABOUT THE NOTIFIER

**1a. Notifier**

| Name of Contact Person | Position or Title |
|---|---|
| Brian Furey | Director of Regulatory Affairs |
| Organization *(if applicable)* | |
| Sustainable Bioproducts, Inc. | |
| Mailing Address *(number and street)* | |
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| City | State or Province | Zip Code/Postal Code | Country |
|---|---|---|---|
| Chicago | Illinois | 60615 | United States of America |

| Telephone Number | Fax Number | E-Mail Address |
|---|---|---|
| N/A | | brian.furey@sustainablebioproducts.com |

**1b. Agent or Attorney *(if applicable)***

| Name of Contact Person | Position or Title |
|---|---|
| Joan Sylvain Baughan | Partner |
| Organization *(if applicable)* | |
| Steptoe & Johnson, LLP | |
| Mailing Address *(number and street)* | |
| 1300 Connecticut Ave, NW | |

| City | State or Province | Zip Code/Postal Code | Country |
|---|---|---|---|
| Washington | District of Columbia | 20036 | United States of America |

| Telephone Number | Fax Number | E-Mail Address |
|---|---|---|
| (202) 429-6417 | (202) 429-3902 | jbaughan@steptoe.com |

## SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Fermented microbial protein (FMP)

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

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

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

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

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

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

## SECTION D – INTENDED USE

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

FMP is intended for use in finished foods to replace or supplement traditional sources of protein and other macronutrients in the human diet. Intended for use in products within; plant protein products, including meat and poultry analogs; dairy product analogs; milk products; beverages and beverage bases; breakfast cereals; fruit and vegetable juices; grain products and pastas; baked goods and baking mixes; soups and soup mixes; and fats and oils. FMP is not intended for use in infant formula or in meat or poultry products regulated by the USDA.

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

*(Check one)*

☐ Yes ☒ No

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

(Check one)

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

## SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

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

☒ Yes ☐ No

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

☒ Yes ☐ No

## SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Sustainable Bioproducts, Inc.
*(name of notifier)*

has concluded that the intended use*(s)* of Fermented microbial protein (FMP)
*(name of notified substance)*

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

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

1452 East 53rd Street, Chicago, Illinois 60615
*(address of notifier or other location)*

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

| 3. Signature of Responsible Official, Agent, or Attorney | Printed Name and Title | Date (mm/dd/yyyy) |
|---|---|---|
| Joan Sylvain Baughan Digitally signed by Joan Sylvain Baughan Date: 2020.01.14 15:19:23 -05'00' | Joan Sylvain Baughan, Partner | 1/14/2020 |

## SECTION G – LIST OF ATTACHMENTS

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

| Attachment Number | Attachment Name | Folder Location (select from menu) (Page Number(s) for paper Copy Only) |
|---|---|---|
| | GRAS Notice | Submission |
| | Fusarium Mycoprotein fermentation product literature survey 21 March 2019 | Submission |
| | Fusarium Mycoprotein fermentation product proteins in vitro digestability study in a simulated gastric fluid (pepsin) assay (pH 2.0) and in simulated intestinal fluid assay 23 March 2019 | Submission |
| | New Fusarium species fermentation product proteins: Bioinformatics to evaluate potential allergenicity 24 March 2019 | Submission |
| | Fusarium Mycoprotein fermentation product overall summary of allergenic risk 23 March 2019 | Submission |
| | | |
| | | |
| | | |
| | | |

# Attachment 2

# Fusarium Mycoprotein fermentation product literature survey 21 March 2019

## STUDY TITLE

*Fusarium* mycoprotein fermentation product literature survey

## AUTHORS

Richard E. Goodman

## STUDY COMPLETED ON

21 March 2019

## PERFORMING LABORATORY

Goodman Laboratory
University of Nebraska
Dept. of Food Science & Technology
1901 North 21St Street
Lincoln, NE 68588-6207, USA

## SUBMITTERS/SPONSORS

Sustainable Bioproducts, Inc. .
1452 E 53rd St
Chicago, IL 60615
USA

## LABORATORY STUDY ID

REG 2019 Sustainable 1

**Study Number:** REG 2019 Sustainable 1

**Title:** *Fusarium* mycoprotein fermentation product literature survey

**Facility:**
Food Allergy Research and Resource Program
Food Science and Technology
University of Nebraska
1901 North 21St Street
P.O. Box 886207
Lincoln, NE 68588-6207
USA

**Principle Investigator:**
Richard E. Goodman
University of Nebraska
Tel: +1 (402) 472-0452

**Study Sponsor:**
Sustainable Bioproducts, Inc.
Brian Furey
Manager of Regulatory Affairs

**Study Start Date:** 10 December 2018

**Study Completion Date:** 14 March 2019

**Records Retention:** All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

**Signature of Final Report Approval:**  24 March 2019

Principal Investigator: Richard E. Goodman Date

Footnote: [1]This taxa ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

## SUMMARY

Sustainable Bioproducts, Inc. developed a fermented food protein product from a new *Fusarium* species related to the *Fusarium fujikuroi* species complex, and deposited in ATCC as No. PTA-10698. This species was identified for use in food products. It is a whole organism with hundreds of proteins. A similar novel food was developed by a different company in the United Kingdom in the 1980's from another species of *Fusarium* that is used to make a commercial food product called Quorn. Quorn has been consumed as a human food source for over 30 years in the UK and the United States.

Sustainable Bioproducts, Inc. asked that the Goodman Laboratory perform an overall allergenicity risk assessment of their product For this safety assessment, we have followed the basic evaluation steps outlined in the *Codex* Guideline in 2003 for the evaluation of potential risks of allergy for genetically engineered (GE) crops, although this is not a GE crop. Following the Codex multifactorial approach, the assessment begins with a literature review of safety of the species to identify any specific food risks, conduct digestive fate studies of the proteins, and evaluate the predicted proteins against the allergens in AllergenOnline.org.

Based on protein sequence similarity matches from genomic DNA sequences of this cultured *Fusarium* taxa by BLASTP, this taxa is most closely associated with the *Fusarium fujikuroi* species complex that includes *Fusarium proliferatum* and *Fusarium mangiferae*. The Quorn fungal source is *Fusarium venenatum*, which is in the *Fusarium sambucinum* species complex. The two species that have been identified as having allergenic proteins are *F. proliferatum* (*Fusaerium fujikuroi* complex) and *Fusarium culmorum* (*Fusarum sambucinum* complex). Most proteins within these related complexes have high sequence conservation and subjects with IgE binding and allergy to these conserved proteins are likely to share responses across these species.

This report is a summary of a literature search that used the PubMed database and internet searches with keywords to search for published scientific reports of allergy to *Fusarium* and Quorn to understand potential risks of food made from this *Fusarium* species. Identified abstracts and potentially relevant publications were read and evaluated for evidence of risk of allergy from the genus and from specific proteins. The reports were evaluated for clinical reactivity from oral (foods), inhalation, and dermal sources of contact. In addition, the WHO/IUIS Allergen Nomenclature database and the AllergenOnline.org database were searched for information on identified allergenic proteins from *Fusarium* and related fungi.

As expected from this broad investigation, a number of references (more than 188) of reports of allergy or allergenicity using keywords to *Fusarium sp*. were found, although most of them were not directly related to reactions following exposure to *Fusarium sp.* Four proteins of *Fusarium sp.*(Fus c 1, Fus c 2, Fus p 4 and Fus p 9) have been accepted as allergens in the WHO/IUIS Allergen Nomenclature database. All four are also listed in the AllergenOnline.org database along with a third putative allergen from *Fusarium culmorum* that has not been recognized by the WHO/IUIS Allergen Nomenclature committee. References for the five allergens were reviewed to understand what is known about reactivity and evidence of possible cross-reactivity or unexpected risks. The results were interpreted in the context of known food

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allergies to major allergen sources and proteins, as well as more common sources and allergenic proteins that present limited risk of food allergy for the typical populations. The goal was to understand any unexpected and or significant new risks this food ingredient might present beyond those expected for those with allergies to *Fusarium sp.* or closely related molds.

The conclusions of the literature review and database searches are that allergic reactions upon consumption of foods containing proteins from various *Fusarium sp.* are rare in published studies. There are some cases of inhalation allergy to the five *Fusarium* proteins in the www.AllergenOnline database and to exposure to crude protein sources using whole extracts of *Fusarium* in laboratory tests reported in a few publications.

There are self-reported reactions from consumption of Quorn as recorded by the Quorn developer, Marlow Foods, as well as by an unrelated non-governmental entity, Center for Science in the Public Interest (CSPI). The data from CSPI was published in 2018. However, the described prevalence and severity of reactions is not remarkable based on the source and amount of food that is consumed in a meal. A number of reactions described by CSPI are not likely to be IgE mediated and may in fact be due to consumption of high concentrations of fiber. From my literature review and evaluation of allergenic proteins and risks from proteins of the new *Fusarium* species, the product does not present more than minimal risks of food allergy to consumers. However, the consumers should be aware that the source is a fungus and those with allergies to *Fusarium sp.* or closely related fungi should use caution if they consume products made from this strain.

Footnote: [1]This taxa ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

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Footnote: [1]This taxa ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

## 1. Introduction

Sustainable Bioproducts, Inc. developed a new fermented food source of a newly discovered *Fusarium* species[1]. The Fusarium mycoprotein is grown as a hyphal mass in controlled culture conditions. The Goodman Laboratory, Food Allergy Research and Resource Program (FARRP), of the University of Nebraska Lincoln has conducted an overall allergenicity risk assessment to evaluate potential risks of food allergy that might occur from consumption of the *Fusarium* mycoprotein based food. The current risk assessment follows the *Codex Alimentarius* Commission guideline (2003) that was written with the intent of evaluating potential risks of food allergy and consideration of risks of toxicity and nutritional properties of a genetically engineered food crop that has had an addition of one or a few genes.. It is important to note that while the guidelines were followed, the reviewed mycoprotein is not a genetically engineered food crop. Following the Codex multifactorial approach, the current assessment includes the following research components:

a) A literature search looking for evidence of allergy to foods and environmental source of proteins from various *Fusarium* species; a search for evidence of IgE binding and allergy to specific proteins of these sources or of cross-reactivity to other sources that have high sequence identity to *Fusarium sp*. Proteins (the topic of this current report);
b) Testing the stability of the proteins in the strain in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) assays (results are provided in a separate report) and
c) Evaluation of genomic sequences of this strain to predict possible proteins that were all compared to AllergenOnline.org version 18B for identity matches with allergens (results are provided in a separate report).

This multifactorial assessment provides a likelihood of allergic response by considering the totality of the evidence. The fourth and final report will summarize the totality of the evidence based on the evidence of 3 areas of research identified above.

Sequence comparisons from proteins predicted from the genome helped identify close sequence homology to proteins in a number *Fusarium* species. Based on the highest identity matches, this strain is predicted to be most closely related to members of the *Fusarium fujikuroi* species complex. The genomic sequencing of this strain was performed by a sequencing company, Igenbio ERGO, under contract by Sustainable Bioproducts, Inc. We (i.e. Goodman Laboratory) analyzed the annotated genome and found that there are 14,239 potential proteins from sequence predicted genes of this *Fusarium sp.* The predicted coding regions were evaluated for matches to allergenic sequence in our www.AllergenOnline.org database version 18B, which was installed at the University of Nebraska Holland Computing Center server by Mohamed Abdelmotelb. Mr. Abdelmotelb ran a batch comparison using BLASTP and also compared the sequences to the NCBI non-redundant protein database. As noted above, those data will be discussed in a separate report on bioinformatics.

The Codex document recommends examining published scientific studies of potential allergenicity of new proteins against peer-reviewed published studies related to allergy and allergenicity of the source (*Fusarium sp.*) and the proteins expressed in the source. This document provides the results of those searches and an overall conclusion of potential risks of allergy based on those reports.

Footnote: [1]This taxa ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

## 2. Literature review.

The PubMed database was queried after gathering information about the genus and strain of *Fusarium* and after work comparing the genomic sequence against those in the NCBI Protein database. The taxonomy of this genus is complex as *Fusarium* is divided into a number of species complexes based on the ecology of the species and predictions of evolution based on genomic sequences. The majority of species live primarily on tissues of certain plant types or in soil and are important in decomposing plants. A few groups like the *Fusarium fujikuroi* species complex are important plant pathogens that are restricted to certain host species (Niehaus et al., 2016). Some species or strains are able to infect humans or other mammalian tissues and some are known to grow in the lungs of humans, causing pulmonary disease similar to that caused by certain *Aspergillus* species. The search identified over 188 publications using *Fusarium* AND allergy OR allergen. However, only a few of the publications showed clear connections with proteins from *Fusarium* and with specific proteins. A publication by Levetin et al., (2016) described an overview of taxa of fungi that have been verified to cause allergies including those due to inhalation, ingestion or infection. A major change in characterization of fungal species is the use of DNA sequence analysis. Publications following the initial description (below) were selected from the PubMed search and from references within the papers that plausibly evaluated proteins identified in the WHO/IUIS Allergen Nomenclature database, within AllergenOnline.org or within references that described plausible reactivity to Quorn.

**2.1 Description of *Fusarium* sp. developed by Sustainable Bioproducts, Inc.** Sustainable Bioproducts, Inc., provided information regarding the strain of *Fusarium* and that it is intended to be used as a cultured product grown under defined conditions. The important thing to consider for risks of allergenicity are the similarity or differences of the proteins to those of other allergenic sources. With redefinition of species of fungi into species clusters that share common genes and proteins. Sustainable Bioproducts, Inc. provided the full-genomic sequences for our bioinformatics searches against AllergenOnline.org. The data comparisons demonstrated that the strain seems to be most closely related to the *Fusarium fujikuroi* species complex which is a relatively broad group of plant pathogens and decomposers.

**2.2 Genomic sequences supplied by Sustainable Bioproducts, Inc.** The genome of this strain was sequenced by Igenbio, Inc., and annotated by them using both illumine sequencing and long-reads. The annotations include approximately 14,239 genes. The results of our bioinformatics evaluation will be described in another report.

**2.3 GRAS Determination for Quorn by Marlow Foods.** The GRAS NOTIFICATION (GRN 91) was reviewed from the online posting for mycoprotein. The report was reviewed for indications of allergy and adverse reactions since the source of that product, *Fusarium venenatum* is a taxa that is closely related to *Fusarium fujikuroi* and *Fusarium proliferatum*. Based on the information in GRN 91, it appears that Quorn mycoprotein has been mixed with egg albumin or potato protein isolates as binding agents. It may also be made without those protein binding agents. Quorn was developed in the 1970's and was tested for nutritional properties and evaluated for safety and suitability for human consumption. In 1986 an initial submission was made to the US FDA for approval as a food additive (FAP 6A3930). Subsequently, Marlow

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Foods notified FDA of is GRAS conclusion (GRN 91), for which FDA had no questions (FDA, 2002). The chemical composition of Quorn was reported in the document and the cell walls are chitin and beta-glucans. The protein content is approximately 40%-50% on a dry matter basis. Fatty acid components are more like vegetable fats than animal fats in composition with low unsaturated and high levels of mono-, di- and tri-unsaturated positions. A variety of animal feeding studies were performed in rodents, rabbits, dogs, baboons and humans at from 20% to more than 40% of their diets. No significant adverse effects were noted. The product was introduced into US food markets in 2002 following the GRAS conclusion.

**2.4 Wikipedia review of Quorn.** Wikipedia has a public view document with an important and thorough historical description of Quorn including the objections and claims of Center for Science in the Public Interest (CSPI) of a high rate of allergic reactions and other food consumption symptoms (emesis being the chief complaint). The Wikipedia story indicates that in 2011 Quorn became recognized as a vegan product, following the removal of egg albumin from the product. The Wikipedia article discusses the difference in CSPI's reported claim that Quorn sickens 4.5% of consumers, but the post-market monitoring by Marlow Foods of the Quorn product suggests 0.007% of consumers show intolerance.

**2.5 CSPI online consumer self-reported reactions to Quorn.** The CSPI started a website asking consumers to self-report adverse reactions to consumption of Quorn in 2002, shortly after the introduction into the U.S. market (Jacobson and DePorter, 2018). CSPI published their results in 2018 and show very high rates of reactivity (4.5%), which are much higher than Quorn's post-market monitoring data (0.007%), and without documentation of reactivity for most cases. The CSPI publication includes a statement that two consumers died from anaphylaxis after consuming Quorn, but the reference in the paper is to a news media website (https://www.dailymail.co.uk/health/article-4861862/Quorn-s-main-ingredient-MOLD.html). It is therefore not possible to confirm the deaths or the cause of death. It is certainly not clear that their deaths were due to an IgE mediated reaction to Quorn proteins.

**2.6 Fus c 1 Ribosomal protein P2.** PMID 12743577, Hoff et al., 2003a present a case study of an asthmatic subject who reported severe asthmatic responses following consumption of Quorn, which is made as a fermentation product. They produced a recombinant P2 protein and verified the identity. Importantly they noted broad cross-reactivity of IgE binding for this protein with many other fungal Ribosomal P2 proteins although it is most commonly noted as asthmatic responses to various fungal species. Based on their studies, the Quorn product was used as an inhibitor of IgE binding to purified Ribosomal protein P2, but Quorn only inhibited IgE binding by 65%, thus showing partial cross-reactivity. This ribosomal protein is recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee and by AllergenOnline.org.

**2.7 Fus c 2 Thioredoxin**. Hoff et al., 2003b performed molecular analysis and an IgE binding study to evaluate an evolutionarily conserved protein in *Fusarium culmorum*. The investigation used 52 allergic human sera from subjects with allergy to various molds to investigate cross-reactivity. The protein sequence was evolutionarily conserved, from Ascomycetes to Basidiomycetes. However, there were no complaints of allergic reactions to any food product.

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2**.8 Putative Fus c 3 named helix-loop-helix.** The protein identified by Hoff et al., (2003b) was called a helix-loop-helix protein in the NCBI Protein database accession AAN73248. The publication shows IgE binding to a cloned protein at > 45 kDa in SDS-PAGE, and with stated binding by four of 26 mold allergic subjects by direct IgE binding in a laboratory developed EAST assay. They did not verify specificity by inhibition as they did with Fus c 1 and Fus c 2.

**2.9 Fus p 4 Transaldolase.** Chou et al., 2014 identified a transaldolase as a novel allergen of *Fusarium proliferatum* and demonstrated IgE binding to human homologue as well as to a homologous protein in *Cladosporium cladosporioides*. They speculated on the relevance of IgE binding to the human protein as a potential contributor to the atopic disease. Yet, such responses have not been published with verifiable data.

**2.10 Fus p 9 Vacuolar serine protease.** Yeh et al. (2016) identified the 36.5 kDa serine protease of *Fusarium proliferatum* as an important allergen following binding of a mouse antibody against a cDNA clone of *C. proliferatum*. The data was submitted to the WHO/IUIS Allergen Nomenclature Sub-Committee and was accepted. The protein was determined to be cross-reactive for some with allergies and IgE bound to *Penicillium chrysogenum* (Pen ch 18) as well as the *Fusarium* protein. The results were not fully cross-reactive, so that species specificity was noted. The authors indicated that it appears to be a major allergen for some individuals who are allergic to *Fusarium proliferatum*.

**2.11 Immediate allergic reaction upon ingestion of Quorn in a 27 year old subject.** A letter of correspondence to the Journal of Clinical Pathology (Katona and Kaminski, 2002) reported an immediate allergic response of oral angioedema and breathlessness in a 27 year old subject who ate a bite of a Quorn burger. She was tested for allergies to mold by skin prick tests with extracts including one of Quorn. The weal to Quorn was 7 mm in diameter, the largest reaction in the tests. This was her first known consumption of Quorn and she had been diagnosed previously with IgE mediated allergies to some other mold species. The investigation did not identify the protein(s) that bound IgE.

**2.12 Investigation of possible allergic reactions to mycoprotein of Quorn.** Performed in 1993 by Tee et al. in factory workers and in 10 patients referred for investigation following consumption of Quorn. In 1985, none of the 10 factory workers had measurable IgE to Quorn proteins. In 1987, two workers (but not clear if these were previously negative) had modest IgE binding to Quorn by RAST Inhibition, but no symptoms. The RAST inhibition for some to mycoprotein was similar to inhibition by *Aspergillus fumigatus*. They also tested RAST Inhibition and Skin prick tests to extracts of mycoprotein, Quorn from 10 subjects who complained of reactions to consumption of Quorn. RAST results were not remarkable. Two subjects had SPT of 2 mm to extract of mycoprotein and freeze dried Quorn. Those wheals are not considered to be diagnostically important for foods by current standards of skin prick testing. The authors indicated that none of the complainants had remarkable IgE binding based on RAST tests.

**2.13 *Fusarium*, allergen of the month**. Richard Weber and E. Levetin. 2014. Remarked on the common exposure to environmental allergens of *sp.* in different regions of the world, with most related to mold spore exposure in the evenings. They discussed the four known allergenic

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proteins of Fusarium. Also infections of humans by various species. The "sensitization" rate is over-stated with respect to clinical reactivity as that means only that specific IgE binding can be demonstrated, but typically without major inhibition by a variety of allergenic sources. Obviously then, cross-IgE binding can provide false information. No new allergens were identified and there was no demonstration of food allergy associated with this paper.

**2.14** ***Fusarium equiseti* 65 kDa allergen.** Verma et al., (1998) determined that a 65 kDa protein is likely the most important allergen in *Fusarium equiseti* in India. The authors purified the protein and demonstrated IgE binding from most of the 10 Fusarium sensitized subjects used in their tests. However, they did not provide a sequence of the protein and did not characterize it further. The patients were identified as asthmatic subjects with positive responses in intradermal tests to extracts of *Fusarium equiseti.*

**3.0** **Summary.** The literature search for evidence of allergy to *Fusarium sp.* proteins demonstrated that there are some people who do have allergic reactions to a five proteins of this species when inhaled, when the organism has invaded their tissues, most commonly their lungs or sinuses, or based on evidence from reported reactions to Quorn by Marlow Foods and by CSPI, from ingestion of food that is made of high concentrations of proteins in food from that related species. That does not come as a surprise as most food sources that contain significant amounts of protein do cause allergies in some consumers. It appears that some of the reactions are cross-reactive in the sense that consumers have allergies to related proteins from inhalation of homologous proteins from *Aspergillus sp.* or *Penicillium sp*. Five proteins have been identified from two species of *Fusarium* as allergens that are recognized by the WHO/IUIS Allergen Nomenclature Sub Committee and are in the AllergenOnline.org database. To evaluate potential risks of allergy from foods made from this *Fusarium sp.* product, a bioinformatics study was performed looking at predicted proteins from this strain of *Fusarium* sp. That study may provide further insight into possible risks. Essentially all foods present some risks of allergy that are specific to the proteins in the food sources. Consumers must avoid foods that they are allergic to in order to avoid reactions. It is highly likely that fungal strains that are species of *Fusarium* will cross-react if consumed. It is important to consider species of other fungi that are closely related to *Fusarium* based on high identity matches to similar fungi. Less commonly shared protein sequences are less likely to share IgE binding and more diverse organisms are less likely to share cross-reactivity.

**4.0** **Conclusions**. From this literature search, there is no evidence suggesting food products containing *Fusarium* strain represent a major risk of allergy for consumers. It seems that allergic reactions reported to Quorn, a product from a different strain of *Fusarium* are rare.

Footnote: [1]This taxa ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

## 5.0 References

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Footnote: [1]This taxa ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

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# Attachment 3

# Fusarium Mycoprotein fermentation product proteins in vitro digestability study in a simulated gastric fluid (pepsin) assay (pH 2.0) and in simulated intestinal fluid assay 23 March 2019

## STUDY TITLE

*Fusarium* mycoprotein fermentation product proteins *in vitro* digestibility study in a simulated gastric fluid (pepsin) assay (pH 2.0) and in a simulated intestinal fluid assay (pH 7.5)

## AUTHORS

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## STUDY COMPLETED ON

23 March 2019

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## LABORATORY STUDY ID

REG 2019 Sustainable 2

**Study Number:** REG 2019 Sustainable 2

**Title:** *Fusarium* mycoprotein fermentation product *in vitro* digestibility study in a simulated gastric fluid (pepsin) assay (pH 2.0) and in a simulated intestinal fluid assay (pH 7.5)

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Manager of Regulatory Affairs

**Study Start Date:** 20 December 2018

**Study Completion Date:** 23 March 2019

**Records Retention:** All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

**Signature of Final Report Approval:** [redacted] **23 March 2019**

Principal Investigator: Richard E. Goodman Date

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## SUMMARY

Sustainable Bioproducts, Inc. has developed a fermentable protein product of a new *Fusarium* species related the *Fusarium fujikuroi* species complex, and deposited in ATCC as No. PTA-10698. This species was identified for use as a cultured fungus for inclusion in food products. It is a whole organism with hundreds of proteins. Two samples of product were supplied to my laboratory as dried pellets. One was minimally dried at 25% solids and lighter color than the other, more dried, 95% solids and darker in color as the final product. These samples represent dried mycelial mats from a cultured new *Fusarium* species and are intended for use as a food ingredient.

We dissolved some of the material lots and measured the protein concentrations. We tested the detection limit of visible bands to 10% and 20% of residual samples as controls for digestion.

Stability of the proteins was tested in a pepsin assays using a simulated gastric fluid (SGF) model with porcine pepsin to digest the material at pH 2, followed by removal of timed samples from time 0 minutes out to 60 minutes. The samples were quenched upon removal and all samples were run in SDS-PAGE reducing gels followed by staining with Coomassie Brilliant Blue to evaluate the disappearance of detectable protein bands. The undigested sample bands ranged from nearly 125 kDa to approximately 5 kDa in size. At 30 seconds of digestion the higher MW bands were no longer visible and bands up to 30 kDa diminished over time with the smallest bands barely detectable at 13 kDa after 60 minutes of digestion. The dried sample with 95% solids, was more visible in the Coomassie stained gel and detectable longer than the 25% solids sample.

Stability in simulated intestinal fluid (SIF) was assessed using porcine pancreatin at pH 7.5 in a timed assay out to 120 minutes of digestion. A sample of spinach leaf Rubisco was used as a digestion control protein in parallel with the *Fusarium* sample. Samples were removed at specific times and the reactions quenched. All samples were evaluated in an SDS-PAGE reducing gel after staining with Coomassie Brilliant Blue. As in the SGF assay, the undigested samples showed protein bands from 125 kDa down to 5 kDa. Those bands disappeared rapidly during digestion, without visible bands after 2 minutes.

The results of these assays demonstrated that the proteins in extracts of this new *Fusarium* species digested moderately well in SGF, leaving only 20% residual stained bands based on densitometry by five minutes of digestion. The samples digested more rapidly in SIF with less than 10% residual stained bands based on densitometry that were visible by 30 seconds of digestion.

While these assays do not predict the fate of proteins in the human digestive tract due to large variations in digestive responses between humans and under diverse circumstances, they do show that most of the proteins in this species are quite digestible by the enzymes and conditions common for human digestion. This suggests a relatively low risks for dietary allergenicity.

Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as Fusarium mycotprotein throughout this report

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**Figures**



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## Abbreviations

| | |
|---|---|
| aa | amino acid |
| $A_{280\ nm}$ | Absorbance of light at a wavelength of 280 nm |
| BSA | Bovine serum albumin |
| D0-60 | Digestion samples (target *Fusarium* protein extracts or RUBISCO plus pepsin or pancreatin) from time 0 (quenched prior to digestion) to time 60 min |
| Dmix | Digestion sample mixture pancreatin plus protein (*Fusarium* or RUBISCO) |
| E0 | Experimental control enzyme (pepsin or pancreatin) without the target protein, time 0 |
| E60 | Experimental control enzyme (pepsin or pancreatin) without the target protein, 60 min |
| E-SIF | Pancreatin in SIF |
| kDa | kilodalton |
| LOD | Limit of detection |
| LSB | Laemmli solution buffer |
| mg | milligram |
| ml | milliliter |
| mM | millimolar |
| µl | microliter |
| na | Not applicable |
| ng | nanogram |
| P0 | Experimental control protein without enzyme, time 0 |
| P60 | Experimental control protein without enzyme, 60 min |
| P120 | Experimental control protein without enzyme 120 min |
| P1/10 | Experimental control protein at 10% loading |
| PAGE | Polyacrylamide gel electrophoresis |
| Pmix | Protein (Fusarium or RUBISCO) without pancreatin |
| SDS | Sodium dodecyl sulfate |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| SOP | Standard operating procedure |
| TCA | Trichloroacetic acid |
| Tris | Tris(hydroxymethyl) aminomethane |
| v/v | solute volume to solution volume |
| w/v | solute weight to solution volume |

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## 1. Introduction

Sustainable Bioproducts, Inc. has developed a novel cultured food product based on a new *Fusarium* species[1]. The product is intended to be a macroingredient for food that can be used in the human diet. The product is harvested as a hyphal mass that includes a high protein content and chitin as a major fiber source. This strain is within the *Fusarium fujikuroi* species complex. Other closely related species are *Fusarium venenatum* and *Fusarium proliferatum*. Goodman Laboratory, Food Allergy Research and Resource Program (FARRP), of the University of Nebraska in Lincoln has completed a literature review,evaluated the genome for a bioinformatics comparison with allergens, and completed an in vitro digestibility study as part of the safety evaluation.

The *Codex Alimentarius* Commission guidelines for assessing the allergenicity of novel foods (2003) recommends assessing the novel food ingredient for stability in pepsin at acidic pH using standard conditions as an assay to help evaluate whether the novel ingredient is likely to increase the rate of sensitization or increase the likelihood of eliciting an allergic response in consumers. In addition, the stability of the mycoprotein was tested using a simulated intestinal fluid digestion assay, a method was recognized by the Codex as useful to consider potential risks of allergy in the safety assessment of foods from novel protein sources. The test method for both the stability in simulated gastric fluid (pepsin) and the stability in simulated intestinal fluid (pancreatin) assessment were first described by Astwood *et al.* (1996). These assays are not meant to predict whether a given protein will always be digested in the stomach of the human consumer, but the assays do provide a simple *in vitro* correlation to evaluate protein digestibility and increased risks of food allergy. Investigation of proteins that have been tested suggest a marked positive predictive value that food allergens causing systemic reactions are relatively stable in the assay, while non-allergenic food proteins are typically digested relatively quickly (Bannon *et al.*, 2002). Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas *et al*., 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but markedly less active at pH 3.5 and irreversibly denatured at pH 7.0 (Collins and Fine, 1981; Crevieu-Gabriel *et al*., 1999). The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. A pure form of pepsin was used for this assay from Worthington Biochemical Co., pepsin A, product LS003319.

The original SGF assay described by Astwood *et al.* (1996) recommended performing the digestion at pH 1.2, however, the FAO/WHO (2001) suggested using two pH conditions (pH 1.2 and pH 2.0). In comparing pH 2.0 vs. pH 1.2, Thomas *et al*. (2004) showed that protein digestion at pH 2.0 resulted in slightly slower rates of full-length protein and fragment degradation, but did not alter the overall sensitivity of a protein to digestion. Results at pH 1.2 were more consistent than at pH 2.0, with 91% and 77% agreement between laboratories, respectively. However, more recently, The Goodman Laboratory has digested a number of proteins at both pH 1.2 and 2.0 and have not demonstrated significant differences (Ofori –Anti et al., 2008). Therefore, in this study we only evaluated stability of the protein at pH 2.0.

A review of the digestibility assay by Bannon *et al.* (2002) and by Thomas *et al.* (2004) indicates that most of the non-allergenic food proteins that have been tested are digested in less than 2 minutes in

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pepsin, while many major food allergens are stable, or produce pepsin-stable fragments that are visible for eight to 60 minutes in this assay.

Assay parameters used in this study included verification of pepsin activity, established limit of detection of the protein in the stained gel (at 10% total stainable protein) and use of an objective measurement of the time of digestion required to reach 90% digestion as described by Ofori-Anti et al. (2008). The activity of the pepsin in SGF was tested on each day of the assay based on digestion of bovine hemoglobin, as described by Worthington to ensure that it is within a tolerance interval reported by Worthington for that lot of enzyme. A second important criterion included in our standard operating procedure (SOP) is an objective measured level of residual test protein that must be reached in determining the time of digestion as discussed in Ofori-Anti (2008).The Goodman Lab has previously defined the time of digestion required to achieve 90% reduction in stained band intensity as the time-point when the residual is less than or equal to 10% of the amount of test protein in the initial sample. To accomplish that, a dilution series of test protein is tested in the same SDS-PAGE and colloidal blue staining system as the digests are analyzed to evaluate a limit of detection (LOD) as described by Ofori-Anti et al, (2008). In this study we chose an LOD of 20% of the starting sample was the limit that was detected in replicates with the protein of *Fusarium sp* extracts.

The simulated intestinal fluid (SIF) assay is performed using a buffer of 0.685 g KH2PO4 + 1.1 ml 2 N NaOH, adjusted to pH 7.5 using 2N NaOH. Pancreatin from MP Biomedicals (Cat.# Cat.#102557, lot # Q9645) is a crystalline looking solid powder that includes trypsin, amylase, lipase, ribonuclease and protease, collected from the pancreas. SIF was made by adding 0.143 g of pancreatin to 10 ml of 0.05M KH2PO4, pH 7.5. The solution was used within 24 hours. When samples of digesta are removed, they are rapidly heated at 85 °C in a water bath after adding SDS-PAGE loading buffer to stop the reaction.

## 2. Materials

### 2.1 Test Substance

The test substances for this study were samples of hyphae from cultures of the new *Fusarium* species were supplied by Sustainable Bioproducts, Inc. The first, dubbed "Sample A" for this study was lightly dried, to 25% solids and light colored, the second, dubbed "Sample B" was more vigorously dried to 95% solids and dark tan. The samples were prepared by Sustainable Bioproducts and shipped to The Goodman Laboratory on dry ice, then stored at -80 °C until prepared for extraction. Once extracted and clarified, the solutions were aliquoted into vials were stored at -80 °C.

### 2.2 Control Substance

The activity of pepsin in SGF was tested using a protocol set up to digest hemoglobin in a timed assay that is the same as that used to validate the Sigma enzyme by the company. There is no activity assay for SIF, therefore a control digest of RUBISCO from spinach was used as a negative control to verify that the pancreatin was not able to digest RUBSICO as we have shown in previous assays, while the SIF was able to digest most of the *Fusarium* proteins. The RUBISCO used in this assay was from Sigma Aldrich, product number R8000.

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**2.3 Reference Substance**

There was no reference substance for this study. Analytical reference standards (e.g., molecular weight markers) used in this study were documented in the data and are described in this report.

**2.4 Characterization of Test, Control, and Reference Substances**

The concentration of the extracts of the two hyphal samples were performed first using a BioRad Protein assay kit, but preliminary gels showed very low concentrations of the protein mixture when loaded based on calculated concentrations. The concentrations were re-tested using a 2D-Quant assay from Sigma-Aldrich. In that kit the soluble proteins are precipitated with trichloro acetic acid and resolubilized to compare to a standard dilution curve of BSA. The protein concentrations were then measured at 0.52 micrograms per ml for the extract of Sample A and 0.57 micrograms per ml for the extract of Sample B. When separated in SDS-PAGE and stained with Coomassie Brilliant Blue, they appeared almost equal and as mixtures of complex patterns of equivalent binding patterns.

**2.5 Critical Analytical Reagents**

- Pepsin A, Worthington Biochemical Corporation, cat #LS003319, lot #R7C17404, certified as having 2,530 activity units per mg solid.
- SGF without pepsin: A 35 mM NaCl solution is adjusted in pH to 2.0 as measured with a calibrated pH meter, using 6.1 N HCl.
- SGF plus pepsin 1260 U: Dissolved the mass of powdered pepsin in SGF to achieve a final activity of 1260 units per 1.52 mL of SGF, based on the activity units from Worthington, which is 10 units activity per 1 µg of tested protein.
- BioRad DC Protein Assay. Reagent A: cat #500-0113, control #64089272; Reagent B: cat #500-0114, control #64092061.
- 2-D Quant protein determination kit GE80-6483-56
- Hemoglobin from bovine blood, Sigma Chemical Co., product #H2625-25G, lot #SLBD9300V.
- Bovine Serum Albumin (BSA) from Sigma Chemical Co., product #A9647-100G, lot #SLBT0167.
- RUBISCO, Sigma-Aldrich, R8000, D-Ribulose 1,5-Diphosphate Carboxylase from spinach
- Limit of detection determination diluent: Mixed 40 ml of SGF, pH 2.0 with 14.7 ml of carbonate buffer, pH 11.0. $NaHCO_3$, Fisher Scientific, cat #S78284, lot #AD-10033-32.
- Pepsin quenching solution: 200 mM $NaHCO_3$, pH 11
- 6X Laemmli buffer, Boston BioProducts, CAS #BP-111NB, lot #J20Z4R.
- β-mercaptoethanol, BioRad #161-0710, lot #210009868
- Precision Plus Protein™ Dual Xtra Standards from BioRad, product #161-0377, control #64130781.
- Novex™ WedgeWell™ 10-20% tris-glycine gel, 1.0 mm, REF #XP10205BOX, lot #18021440.
- Novex™ Tris-Glycine-SDS 10 x running buffer, cat #BP1341-4L, lot #153375.
- Gel fixing solution: $H_2O$, methanol, and acetic acid in a ratio of 43/50/7 (v/v/v).

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- Coomassie Brilliant Blue R-250 staining solution from BioRad, cat #161-0436, control #200008120.
- Destaining solution for Coomassie Brilliant Blue R-250: H2O, methanol, and acetic acid in a ratio of 65/25/10 (v/v/v).

## 3. Test Systems.

**3.1 SGF (Pepsin) Digestion test system:** This study was an *in vitro* digestion model using pepsin in simulated gastric fluid (SGF). The digestion was performed at 37 °C, samples are removed at pre-determined times, and the activity of pepsin is quenched by neutralization with carbonate buffer and Laemmli loading buffer. The samples are then heated to more than 85°C for 10 minutes. The timed digestion samples are separated by SDS-PAGE and stained with Coomassie Brilliant Blue to evaluate the extent of digestion. Standard Operating Procedures (SOPs) for preparation of the SGF, determination of the detection limit assay, pepsin activity assay, digestion assay, SDS-PAGE and gel staining are on record in the laboratory are as described in Ofori-Anti et al., (2008). The SGF preparation and digestion procedures were based on the methods described by Thomas *et al*. (2004) as modified by Ofori-Anti et al., (2008).

The pepsin activity assay was based on the method described by Worthington for determining the activity of pepsin. An appropriate mass of pepsin powder was dissolved in prepared SGF, pH 2.0 to provide 0.9 mg/ml as a 30 x stock, which was then diluted to 1 x with SGF. Acidified bovine hemoglobin at 2% w/v was prepared and digestions to evaluate the labeled pepsin activity were performed in triplicate (1.25 ml per tube).

The amount of pepsin powder used to prepare SGF was calculated from the specific activity labeled on the product as 2,530 units /mg solid pepsin product. One-unit activity is defined as a change in $A_{280\ nm}$ of 0.001 at 37 °C, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as the substrate. The assay was designed for fixed volumes and a fixed amount of test protein so the amount of pepsin diluted in SGF is adjusted to provide the appropriate ratio of 10 units of pepsin activity per microgram of test protein in the digestion mixture. The appropriate amount of solid pepsin was added to SGF to provide 1260 units in 1.52 ml (for 10 units per microgram test protein). The pepsin/SGF reaction mixture was preheated to 37°C in a water bath before adding 200 microliters of test protein (0.36 mg/mL) for a total volume of 1.6 mL, providing 10 units per µg test protein.

Once the pre-heated (37ºC) test protein solution was mixed with pre-heated pepsin-SGF, a 200 µl volume was withdrawn at each predetermined time (between 0.5 and 60 minutes) and added to a sample tube containing 70 µl neutralizing (carbonate buffer, pH 11) and 70 µl of denaturing reagents (reducing Laemmli buffer) and immediately heated to 95°C, which stopped the digestion. Samples were then cooled in an ice-bath and then heated to > 85°C before running in SDS-PAGE. All samples from a single digestion were applied to wells of the same SDS-PAGE gel along with molecular weight markers, undigested test protein equivalent to the initial undigested test protein sample, a 10% test protein sample, and pepsin alone (to assess pepsin stainable protein bands). Samples were separated by electrophoresis, fixed with fixing solution for 30 min, stained with

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Coomassie Brilliant Blue R-250 solution overnight (at least 15 hr), and destained in R-250 destaining solution, and the stained gels were captured using a UVP BioSpectrum 815 Imaging System, Analytik Jena Company (Upland, California). The stability of the protein was defined as the time required to achieve 90% digestion, which was estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard well (P1/10). Any new bands above approximately 3,000 MW, which were generated as intermediate products of digestion, were noted as stable (or partially stable) intermediate proteolytic fragments and were considered based on stability. If those bands were also in the pepsin only controls (time 0 and time 60 mins), they were judged to be from pepsin. Otherwise, if stable for more than 5 mins, they would be analyzed by proteomic methods to determine whether they were from the test protein.

Proteins with more than 10% stainable full-length protein band remaining at 60 minutes were considered stable. Proteins reduced to < 10% stainable band at 5 to 30 minutes were considered of intermediate stability. Proteins reduced to < 10% stainable band by 2 minutes were considered labile (rapidly digested).

**3.2 SIF (Pancreatin) Digestion test system**: This study was an *in vitro* digestion model using pancreatin in simulated intestinal fluid (SIF) as described by Fu et al. (2002). Standard Operating Procedures (SOPs) for preparation of the SIF, determination of the detection limit assay, pepsin activity assay, digestion assay, SDS-PAGE and gel staining are on record in the laboratory. The SIF preparation and digestion procedures were based on the methods described by Fu et al. (2002) to measure stability of dietary proteins in simulated intestinal fluid with crude pancreatin from pigs as an enzyme mixture would improve predictions for potential food allergy risks. More recent work by Yu et al., (2013) extended the evaluation beyond testing individual proteins, such as arginine kinase of crab to testing crude extracts of muscles of crab meat, but comparing digestion in individual assays using pepsin, trypsin or chymotrypsin. This assay is not as well validated as the pepsin assay, but it is useful in safety assessment.

**3.3 Justification for Selection of the Test System**

*In vitro* digestion models are used commonly to assess the digestibility of ingested substances. Stability in pepsin in the SGF assay is the usual applied assay. Previous studies have used this simple, *in vitro* assay to evaluate potential risk of food allergy, and demonstrated that stability in pepsin is a risk factor for food allergy, which might be related to initial sensitization or to elicitation once the individual is sensitized (Astwood *et al.*, 1996 and del Val *et al.*, 1999). In this analysis, digestion was performed at pH 2.0 as a conservative approach as some authors have claimed a lack of predictive value for the digestion assay in pepsin at pH 1.2 (Fu *et al*., 2002; Yagami *et al*., 2000). However, Bannon *et al.* (2002) reviewed a broad range of published representative pepsin digestion studies and found a strong positive predictive value when comparing the stability of allergenic and non-allergenic dietary proteins, although that is primarily testing stability of a purified protein rather than a complex mixture of protein in an extract of a food source. This assay is not meant to be a stand-alone determinant in evaluating the potential allergenicity of the protein or protein mixture. It is not intended to predict the fate of proteins in the digestive tract of consumers but a measure of the resistance of the protein to proteolysis in a test tube assay as defined by Codex (2003). Some investigators use SIF to provide

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a more thorough image of digestibility of proteins, especially when whole foods are evaluated for digestibility. Some investigators do a complete SGF digestion of a test substance and neutralize the pH, then put the sample through digestion with SIF. We used separate assays to test the stability of the proteins of the new *Fusarium* species in this study.

### 3.4 Experimental Controls

Controls in this study were used to ensure assay reliability and include:

- Measurement of the activity of pepsin in SGF.
- Evaluation of the sensitivity of the staining properties of the test protein from serially diluted samples, in a separate, but similar SDS-PAGE gel.
- Inclusion of samples of pepsin without test protein at times zero and 60 minutes to determine whether any stainable protein bands observed in digestion samples with test protein are from the test protein, contaminants in pepsin or from pepsin autocatalysis.
- Inclusion of protein in SGF without pepsin at times zero and over 60 minutes to evaluate the effect of acid and heat alone.
- Digestion of Rubisco in the SIF assay as a measure to ensure enzyme activity.
- Sample limit of detection. Samples of *Fusarium* protein extracts were diluted to determine the limit of detection in stained SDS-PAGE gels used to read the results of digestion (step 4.1 below). The original measure of proteins using a Lowry detection method provided an inaccurate over-estimate of protein concentration as judged by Coomassie staining of protein samples in preliminary gels (not shown). Therefore, the protein samples were retested using a 2D Quant assay that precipitates proteins which are then re-solubilized. The lower protein estimates upon detection demonstrated that small molecular weight colored compounds were adding to detection in the Lowry assay. Repeated testing with 2D Quant demonstrated stability and Coomassie staining of samples separated by SDS-PAGE were more highly visible. Therefore we changed to a different protein determination assay to measure and adjust concentrations of sample used in the digestion tubes.

### 3.5 Sample Retention

Samples of test protein and digested samples were numbered to distinguish assay time points and assay replicates by date. Residual samples were stored at -20°C and will be discarded approximately six months after the completion of the study.

## 4. Detailed Study Methods.

This study evaluated the stability of new *Fusarium* species proteins in SGF at pH 2.0 and in a separate assay with SIF at pH 7.5. A number of control steps were performed to ensure study validity. A detailed description of the study is presented here. Laboratory records and protocols are on file in the Goodman laboratory, Dept. of Food Science & Technology, University of Nebraska, Lincoln, USA.

**4.1 Extraction of *Fusarium* pellets, protein concentrations and verification of detection.** Initially samples were prepared by grinding frozen dry pellets of Sample A and B with approximately 1 g in mass in clean mortar and pestles. Samples were extracted at a ratio of 1:5 w/v in phosphate buffered saline, and protein concentrations were attempted using a BioRad dye-binding assay (Bradford). However, a test of proteins stained with Coomassie Blue in SDS-PAGE gel showed

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the proteins were barely visible. Therefore a new extraction was performed using 1:3 protein:buffer ratio with 1 g pulverized *Fusarium* for Sample A and B, extracted with 2985 µl PBS with 15 µl Triton X 100 to solubilize proteins. The mixture was shaken for 2 hours at room temperature, then centrifuged for 30 minutes at 18,514 x g at 4 °C to clarify the solution. The protein content of the solutions were determined using a 2D-Quant assay that requires precipitation of soluble proteins with trichloroacetic acid (at -20 °C). The precipitated proteins were solubilized and tested against a standard curve to estimate the protein content Sample A was 0.52 mg/ml and Sample B was 0.57 mg/ml. Samples of the extracts were prepared and separated in SDS-PAGE in dilution series, stained with Coomassie blue and evaluated for detection limits. The gels were destained four times, 30 min each with destaining solution until the background was clear. The image was captured using the UVP imager (BioSpectrum® 815 imaging system (UVP), CA).

**4.2 Preparation of SGF Plus Pepsin.** The simulated gastric fluid (SGF) reaction buffer was prepared by adding 122.8 mg of NaCl to 59.94 mL of distilled water. The pH of the solution was adjusted to pH 2.0 using approximately 60 µl of 6.1 N HCl and water. The HCl content was approximately 0.084 N, and the salt concentration was 35 mM NaCl. The certified activity of pepsin A from Worthington was used to calculate the amount of solid pepsin that was dissolved in 1.52 mL of SGF. For this lot, the certified value was 2,530 units per mg of pepsin solid material. The target was 1260 units of activity per 1.52 ml solution which is 10 units pepsin activity per 1 µg tested protein. Based on the Worthington analysis, the concentration of pepsin A used in the assay was 0.3 µg/µl, which is 10 x dilution of the previously made 2.29 µg/µl stock with SGF. After thoroughly dissolved and mixed, the pepsin solutions were stored at 4 °C and assayed for activity and used within 24 hours.

**4.3 Pepsin Activity Assay.** The activity of the pepsin and the digestion assay were completed within 24 hours of the SGF preparation. The purpose of performing the activity assay was to ensure that the pepsin was active within a pre-defined range around the certified claim of activity by Worthington. This product has a labeled activity of 2,530 units per mg of solid material. The activity assay used was similar, but not identical to that used by Worthington. The tolerance was +/- 23% of the target units per mg compared to the Worthington certified claim. The SGF plus pepsin was freshly prepared and stored at 4 °C just before use, and then warmed to 37 °C before the addition of the target protein. The procedure was performed as follows:

4.3.1 A solution of 2% acidified bovine hemoglobin was prepared by dissolving 0.5 g of hemoglobin (Sigma # H2625) in 20 mL of distilled water, then mixing with 5 mL of 300 mM HCl.

4.3.2 Three polypropylene screw-top centrifuge tubes were labeled as Test (#1-3), three were labeled as Blank (#1-3), each received 1.25 mL of 2% acidified hemoglobin and all were preheated to 37 °C for 10 min.

4.3.3 At a timed interval (~ 1 min.), each of the test tubes in turn received 0.25 mL of SGF plus pepsin, was mixed by gentle vortex and returned to the incubator. As each test tube reached 10 minutes incubation time, 2.5 mL of 5% TCA (Sigma 6.1 N product T0699, diluted 1:20 with distilled water) was added to stop the reaction, the tube was mixed briefly by multiple inversion and then placed on ice to cool down. Then insoluble material

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(undigested hemoglobin) was removed using syringes (LuerLok BD 309646, 5 ml) and syringe filters (Corning Incorporated, 0.45 μm PTFE, product #431220).

4.3.4 Blank tubes were interspersed with the Test tubes. Blank tubes (with 1.25 mL of hemoglobin) received 2.5 mL of 5% TCA, multiple inversion, then 0.25 mL of SGF plus pepsin. After 10 minutes incubation time, these tubes were also placed on ice and then filtered to remove insoluble material.

4.3.5 The absorbance at 280 nm was measured on a spectrophotometer (Spectronic Genesys 5, Milton Roy). The activity units of pepsin per mL were calculated as the mean net absorbance (A280 nm hemoglobin – A280 controls) multiplied by a conversion factor of 1,000 to yield units of activity per mg of solid pepsin.

**4.4 Test Protein Digestion in SGF with pepsin.** Protein solutions of Sample A and B *Fusarium* samples were prepared and aliquoted, then stored at − 80 °C until just before use. The samples were thawed to room temperature for each assay.

4.4.1 Sample tube preparation. 2.0 ml centrifuge tubes were labeled as P1/10, P0, P60, D0, D0.5, D2, D5, D10, D20, D30, D60, E0, E60.

4.4.2 70 μl of pepsin quenching solution (carbonate buffer) and 70 μl of 5X Laemmli, reducing buffer were added to each tube in 4.4.1.

4.4.3 An aliquot of sample protein solution in a tube labeled as P was prepared.

4.4.3 P1/5: 190 μl of SGF plus pepsin was added, quick heated at 85°C, then 40 μl 1/5 diluted *Fusarium* solution was added. Solution was vortexed and then heated at 85°C for 10 minutes.

4.4.4 Label a tube **Pmix** (no pepsin, protein control): 350μl out of tube P and then 1.52 ml SGF were added and mixed.

4.4.4.1 Immediately 200 μl into the P0 tube were removed, mixed and heated at 85°C for 10 minutes.

4.4.4.2 After 60 minutes at 37°C water bath, 200 μl into the P60 tube were removed, mixed and heated at 85°C for 10minutes.

4.4.5 Label a tube **Emix** (pepsin enzyme, no protein control): 350 μl distilled water was added to 1.52 ml SGF plus pepsin, and then were mixed.

4.4.5.1 Immediately 200 μl into the E0 tube were removed, mixed and heated at 85 °C for 10 minutes.

4.4.5.2 After 60 minutes at 37 °C water bath, 200 μl into the E60 tube were removed, mixed and heated at 85°C for 10 minutes.

4.4.6 Label a tube Dmix (digestion mixture): 350 μl out of tube P was added to 1.52 ml SGF plus pepsin and mixed, then placed in 37°C water bath.

At 0.5, 2, 5, 10, 20, 30, 60 minute internals, 200 μl of digestion mixture were withdrawn into

D0.5, D2, D5, D10, D20, D30, D60 quenching tubes. (e.g. D 0.5 at 30 sec., D2 at 2 minutes),

each sample tube was heated to 85°C for 10 minutes.

4.4.7 P0: 190 μl of SGF plus pepsin was added, quick heated at 85 °C, then 40 μl out of tube P was added. Solution was vortexed and then heated at 85 °C for 10 minutes.

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**4.5 Preparation of SIF with Pancreatin.** SIF buffer was prepared as described in the US Pharmacopeia 26, in 100 ml. In brief, 0.685 g of $KH_2PO_4$ was added to 90 ml of $H_2O$. The pH of the solution was adjusted to 7.5 by adding approximately 1.1 ml of 2 N NaOH to the solution while measuring the pH. Make **E-SIF** by adding 0.143 g of pancreatin to 10 ml of **SIF** fluid with stirring until the solution was visibly clear.

4.5.1 Switch on the water bath to 37 °C

4.5.2 Weigh pancreatin and dissolve it in SIF (buffer) to produce **E-SIF** (at 10 mg/mL)

4.5.3 Prepare three 2 ml microfuge tubes and label them as **Pmix** (protein control mix, no enzyme), **Dmix** (test protein plus enzyme) and **Emix** (enzyme control, no protein)

4.5.4 Label 13 microfuge (1.5 ml) tubes as follows (number equals incubation times)

4.5.4.1 Protein sample control tubes were labeled (P0 and P120)

4.5.4.2 Digestion sample tubes ((D0, D2, D5, D10, D20, D30, D60 and D120)

4.5.4.3 P0-1/10 for protein control at 1/10 protein level

4.5.4.4 Enzyme controls were labeled (E0, E120)

**4.6 Protein control tubes.** Test proteins (*Fusarium* Sample B extract and **RUBISCO**)

4.6.1 **Pmix** tube. Pipet 50 µl of Fusarium Dark extract or RUBISCO protein (0.5 mg/ml) into 190 µl of **SIF** (without) enzyme, mix

4.6.2 **P0**: Pipette 40 µl of **Pmix** into tube **P0** and add 10 µl of **LSB** (Laemmli Solution Buffer), mix and heat at 100 °C for 5 mins, store on ice until ready to run gel

4.6.3 **P120:** Pipette 40 µl of **Pmix** into tube **P120**, mix and put at 37 °C water bath for 120 min before adding 10 µl **LSB**, then mix, heat at 85 °C for 5 min and store on ice until ready to run the gel

4.6.4 **P0-1/10**: Pipette 36 µl of **E-SIF** (with enzyme) into tube **P0-1/10**, add 10 microliters of **LSB,** mix, heat at 85 °C for 5 min and store on ice until cool and add four µl of **Pmix** as the undigested 1:10 control. Store on ice until ready to run the gel

**4.7 Enzyme control tubes**

4.7.1 **Emix** (pancreatin enzyme, no protein control): in a 1.5 ml microfuge tube, 50 µl $dH_2O$ was added to 190 µl **E-SIF** and then were mixed.

4.7.2 **E0**: in a 1.5 ml microfuge tube, 40 µl of **Emix** was added and then 10 µl **LSB** was added and mixed, then heated to 85 °C for 5 mins. This was stored on ice until ready to run the gel

4.7.3 **E120**: in a 1.5 ml microfuge tube, 40 µl of **Emix** was added and placed in a 37 °C water bath for 120 minutes before adding 10 µl of **LSB**, mixing and heating for 5 min in 85 °C, then icing until running the gel.

**4.8 Digestion time zero control: D0**: Pipetted 38 µl of Emix into two 1.5 ml microfuge tubes containing 10 µl **LSB**. This was heated at 85 °C for 5 mins then stored on ice.

4.8.1 **D0 Fusarium**, pipetted 10 µl Fusarium protein sample (0.5 mg/ml) into 38 µl **Emix** stopped.

4.8.2 **DO RUBISCO**, pipetted 10 µl of RUBSICO sample (0.5 mg/ml) into 38 µl **Emix** stopped.

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**4.9 Digestion samples**

4.9.1 **Dmix** was prepared by adding 380 µl of **E-SIF** into two microfuge tubes, and pre-heating to 37 °C in a water bath

4.9.2 Digestion of Fusarium protein extract with 100 µl of 0.5 mg/ml Sample B extract to 380 µl of **E-SIF** in tube labeled **Fusarium**, mixed and returned to the 37 °C water bath.

4.9.3 Digestion of RUBISCO protein with 100 µl of 0.5 mg/ml RUBISCO added to 380 µl of **E-SIF** in tube labeled **RUBISCO**, mixed and returned to the 37 °C water bath.

**4.10 Prepared digestion stop tubes** for **Fusarium** and for **RUBSCO.** Tubes labeled D2 to D120 were marked with **Fusarium** or **RUBISCO** and the numbers 2, 5, 10, 20, 30, 60 and 120. Each tube received 10 µl of **LSB**

**4.11 Stopping reactions**. At the timed intervals, 40 µl of the appropriate digestion mixture (Fusarium or RUBSICO) was pipetted out of the 37 °C incubation tube and added quickly to the appropriately marked digestion stop tube. Each was quickly vortexed and placed in an 85 °C water bath to stop the reactions. After five min the tubes were removed and put on ice until the SDS-PAGE gels were ready to run with all samples at the same time.

**4.12 SDS-PAGE gels.** All samples from one digestion assay (SGF: Sample A and B) or SIF (Sample B or RUBISCO) were evaluated on a single gel. All gels were Novex 10-20% tris-glycine gels using SDS-PAGE buffer. After separation, gels were fixed and stained in Coomassie Blue with acetic acid. Then destained using 10% methanol with acetic acid before images were captured in the UVP-light box.

4.12.1 Limit of Detection. Samples of both A and B were diluted and mixed with LSB to load samples from 20 µg/well (100% of loading digestion samples), down to 2 µg/well (10% of loading digestion samples). The results are shown in Figure 1.

4.12.2 Digestion samples SGF (pepsin). Both Sample and B digests were prepared and separated in SDS-PAGE gels with reducing loading buffer and a BioRad Precision Plus molecular weight standard.. The results are shown in Figures 2 and 3.

4.12.3 Digestion samples of SIF of Sample B of *Fusarium* and the RUBISCO samples were prepared and separated in SDS-PAGE gels with reducing loading buffers and a BioRad Precision Plus molecular weight standard. The results are shown in Figures 4 and 5.

**4.13 Image Analysis.** The destained gels were visualized in a UVP imager. The images were captured and the image intensity adjusted to optimum background and band intensities. The raw image was saved as an archival file. Images are shown in Figures 1-5.

**5. Results.** The results of the SGF and SIF digestion studies are presented for *Fusarium* strain. The Sample A was less detectable than Sample B due to differences in total protein estimation in the protein assay. The patterns in the gels were the same in band positions and intensities. Therefore Sample B was the focus of the study for both SGF and SIF.

**5.1 Limit of Detection**. The stained gels of the dilution series for Sample A and B from the new *Fusarium* species 7 extracts are shown in **Figure 1**. The minimum amount of protein that was

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reliably detected was 1 µg sample protein. Based on these data, the limit of detection was approximately 20% of sample protein loaded in digestion samples.

**5.2 Pepsin Activity.** The certified activity of the lot of pepsin from Worthington used in this study was labeled as 2,530 units per mg of solid pepsin. The mean value from triplicate analysis was 2,406 units per mg, which is acceptable according to our protocol.

**5.3 SGF Digestion results for the new *Fusarium* species.** Stained gels of digestion of *Fusarium* strain extracts are presented in Figures 2 and 3.

5.3.1 Digestion of Sample A pellets of the new *Fusarium* species resulted in lightly stained bands that were difficult to detect after 30 seconds of digestion **Figure 2**. High MW bands were clearly seen above 37 kDa in protein only samples lanes 1 and 2 at time equal to 0 and time 60 mins as well as protein plus stopped pepsin (lane 3. Bands at 15 kDa were visible as dominant bands but were markedly reduced at 30 seconds of digestion and barely visible through most times. However, the sample in lane 13 (with pepsin stopped, did not show bands, and sensitivity was thus low.

5.3.2 Digestion of Sample B pellets of the new *Fusarium* species were more clear **Figure 3**. Again, lanes 1-3 showed high MW bands clearly present at ~ 125 kDa and 100 kDa as well as 58 kDa and 50 kDa. Those bands were not visible after 30 seconds of digestion. Instead a smear of stained protein was visible below 18 kDa in lanes 4 and beyond, but at reduced intensity, and reduced size until 60 mins of digestion (lane 10) where the only visible smear is just above 10 kDa.

**5.4 SIF Digestion results for the new *Fusarium* species and RUBISCO.** The higher protein content Sample B was digested with pancreatin (**Figure 4**). RUBISCO was also digested as a control (**Figure 5**). The no enzyme wells (lanes 1, 2 and 3 of Fig 4) show band patterns similar to the SGF gel, with high MW bands visible above 50 kDa. The band patter below 50 kDa is primarily of papain, which is a complex of enzymes including trypsin, lipase, RNase and other proteases as confirmed by lanes 12 and 13 that do not include any *Fusarium* sample. Close examination of lanes 4-10 do not show bands that correspond to protein only bands of lanes 1 and 2. Lane 11 is enzyme stopped before addition of 1/10 the protein load of lanes 1 and 2. There are only a few possible faint bands visible, one at 125 kDa, and another at 40 kDa that appear the same as corresponding bands in lanes 1 and 2. These results indicate that pancreatin was able to fully digest the mixture of proteins from *Fusarium* strain. RUBISCO was used as a control protein (Figure 5) that is easily digested in an SIF assay. Unfortunately the main band is nearly exactly the same as a pancreatin protein at 50 kDa. There is a small mw protein at 13 kDa that is lightly stained in lanes 1-3 of Fig. 5. It is barely visible in lane 11 the killed enzyme control.

**6.0 Conclusions.** Digestion assays in SGF (pepsin) have been part of the safety evaluation process for genetically engineered crops since 1996 (Astwood et al., 1996). Digestion assays in SIF (pancreatin or trypsin & chymotrypsin, Fu et al., 2002 and Yu et al., 2013) are less commonly used for evaluating possible safety questions related to dietary allergenicity or toxicity and the value of the stability in SIF is not as clear as it is for stability in pepsin (Bannon et al., 2002). However, the SIF assays have been more commonly used in evaluating whole food or whole crop safety or in evaluating nutritional properties of whole foods. One of the confounding factors seems to be the

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whole foods often include one or more proteins or small molecular weight factors that inhibit the digestive ability of pepsin to digest proteins such as beta-lactoglobulin (Nacer et al., 2004) The proteins in cultured product from this new *Fusarium* species are not all rapidly digested in SGF but they do appear to be rapidly digested in SIF. The results are useful as part of the overall food safety evaluation of this strain of fungus for use in foods. It appears that the proteins are relatively labile to enzymes from the human digestive tract. Since it is likely that few people may have allergies to proteins in *Fusarium* fungi, digestion of the proteins are unlikely to alleviate all risks. Foods made using this strain of fungus as a major protein source should be labeled as to the source so that people who do have allergies associated with this source can avoid consumption of those products if they are found to have reactions upon exposure.

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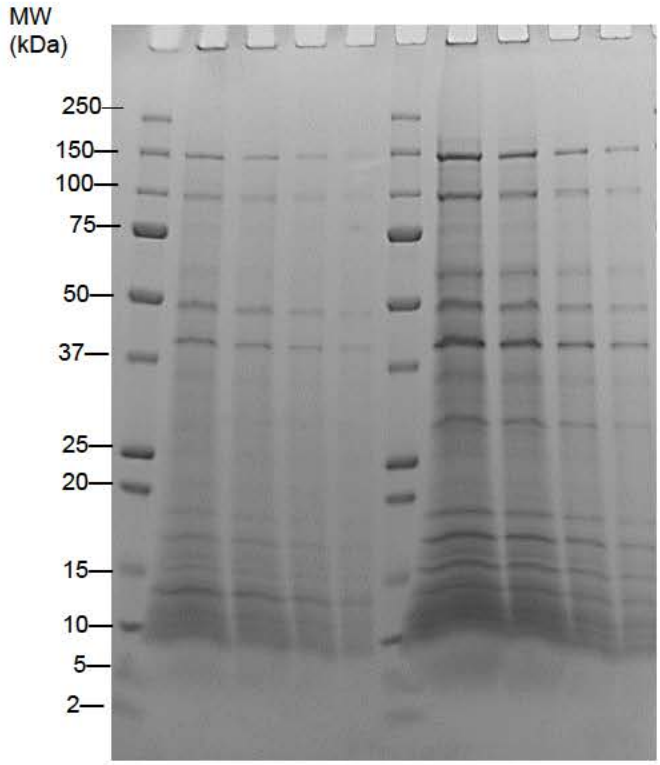


**Figure 1. Coomassie Brilliant Blue Stained SDS-PAGE Gel showing the serial dilution of the new *Fusarium* species proteins starting from 100% of total protein.** Proteins in extracts of Sample A and B pelleted proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel and stained with Coomassie Blue. Lanes 1-4 represent diluted extracts from Sample A ranging from 10 ug (lane 1) to 1 ug (lane 4). Lanes 5-8 represent diluted extracts from Sample B ranging from 10 ug (lane 5) to 1 ug (lane 8). M represents the BioRad Precision Plus markers.

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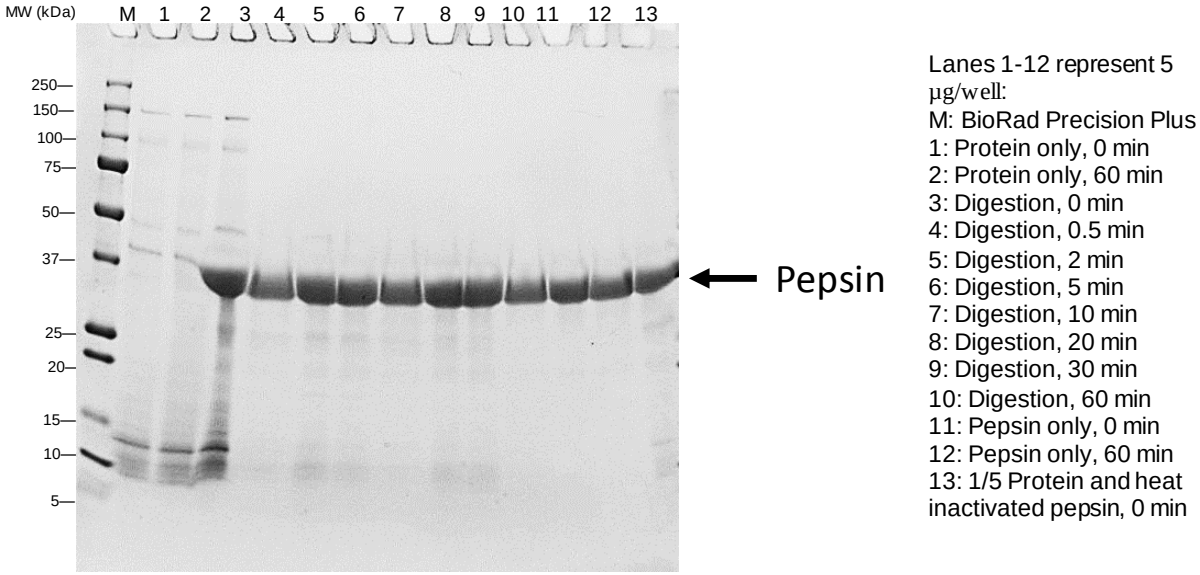


**Figure 2. Coomassie Brilliant Blue stained SDS-PAGE gel showing SGF digestion of proteins extracted from Sample A from the new *Fusarium* species in simulated gastric fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel with extract equivalents of 5 µg per lane based on pre-digestion concentration (pH 2.0).

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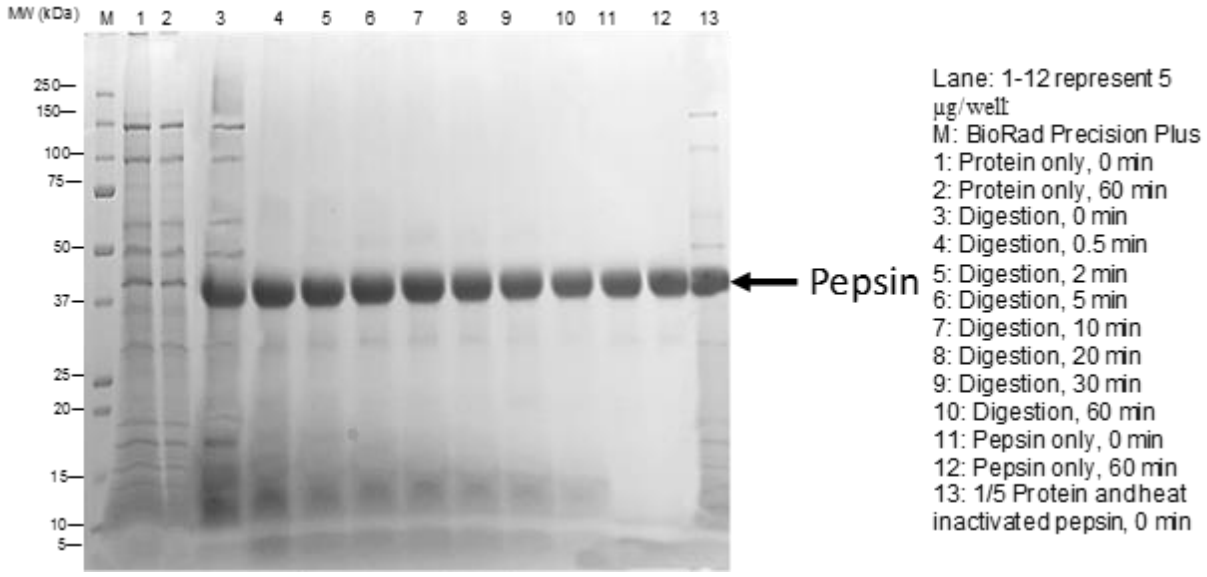


**Figure 3. Coomassie Brilliant Blue stained SDS-PAGE gel showing SGF digestion of proteins extracted from Sample B from the new *Fusarium* species in simulated gastric fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel with extract equivalents of 5 µg per lane based on pre-digestion concentration (pH 2.0).

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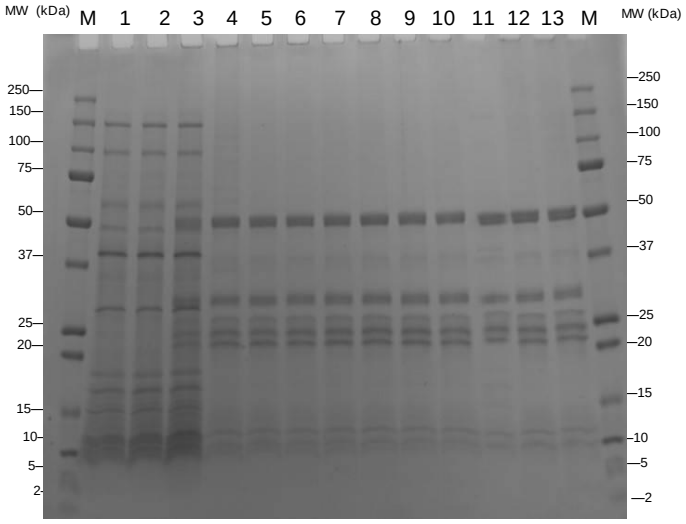


Lane: Samples loaded at 5 µg protein per well.
M: BioRad Precision Plus
1: Protein only, 0 min
2: Protein only, 120 min
3: Digestion, 0 min
4: Digestion, 2 min
5: Digestion, 5 min
6: Digestion, 10 min
7: Digestion, 20 min
8: Digestion, 30 min
9: Digestion, 60 min
10: Digestion, 120 min
11: 1/10 Protein and heat inactivated pancreatin, 0 min
12: Pancreatin only, 0 min
13: Pancreatin only, 120 min

**Figure 4. Coomassie Brilliant Blue stained SDS-PAGE gel showing the SIF digestion of proteins extracted from Sample B from the new *Fusarium* species in simulated intestinal fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel with extract equivalents of 5 µg per lane based on pre-digestion concentration (pH 7.5).

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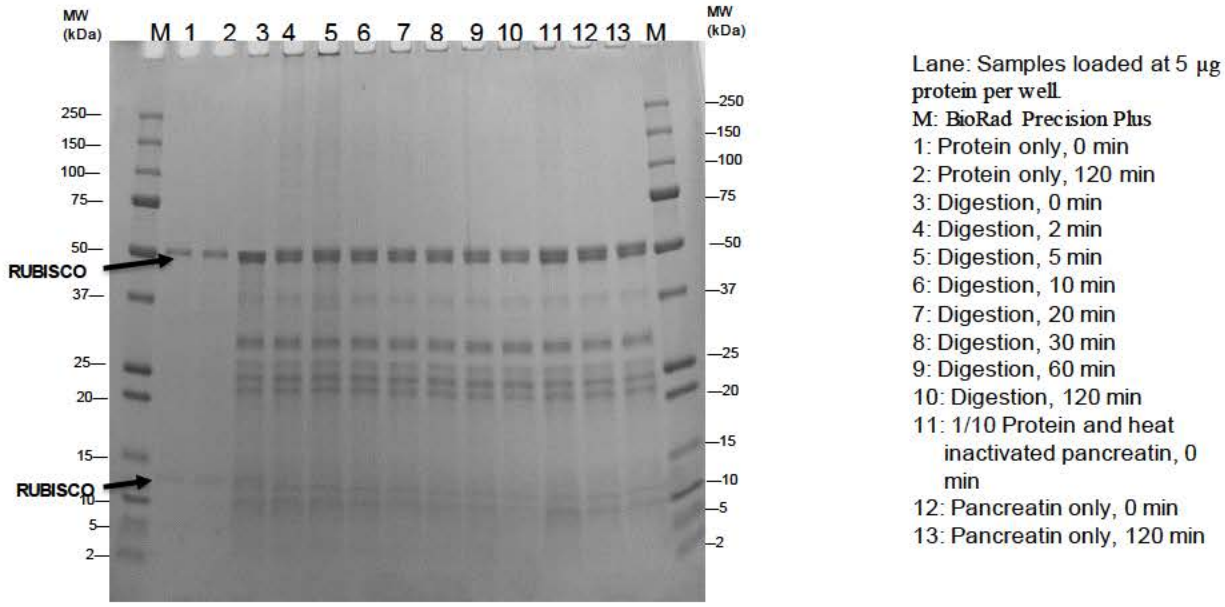


**Figure 5. Coomassie Brilliant Blue stained SDS-PAGE gel showing the SIF digestion of RUBISCO protein in simulated intestinal fluid.** Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel with extract equivalents of 5 µg per lane based on pre-digestion concentration (pH 7.5).

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# Attachment 4

# New Fusarium species fermentation product proteins: Bioinformatics to evaluate potential allergenicity 24 March 2019

## STUDY TITLE

New *Fusarium* species fermentation product proteins: Bioinformatics to evaluate potential allergenicity

## AUTHORS

Richard E. Goodman

## STUDY COMPLETED ON

24 March 2019

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## LABORATORY STUDY ID

REG 2019 Sustainable 3

**Study Number:** REG 2019 Sustainable 3

**Title:** New *Fusarium* species fermentation product proteins: Bioinformatics to evaluate potential allergenicity

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**Study Sponsor:**
Sustainable Bioproducts, Inc.
Brian Furey
Manger of Regulatory Affairs

**Study Start Date:** 10 December 2018

**Study Completion Date:** 24 March 2019

**Records Retention:** All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

**Signature of Final Report Approval:**  24 March 2019

Principal Investigator: Richard E. Goodman Date

## SUMMARY

Sustainable Bioproducts, Inc. developed a fermented food protein product from a new *Fusarium* species related to the *Fusarium fujikuroi* species complex and deposited in ATCC as No. PTA-10698. The product is a fermented hyphal mass that is intended for use in food products. It is a whole organism with hundreds of proteins that were identified based on a full-genomic sequence analysis. A similar novel food was developed by a different company in the United Kingdom in the 1980's from another species of *Fusarium* that is used to make a commercial food product called Quorn. Quorn has been consumed as a human food source for over 30 years in the UK and the United States.

Sustainable Bioproducts, Inc. asked the Goodman Laboratory to perform an overall allergenicity risk assessment of their product, beginning with a literature review of allergen safety of the species to identify any specific food risks, as well as conducting digestive fate studies of the proteins and evaluation of predicted proteins against the allergens in AllergenOnline.org. Those two studies are presented in other reports. In addition, we performed a bioinformatics evaluation of the 14,239 predicted proteins that were annotated as possible translation products based on genomic identification and theoretical translation of the genes performed for Sustainable Bioproducts by Igenbio's ERGO ™ Bioinformatics platform that was submitted to us on 30 November 2018. The annotated data was converted to a FASTA file that represents the expected full proteome of hypothetical proteins of the new *Fusarium* species,predicted by Igenbio ERGO™. The FASTA file was searched against the AllergenOnline.org database.

The predicted proteins were compared to our publicly available www.AllergenOnline.org version 18B database (original posting was 23 March 2018) in December, 2018. The allergen database was loaded in the University of Nebraska's Holland Computing Center server and sequences were performed using the FASTA3 search that was used to search the Public database. The resulting alignments were recorded and evaluated for potential risks based on how unique the proteins were that had identity matches of greater than 35% over aligned lengths of at least 80 amino acids (AA). The full-length alignments and the *E* score statistic for uniqueness were evaluated for each alignment and reported here. There were 504 identity matches above the minimum criteria of 35% identity over 80 AA. On 10 February 2019 an updated version 19 of the AllergenOnline database was posted an included the *Fusarium proliferatum* protein 9.0101 as an allergen. Therefore the sequence of Fus p 9.0101 was searched against the predicted proteins and a 100% identity match was found to predicted protein RFSUS69279, that had a match of 72.6% identity to *Cladosporium* sp. protein as identified from version 18B. Thus, the number of matches was still 504. Due to the large number of matches of these predicted proteins to allergenic proteins, we asked Sustainable Bioproducts if it would be appropriate to test the public genomic sequences of Quorn, *Fusarium venenatum* against the AllergenOnline database for relative comparison. Sustainable Bioproducts agreed and the findings of this evaluation identified 478 proteins with matches of 35% identity over 80 or more AA for Quorn with most matched proteins having nearly identical hits compared to the genomic matching evaluation of the new *Fusarium* species with AllergenOnline. Our conclusion of the two bioinformatics evaluations is that there is a high number of identity matches above the CODEX guidelines, but further evaluation of the matches show that the proteins are not major allergens and are unlikely to be clinically relevant matches

beyond *Fusarium* sp. We noted a lack of high sequence identity matches to major allergens in unrelated sources (e.g. peanut, tree nuts, egg, milk, soybeans, or mustard). The identified allergens were also compared to sequences in the PubMed Protein database to understand the relevance of these proteins to proteins in other common sources. Those identity matches were higher in identity than the alignments to AllergenOnline proteins. The results are discussed in this report.

The conclusions of this evaluation are that foods containing proteins from the new *Fusarium* species are unlikely to cause significant allergic reactions due to cross-reactivity. The number of consumers who might experience allergic reactions from these proteins is expected to be low and most would be allergic to other *Fusarium* sp. or closely related fungi. These foods should not be seen as a major allergen source.

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## 1. Introduction

Sustainable Bioproducts, Inc. developed a new fermented food source of a newly discovered *Fusarium* species that is grown as a hyphal mass in controlled culture conditions. The Goodman Laboratory, Food Allergy Research and Resource Program (FARRP), of the University of Nebraska has conducted an overall allergenicity risk assessment to evaluate potential risks of food allergy that might occur from consumption of the Fusarium based food. The risk assessment includes:

a) A literature search looking for evidence of allergy to foods and environmental source of proteins from various *Fusarium* species; a search for evidence of IgE binding and allergy to specific proteins of these sources or of cross-reactivity to other sources that have high sequence identity to *Fusarium sp*. Proteins (results are provided in a separate report)
b) Testing the stability of the proteins in the strain in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) assays (results are provided in a separate report) and
c) Evaluation of genomic sequences of this strain to predict possible proteins that were all compared to AllergenOnline.org version 18B for identity matches with allergens. (The topic of this current report)

The purpose of this assessment is to identify any potential extra risks of food allergy that might occur from consumption that could be related to consumers who are allergic to other sources of proteins. Clearly most food sources pose some risks of food allergy, so the outcome of this assessment is not expected to show a lack of risks of any allergic response, but to identify unexpected risks such as protein sequence identity matches.

The genomic sequencing of this strain was performed by a company, Igenbio, Inc., under contract by Sustainable Bioproducts, Inc. Their annotated genome was provided to us and our analysis indicates there are 14,239 proteins that may be expressed from this genome based on annotation of the DNA sequences. The predicted amino acid coding regions were evaluated for matches to sequences of allergens in our public www.AllergenOnline.org database version 18B (AOLv18B) on October 23, 2018. The AOLv18B was installed at the University of Nebraska Holland Computing Center server by Mohamed Abdelmotelb to test whole predicted proteomes like this one. Mr. Abdelmotelb ran a batch comparison using FASTA-36.3.8 to provide matches of >35% identity over 80 or more amino acids. Mr. Abdelmotelb also compared the sequences to the NCBI non-redundant protein database to provide an overall identity match using BLAST+2.7.1 (October 23, 2017).

*Codex Alimentarius* Commission guideline (2003) that was written with the intent of evaluating potential risks of food allergy and consideration of risks of toxicity and nutritional properties of a genetically engineered food crop that has had an addition of one or a few genes. It is important to note that while the guidelines were followed, the reviewed mycoprotein is not a genetically engineered food crop. It was not written to evaluate new organisms intended to be a novel food source. The Codex was followed for this assessment, evaluating this novel food macroingredient to ensure that there were not unexpected high identity matches to proteins that have very high identity matches to severe allergens from highly allergenic sources (e.g. Ara h 1, Ara h 2, Ara h 3 or Ara h 6 of peanut; tropomyosin from shrimp; or beta-lactoglobulin from cow's milk). The Codex document recommends examining published

scientific studies of potential allergenicity of new proteins against peer-reviewed published studies related to allergy and allergenicity of the source. The highest identity matches reported here are to proteins of *Fusarium sp.* and the reports of allergy to *Fusarium* and other related species were already discussed in the literature review study. Those reports are of rare cases of IgE mediated allergy and no obvious severe reactions to foods that would not be associated with the mycoprotein source. This investigation provides a clear comparison to provide a full assessment of risk.

**2. Proteins predicted from the genome analysis of *Fusarium* strains.**

The 14,239 proteins predicted from the genomic sequences of the new *Fusarium* species were evaluated for amino acid sequence identity matches >35% to allergens in www.AllergenOnline.org version 18B. These *Fusarium* protein sequences have not been demonstrated to be expressed in the cultured fungus, but the DNA sequences predict that they may be expressed. The predicted sequences were used as the entire set of proteins to evaluate for potential identity matches to known allergens.

In addition, since the genome is complex and the assumption is that food produced from cultures of this organism would produce a very complex set of proteins, we decided to evaluate sequences of related organisms for evidence of allergy. The genus is complex as *Fusarium* is divided into a number of species complexes based on the ecology of the species and predictions of evolution based on genomic sequences. The majority of species live primarily on tissues of certain plant types, or in soil and are important in decomposing plants. A few groups like the *Fusarium fujikuroi* species complex are important plant pathogens that are restricted to certain host species (Niehaus et al., 2016). Some species or strains are able to infect humans or other mammalian tissues and some are known to grow in the lungs of humans, causing pulmonary disease similar to that cased by certain *Aspergillus* species. The literature search identified over 188 publications using *Fusarium* AND allergy OR Allergen. However, only a few of the publications showed clear connections with proteins from *Fusarium* and with specific proteins. A publication by Levetin et al., (2016) described an overview of taxa of fungi that have been verified to cause allergies including those due to inhalation, ingestion or infection. A major change in characterization of fungal species is the use of DNA sequence analysis. The WHO/IUIS Allergen Nomenclature database was searched for proteins that have been identified as possible allergens. Within AllergenOnline.org there are five proteins that have been identified as possible allergens in this genus. Furthermore, since a similar species, *Fusarium venenatum*, has been consumed as Quorn, a protein source in foods in the United Kingdom since the mid-1980's and in the US since 2002, we also collected the sequences of all proteins from this species for comparisons to AllergenOnline.org as well as the new *Fusarium* species being evaluated.

**2.1 Proteins from the new *Fusarium* species.** Sustainable Bioproducts, Inc. provided the genomic sequences that were performed by MrDNA and evaluated by IgenBio. According to our communications with Sustainable Bioproducts, the sequencing was performed using Pacbio (for long-reads) and Illumina (for short-2x250 bp reads) of the cultured species and the genomic sequence compiled for highest accuracy and completeness. The quality of the sequences that included 340k reads after trimming and correcting from Pacbio, and 56.5 M read pairs from Illumina. The reads were evaluated using FASTQC. Sequences were compiled using assemblers

MaSuRCA (Zimin et al., 2013) and SPAdes (Bankevich et al., 2012) used K-mers of 21, 33, 55, 77, 99 and 127. Post assembly polishing used Pilon (Walker BJ et al., 2014). Assembled sequences included 89 contigs, with the largest contig being 4.9 MB, N50 for 3.2 MB, N75 for 2.3 MB and L50 6, L75 10 and 0 Ns with a GC content of 48.3%. Pacbio reads mapped at 99.95% using Minimap2 software. Illumina reads mapped at 99.81% using Bowtie2 software. Genes were predicted using the *Fusarium* model from Augustus (King et al., 2015), mitochondrial genes were predicted using Prodical (Hyatt et al., 2010), tRNA were predicted using tRNA scan-SE (Lowe and Chan, 2016) and rRNA were predicted using Barrnap software (Torsten Seemann, https://github.com/tseeman/barrnap/). Functional annotation was done using the ERGO software package of IgenBio. The overall sequence completeness was further evaluated by comparison to the genomes of strains of *Fusarium* which had been characterized before and provide a good framework for understanding completeness (Niehaus et al., 2016).

The important things to consider for risks of allergenicity are the similarity or differences of the proteins to those of other allergenic sources. Essentially all foods present some risks of allergy that are specific to the proteins in the food sources. Consumers must avoid foods that they are allergic to in order to avoid reactions. It is highly likely that fungal strains that are species of *Fusarium* will cross-react if consumed. It is important to consider species of other fungi that are closely related to *Fusarium* based on high identity matches to similar fungi. Less commonly shared protein sequences are less likely to share IgE binding and more diverse organisms are less likely to share cross-reactivity. With redefinition of species of fungi into species clusters that share common DNA and protein sequences, the genus Fusarium has been segmented into groups of species called complexes. Sustainable Bioproducts, Inc. provided the full-genomic sequences for our bioinformatics searches against AllergenOnline.org. The data comparisons demonstrated that this *Fusarium* species, which was deposited in the ATCC collection as No. PTA-10698, seems to be most closely related to the *Fusarium fujikuroi* species complex which is a relatively broad group of plant pathogens and decomposers.

The predicted protein sequences were translated from the DNA sequences and annotations included 14,239 expected proteins. Those sequences were compared to the AllergenOnline.org database version 18B by batch comparisons using FASTA 36.3.8 (23 October 2018 by W.R. Pearson, University of Virginia, http://faculty.virginia.edu/wrpearson/fasta/fasta_versions.html) in the Holland Computing Center at the University of Nebraska. Our public AllergenOnline.org database currently uses FASTA 35.04, which provides the same matches as 36.3.8. BLASTP was used to compare these sequences to other NCBI Proteins.

**2.2 Proteins predicted for Quorn strain *Fusarium venenatum*.** The genome of the Quorn strain is available online at https://www.ncbi.nlm.nih.gov/genome/69468 . It was performed by Rothamsted Research in the United Kingdom and deposited at NCBI by R. King on 21 October, 2014 as LOCUS NC_038012 11988928 bp DNA linear CON 12-JUL-2018 DEFINITION *Fusarium venenatum* strain A3/5 genome assembly, chromosome: I. ACCESSION NC_038012. This is a well documented RefSeq. However, determination of probable protein sequences is presented in the Protein file. The total genome translation products predicted from the genome includes 13,930 proteins.

**2.3** ***Fusarium sp*. proteins in the WHO/IUIS Allergen Nomenclature and AllergenOnline databases.** The WHO/IUIS Allergen Nomenclature Database (www.allergen.org) includes four proteins that have received approval as named allergens by the committee. Fus c 1 is a Ribosomal protein P2 of approximately 11 kDa from *Fusarium culmorum*. Fus c 2 is a Thioredoxin-like protein of approximately 13 kDa from *Fusarium culmorum*. (Hoff et al., 2003a). Fus p 4 is a Transaldolase protein of approximately 37.5 kDa from *Fusarium proliferatum*. Fus p 9 is a Vacuolar serine protease of approximately 36.5 kDa from *Fusarium proliferatum.* AllergenOnline also lists a fifth protein that is not given a biological protein name, only helix-loop-helix 450 AA protein from *Fusarium culmorum* that showed IgE binding in IgE immunoblots in 3 of 52 subjects with suspected allergy to this mold although IgE binding was weak and they did not perform basophil activation as was performed for Fus c 1 and Fus c 2 in this publication (Hoff et al., 2003b).

## 3. AllergenOnline.org version 18B database.

The Allergenonline.org version 18B database that was posted on the public website (www.AllergenOnline.org) on 23 Mar 2018. It has 2089 allergenic protein sequences organized into 853 protein groups from 384 total species, was loaded on the Holland Computing Center server along with the FASTA 36.3.8 search program. The database includes proteins from airway, food, injection and contact allergens. The allergens are identified by a minimum of IgE binding from subjects with symptoms consistent with exposure in published studies. This dataset was the basis for sequence searches with the *Fusarium* species predicted proteins. In addition, the sequence of the Fus p 9 allergen was used in a separate search of the *Fusarium* proteins since that protein was not in the Version 18B dataset. It has now been added to the AllergenOnline.org version 19 dataset on 10 February 2019.

## 4. NCBI Protein database.

The non-redundant NCBI Protein database (https://www.ncbi.nlm.nih.gov/protein/) was used as the primary sequence resource to identify proteins that have high identity matches to *Fusarium* proteins and also for comparisons with matched allergens. The database is updated approximately every week. This database can be searched using BLASTP search engine with keywords (e.g. allergen) or for species of protein types. Most comparisons were performed on 4 December 2018 or during evaluation of the current bioinformatics alignments, until 5 March 2019. It is also the source of DNA sequences and the genome of *Fusarium venenatum*. BLASTP searches were conducted to make the comparisons used in this evaluation.

## 5. Bioinformatics methods and results.

**5.1 FASTA 36.3.8 to the new *Fusarium* species to AllergenOnline.org version 18B.** The FASTA program was created by W.R. Pearson at the University of Virginia (http://faculty.virginia.edu/wrpearson/fasta/fasta_versions.html) with descriptions on the University of Virginia website for changes and critical points for alignment comparisons (Pearson, 2016). The results of these comparisons of sequences with AllergenOnline.org version 18B were down-loaded (Appendix 1) and saved as files at the University of Nebraska. Based on the literature review performed by our laboratory, it is clear that some people with asthma have IgE mediated allergy to

inhalation of proteins from *Fusarium sp*. and since it is an evolutionary relative of molds such as *Penicillium* and *Aspergillus*, it should not be surprising to find conservation of some allergens. Comparison of the 14,239 predicted protein sequences from the new *Fusarium* species resulted in 504 alignments to allergens in AllergenOnline.org version 18B that were above the CODEX criteria of >35% identity over 80 or more AA, as shown in Appendix 1. High identity matches over most of the length of proteins (showing true "homology") were common in the top 70 or so alignments with identity matches >50%. Most of those were matches to fungal allergens including very high identity matches (>70%) to 15 sequences of house-keeping proteins, primarily in fungi. These are generally of low abundance and have not been proven to cause clinical disease, only binding to IgE and showing skin prick test reactivity. To get a better understanding of the relevance of matches, the predicted proteins of *Fusarium venenatum* (Quorn source) were also compared to AllergenOnline.org version 18B (Appendix 2). The identity matches of more than 450 proteins from Quorn had very similar alignments to the version 18B allergens (see Table 1 for comparison of the top five aligned allergens to both *Fusarium* sp.). A careful comparison of the two appendices showed that only 44 proteins in of the new *Fusarium* species were not predicted in the Quorn genome, as indicated in Appendix 1, highlighted in yellow. Those sequences were further evaluated using BLASTP searches with the NCBI non-redundant Protein database to understand their overall identity matches (see Appendix 3).

It is important to understand that the CODEX guideline (>35% identity over 80 amino acids) was written to protect highly allergic people from extremely potent allergens such as 2S albumins of peanut and tree nuts, lipid transfer proteins of peach and tropomyosin of shrimp (Goodman, 2006). The low identity matches are thought to severely over-estimate cross-reactivity as suggested by Aalberse in 2000 who noted that proteins sharing more than 70% identity over their full lengths are common cross-reactive allergens, while those sharing less than 50% identity over their full-length rarely show IgE cross-reactivity. Even the dominant evolutionarily conserved allergenic proteins show marked percent identity differences for predicting cross-reactivity. For instance, human tropomyosin is 52% identical to shrimp tropomyosin and people allergic to shrimp do not have auto-reactivity to their own tropomyosin. The 2S albumins of peanut and tree nuts often shares about 35% to 42% identity, and while some of those proteins have been shown to have some IgE cross-reactivity in laboratory tests, few people have shared clinical reactivity across the species of tree nuts except for closely related species (walnut and pecan). Lipid transfer proteins (e.g. Pru p 3 of peach) has very high identity matches across other members of the genus Prunus and related fruits (apple) with >60% identity, but clinically relevant cross-reactivity is probably insignificant below 60% identity for full-length alignments. Yet 69 proteins within AllergenOnline.version 18B have identity scores >35% over 80 amino acids including those that are extremely unlikely to be clinically relevant (wheat, barely, tomato, sunflower and kiwi). Thus, it appears that the criteria should be different for very different proteins to provide a better risk prediction of cross-reactivity for diverse proteins as discussed here.

In the current analysis of the new *Fusarium* species, the top scoring alignments ranged from 100% identity over 323 AA with an *E* score of 4.4e-133 for the transaldolase that is known as Fus p 9, and 92.7% identity to Fus c 1 over 110 AA with an *E* score of 3.9e-36, Thioredoxin of Fus c 2 with 91.7% identity over 121 AA and an *E* score of 2.2e-60 to enolase from *Aspergillus fumigatus* with 85.8% identity over 438 AA and an *E* score of 1.4e-163. Those results indicate that people

who are already allergic to *Fusarium sp*. and closely related fungi (e.g. *Aspergillus* sp.) are likely to react following consumption of foods made from the new *Fusarium* species. The highest scoring 12 alignments were to fungi, followed by a match was identified to tubulin of the *Dermatophagoides farina* house dust mite with 73.2% identity over 451 AA with an *E* score of 1.9 E -150. Tubulin is highly conserved across-eukaryotes and it is an intracellular protein. Tubulin is not a major allergen of house dust mites, as Der p and Der f 1, Der p/f 2 and Der p/f 23 are major allergens of house dust mites that are thought to be the primary causes of asthma for those with significant allergy to house dust mites. It is highly unlikely that there would be clinically important IgE cross-reactivity between the new *Fusarium* species in consumers allergic to house dust mites. The highest scoring match to a plant allergen was to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of wheat (*Triticum aestivum*) with 70.1% identity over 488 AA and an *E* score of 7.4e-101. The GAPDH protein is a highly conserved enzyme across-eukaryotes and it is a minor allergen. GAPDH does not seem to be an important allergen from wheat. It has only shown some IgE binding from people with allergies and has not been carefully tested for relevance. A few other high identity matches are present in Appendix 1, and the identity matches fall to low levels (~ 50% or less) by page 3 of Annex 1. The matched proteins are primarily highly conserved proteins in evolutionary terms, abundance is low and they are mostly intracellular.

**5.2 FASTA 36.3.8 to *Fusarium* venenatum (Quorn) to AllergenOnline.org version 18B.** The results of batch FASTA search of the predicted proteins from this genus which is grown to produce commercially available and consumed Quorn for more than 30 years (Appendix 2) are very similar to identity matches identified using the new *Fusarium* species predicted proteins except for the 44 proteins missing from alignments in this species. Thus allergic reactions to Quorn are likely to be mimicked in subjects consuming the new *Fusarium* species.

Table 1. Comparison of FASTA alignments for the new Fusarium sp. from Sustainable Bioproducts and from Quorn. The alignments of the entire expected protein output are shown in the Annexes of this report. However, for a direct comparison we have summarized the data from the top scoring five alignments for both species.

| | **New *Fusarium* sp.** | | | **Quorn** | | |
|---|---|---|---|---|---|---|
| **Protein** | Identity % | Align length | E score | Identity % | Align length | E-score |
| Transaldolase | 100 | 323 | 4.4e-133 | 96 | 323 | 5.4e-126 |
| Ribosomal P2 | 92.7 | 110 | 3.9e-36 | 90.8 | 109 | 6.7e-36 |
| Thioredoxin | 91.7 | 121 | 22e-60 | 95.9 | 121 | 1.3e-48 |
| Enolase | 85.8 | 438 | 1.4e-152 | 85.4 | 438 | 1.1e-162 |
| Cytrochrome C | 83.5 | 103 | 2.8e-53 | 80.6 | 103 | 3.6e-50 |

**5.3 Consideration of the 44 unique proteins in new *Fusarium* species compared to all proteins in the NCBI Protein database by BLASTP.** The 44 identity matched proteins from the genome of this strain were compared to the NCBI Protein database without a keyword selection. The results are summarized in Appendix 3. For all of the 44 proteins, the top identity matches are too many

species of *Fusarium* fungi, and lower identity matches to other related fungi (Cladosporium and Aspergillus) for some of the 44 proteins. This demonstrates that risk of allergy, if any is within the genus *Fusarium*.

Based on the bioinformatics results it appears that the new *Fusarium* species does not represent a risk of clinical allergy except for consumers who are allergic to *Fusarium sp.*, or closely related fungi.

**6.0 Conclusions.** The results of this bioinformatics comparison of proteins expected as possible translation products from this species of *Fusarium* shows that they are highly homologous to proteins expected from a number of species of this genus. There are a few proteins of moderate identity from other related fungal species. Thus, allergic reactions and cross-reactions are likely to be restricted to highly related species. There were no high identity matches to significant allergens from other sources that would put consumers with allergies to those sources, whether a food or environmental species, at risk.

The literature search for evidence of allergy to *Fusarium sp.* proteins demonstrated that some people do have allergic reactions to a few proteins of this species when inhaled, when the organism has invaded their tissues, most commonly their lungs or sinuses. In addition, based on evidence from Quorn, a few people may have IgE mediated allergy from ingestion of proteins containing Quorn (*Fusarium venenatum*), which is made of high concentrations of proteins in food from that related species. Those reactions should not be a surprise as most food sources that contain significant amounts of protein do cause allergies in some consumers. It appears that some of the reactions are cross-reactive in the sense that consumers have allergies to related proteins from inhalation of homologous proteins from *Aspergillus sp.* or *Penicillium sp*. Four proteins have been identified from two species of *Fusarium* as allergens that are recognized by the WHO/IUIS Allergen Nomenclature Sub Committee and are in the AllergenOnline.org database along with one additional protein that is not recognized by the WHO/IUIS database. An important characteristic of reactions to Quorn are the rare cases of clear IgE mediated allergy. The evidence suggests that there may have been two deaths from consumption of Quorn in meals where the protein was consumed by someone already sensitized to fungi who ate a meat-patty sized meal of Quorn (Jacobson and DePorter, 2018). Based on the sparse information provided in Jacobson and DePorter (2018) and their references, it was not certain that their deaths were due to an IgE mediated reaction to Quorn proteins. The reactions may be due to proteins in the meal from egg or milk whey or to something else. In addition, a number of cases were reported from individuals who have experienced occasional episodes of vomiting or diarrhea from consumption of meals of Quorn that could be due to an intake of bulky food that is harder to digest due to the mass of chitin, a complex carbohydrate of N-acetyl glucosamine. IgE mediated reactions would occur every time a substantial amount of the causative food was ingested and not sporadically (Sicherer and Sampson, 2018).

From this bioinformatics review, there is no evidence suggesting food products containing *Fusarium* species represent a risk of allergy for consumers, except for those

who are allergic to *Fusarium* sp., or closely related fungi. Overall, there is no evidence suggesting such food products represent a major risk of allergy for consumers.

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# 8.0 Appendices

Annex 1 FASTA36 for the new *Fusarium* species proteins by genomic sequences

Annex 2 FASTA36 of the Quorn proteins by genome of *Fusarium* venenatum

Annex 3 BLASTP of 44 proteins unique to the new *Fusarium* species

Annex 4 AllergenOnline.org version 18B

# Attachment 5

# Fusarium Mycoprotein fermentation product overall summary of allergenic risk 23 March 2019

## STUDY TITLE

*Fusarium* mycoprotein fermentation product overall summary of allergenicity risk

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## STUDY COMPLETED ON

23 March 2019

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REG 2019 Sustainable 4

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**Principle Investigator:**
Richard E. Goodman
University of Nebraska
Tel: +1 (402) 472-0452

**Study Sponsor:**
Sustainable Bioproducts, Inc.
Brian Furey
Manager of Regulatory Affairs

**Study Start Date:** 10 December 2018

**Study Completion Date:** 23 March 2019

**Records Retention:** All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.4

**Signature of Final Report Approval:**

Principal Investigator: Richard E. Goodman — Date: 24 March 2019

Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

**Summary review of the safety assessment of protein from new *Fusarium* species**

Sustainable Bioproducts, Inc. asked the Goodman laboratory in the Food Allergy Research and Resource Program at the University of Nebraska-Lincoln to evaluate potential risks of food allergy that might be caused by their new, fermented protein product from a cultured new species of *Fusarium* that has been characterized and submitted to the ATCC as No. PTA-10698. The assessment was designed to consider evidence of allergenicity of the organism based on information from literature searches in the PubMed database and the internet (Google) as well as comparison of sequence information to proteins that have been identified in the NCBI Protein database as well as allergens identified in our www.AllergenOnline.org database. The sponsor had the genome of this species determined by Igenbio's ERGO™ Bioinformatics platform with annotation provided to us. We checked the data for quality and accuracy and used the FASTA35 program to characterize similarity of sequences to www.AllergenOnline.org version 18B. We also used the BLASTP program to search the NCBI Protein database for the best sequence matches to *Fusarium sp.* proteins as well as those in other species. The sponsor also provided two processed lots of hyphal mass of this species that were dried to different levels so that we could test the stability of the *Fusarium* sp. proteins in standard digestion conditions of Simulated Gastric Fluid (SGF) with pepsin as the proteolytic enzyme and with Simulated Intestinal Fluid (SIF) with papain as the standard mixture of pancreatic enzymes. This evaluation was designed to follow the allergenicity evaluation process outlined by the Codex Alimentarius guideline (2009) for evaluating genetically engineered organisms, although the organism is not a GE organism. The overall process evaluation described by Codex was not intended for evaluation of the complex mixture of proteins in a novel food source. However, the steps provide some guidance on evaluating potential risks of a whole food. This assessment provides a scientific basis for considering possible food allergy risks.

The individual evaluation processes, data and conclusions were presented in three reports:

1) *Fusarium* mycoprotein fermentation product literature survey.
2) *Fusarium* mycoprotein fermentation product proteins in vitro digestibility study in simulated gastric fluid (pepsin) assay (pH 2.0) and in simulated intestinal fluid assay (pH 7.5)
3) New *Fusarium* species fermentation product proteins: Bioinformatics to evaluate potential allergenicity.

The goal of the study process was to determine whether there is information that would suggest a potential risk of food allergy to consumers, including potential cross-reactions for those with existing allergies. At this time (2019) there are no methods to accurately predict the probability of *de novo* sensitization to a new food. There are however, good strategies and procedures (e.g. Codex, 2009) for identifying proteins that have evidence of causing allergies due to evidence of allergic reactions to the source, to specific proteins and comparison of the amino acid sequences of proteins known to cause allergies, and thus requiring additional testing with sera from those with specific allergies to understand risks. The US Food and Drug Administration and the US Department of Agriculture have been involved in managing foods for risks of allergy for decades. Many allergenic foods (peanut, milk, eggs, tree nuts and others) are used in foods produced and consumed today and as long as they are properly labeled as to the source.

Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

The literature review (Study # REG 2019 Sustainable 1) included searching PubMed and Google for publications about *Fusarium* species, based on identification of this new species (from Study # REG 20019 Sustainable 3) as part of the *Fusarium fujikuroi* species complex that includes *Fusarium proliferatom* and *Fusarium mangiferae*. There were 188 published references that included keywords "allergy" or "allergen" and the abstracts were reviewed for evidence of any study related to allergy. In addition information about the approved, consumed species, *Fusarium venenatum* that is that is the major ingredient in Quorn, was identified and reviewed. There are a few reports of allergy to consumption of Quorn that describe case-reports of allergy (Hoff et al., 2003a), Katona and Kaminski 2002 and Tee et al., 1993). There is also a publication by Center for Science in the Public Interest (CSPI) described their public database of consumers who self-reported adverse reactions upon consumption of Quorn (Jacobson and DePorter, 2018). Quorn is a vegetarian high protein meat substitute made from continuous culture of *Fusarium venenatum* and is therefore relevant to this assessment. The Jacobson and DePorter paper presents a few self-reported cases that seem to be legitimate IgE mediated food allergy reports based on time or reaction and symptoms, though scientific data of specific IgE binding or skin prick tests were not reported. It reports one apparent case of fatal anaphylaxis of an 11 year old boy who had severe asthma cause by molds. The clinical data was not reported. It also reports another news report of a fatal reaction. Again, without clinical data. The Jacobson and DePorter report also describes many self-reports of vomiting that occur hours after ingestion and are thus not common for IgE mediated allergy. The cause of these late-reactions is unknown and there were no clinical or laboratory tests to confirm the reports. However, since a polysaccharide, chitin, is a major component of *Fusarium* hyphal mass we also considered information about the impact of chitin in disease. There is evidence that some people have an increased risk of inflammation and asthma due to the presence of chitin from molds or house dust mites by inhalation. Although unproven it seems that these subjects may have genetic defects in chitinase and the presence of chitin correlates with inflammation that leads to increased incidence and severity of asthma (Zaccone, et al., 2007; Mack et a., 2015). Although the tissue involvement is different in the intestine compared to the lung, similar inflammatory cells (macrophages, eosinophils, basophils, T-lymphocytes and neutrophils) are present in the intestinal lamina propria and may be involved in pathology. As reported by Lee (2009), different animal models have demonstrated diverse responses to chitin, partly through induction of chitosan and chitosan like proteins that influence different responses in different cells and presumably under the control of different genetic factors. In reviewing all published reports of reactions to Quorn, there are very few cases of bonafide IgE mediated reactions to consumption of Quorn.

The WHO/IUIS Allergen Nomenclature database (www.allergen.org) and our allergen risk assessment database (www.AllergenOnline.org version 18B) were searched and a total of five *Fusarium* proteins were identified that are known to cause airway allergy in some subjects (Fus c 1, Fus c 2, a helix-loop-helix protein the authors suggest as Fus c 3 and Fus p 4 and Fus p 9). The finding of the literature report is that there might be an expectation of rare instances of allergy due to consumption of food prepared from sufficient doses of these proteins of the new *Fusarium*

Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

sp. However, as long as the food is labeled to be mycoprotein or *Fusarium* protein, the consumers who might have allergies to *Fusarium* species should be warned and able to avoid consumption. Importantly, there are no reports of life-threatening reactions to these proteins. (Chou et al., 2014, Hoff et al., 2003a, Hoff et al., 2003b, Verma et al., 1998, Weber and Levetin, 2014, Yeh et al., 2016).

The results of digestion assays reported in the second report (Study report REG 2019 Sustainable 2) with tests of SGF and SIF show that most proteins in the extract of the new Fusarium sp. are digested to markedly or to smaller protein molecular weights by pepsin, and more efficiently by pancreatin. One protein of ~ 30 kDa was visible through 30 minutes of digestion in pepsin, but apparently gone at 60 minutes. Pancreatin digested all proteins to below 15 kDa. Complete digestion is not required to demonstrate a lack of possible food allergy. It is clear from published studies that some food allergens are rapidly digested and that some non-allergenic proteins of modest abundance are not digested in pepsin or in pancreatin (Fu et al., 2002; Thomas et al., 2004; Ofori-Anti et a., 2008). The results suggest the proteins are generally digestible. Other semi-stable bands were visible above 10 kDa, but as smeared stainable bands, and mostly gone by 60 minutes. The results of the digestion assays are that proteins from this new Fusarium sp. are likely to behave like the protein mixtures of a number of low-allergy foods.

The bioinformatics study (Study No. REG 2019 Sustainable 3) provides complex data as the genome of most eukaryotes include proteins that are highly conserved in evolution. The genetic relationships of various *Fusarium* species were reviewed (King et al., 2015, Niehaus et al., 2016, Yoder and Christianson, 1998). A number of allergens in AllergenOnline.org were identified as matching predicted proteins from this new *Fusarium* species using FASTA3 alignments with identities above the very conservative Codex (2009) criteria of >35% identity over 80 amino acids. In order to understand the potential risks associated with the sequence matched proteins, additional searches were performed including evaluation of the published predicted proteins from *Fusarium* venenatum used to make Quorn. The results showed that nearly all of the proteins in this *Fusarium* sp. were close homologues of the Quorn species proteins. More detailed studies of the 44 different proteins showed that they are homologues of fungi that are evolutionarily close to *Fusaerium* sp. The conclusion of that study is that food produced from this new species is unlikely to represent any additional risk of food allergy beyond that presented by Quorn.

There were no high identity matches to other common and potent allergenic proteins that would be unique to this species. There were no matches to 2S albumins of peanut or tree nuts, no matches to lipid transfer proteins from peach or related fruits. There were no significant matches to vicilins or tropomyosins that are major sources of severe reactions from peanut, tree nut, shrimp, cockroach or house dust mites. The sequence identity matches that were observed to allergenic proteins in AllergenOnline.org, were to moderate allergenic proteins and the identity matches of the matched allergens have higher identity matches of 8% or more to proteins in other sources of foods that do not show evidence of clinical reactivity. Thus there does not appear to be a risk of allergy from those matched proteins.

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Risk management for food allergy in the United States and other countries is to label foods with ingredients that can be recognized by consumers to inform them of the protein sources to enable the consumer to avoid exposure to their allergic source. It also helps food manufacturers understand risks and design appropriate ingredient segregation and control strategies to minimize the chance of accidental carry-over of major allergenic source materials into foods that are not intended to have those ingredients.

Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

**References**

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Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

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Footnote: [1]This taxa, ATCC Accession Deposit No. PTA-10698 will be defined as *Fusarium* mycoprotein throughout this report.

## Viebrock, Lauren

**From:** Baughan, Joan <jbaughan@Steptoe.com>
**Sent:** Wednesday, May 13, 2020 5:24 PM
**To:** Viebrock, Lauren
**Cc:** Attwood, Deborah
**Subject:** RE: Acknowledgement of filing of GRAS Notice No. GRN 000904
**Attachments:** 2020_04_22 Filing Letter GRN 904 Transmittal.pdf

Dear Lauren,

Thank you very much for providing us with the acknowledgment letter for GRAS Notice (GRN) No. 904. Attached to this message please find a letter that: (1) notifies FDA regarding a name change for the company responsible for GRN No. 904; and (2) requests that FDA revise the description of the substance that is the subject of GRN No. 904.

Please let us know if you have any questions or concerns regarding this letter.

With best regards,

Joan

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**From:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Sent:** Wednesday, April 22, 2020 10:13 PM
**To:** Baughan, Joan <jbaughan@Steptoe.com>
**Subject:** Acknowledgement of filing of GRAS Notice No. GRN 000904

Dear Ms. Baughan,

Attached to this email, please find the acknowledgement letter for filing of the GRAS notice you submitted to our office, which has been designated as GRAS Notice No. GRN 000904.

Please let me know if you have any questions.

Regards,
Lauren VieBrock

**Lauren VieBrock**
*Consumer Safety Officer/Microbiology Reviewer*

**Center for Food Safety and Applied Nutrition**
**Office of Food Additive Safety**
**U.S. Food and Drug Administration**
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov



April 22, 2020

Joan Sylvain Baughan
Steptoe & Johnson, LLP
1300 Connecticut Ave, NW
Washington, DC 20036

Re: GRAS Notice No. GRN 000904

Dear Ms. Baughan:

The Food and Drug Administration (FDA, we) received the GRAS notice dated January 14, 2020 that you submitted on behalf of Sustainable Bioproducts, Inc. (SBP). We received this notice on January 15, 2020, filed it as of the date of this letter, and designated it as GRN 000904.

The subject of the notice is fungal protein from cultured *Fusarium* sp.[1] for use as an ingredient in meat and poultry analogs; dairy product analogs; meal replacement products; fruit juices and vegetable juices; grain products and pastas; baked goods and baking mixes; soups and soup mixes; and fats and oils[2] at levels up to 23.3% dry weight. The notice informs us of SBP's view that these uses of fungal protein from cultured *Fusarium* sp. are GRAS through scientific procedures.

In accordance with 21 CFR 170.275(b)(1), the information in this notice described in 21 CFR 170.225(c)(2) through (c)(5) will be accessible to the public at www.fda.gov/grasnoticeinventory. If SBP has any questions about the notice, contact me at lauren.viebrock@fda.hhs.gov or 301-796-7454.

Sincerely,

Lauren VieBrock, Ph.D.
Division of Food Ingredients
Center for Food Safety and Applied Nutrition

[1] SBP states the fungal species is *F. novum. yellowstonensis.* SBP notes that it was deposited at ATCC as *"Fusarium oxysporum:* MK7" under accession number PTA – 10698 on March 2, 2010. Since 2010, further taxonomic characterization and genetic analysis has indicated the organism is a distinct species named *F. novum. yellowstonensis*.

[2] SBP states that fungal protein from cultured *Fusarium* sp. is not intended for use in infant formula or in products subject to regulation by the U.S. Department of Agriculture.

**U.S. Food and Drug Administration**
**Center for Food Safety & Applied Nutrition**
**5001 Campus Drive**
**College Park, MD 20740**
**www.fda.gov**

## Viebrock, Lauren

**From:** Baughan, Joan <jbaughan@Steptoe.com>
**Sent:** Thursday, May 14, 2020 7:51 AM
**To:** Viebrock, Lauren
**Cc:** Attwood, Deborah
**Subject:** RE: Acknowledgement of filing of GRAS Notice No. GRN 000904
**Attachments:** GRN904 Name Change-c2.pdf

Dear Lauren,

I now see that I included the wrong attachment in my message yesterday regarding the name change for GRAS Notice 904. I am attaching the correct letter in that regard to this message. Please forgive the inconvenience. If there are any questions, please let me know.

Thank you for your continued assistance.

Kind regards,

Joan

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**From:** Baughan, Joan
**Sent:** Wednesday, May 13, 2020 5:24 PM
**To:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Cc:** Attwood, Deborah <dattwood@steptoe.com>
**Subject:** RE: Acknowledgement of filing of GRAS Notice No. GRN 000904

Dear Lauren,

Thank you very much for providing us with the acknowledgment letter for GRAS Notice (GRN) No. 904. Attached to this message please find a letter that: (1) notifies FDA regarding a name change for the company responsible for GRN No. 904; and (2) requests that FDA revise the description of the substance that is the subject of GRN No. 904.

Please let us know if you have any questions or concerns regarding this letter.

With best regards,

Joan

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**From:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Sent:** Wednesday, April 22, 2020 10:13 PM
**To:** Baughan, Joan <jbaughan@Steptoe.com>
**Subject:** Acknowledgement of filing of GRAS Notice No. GRN 000904

Dear Ms. Baughan,

Attached to this email, please find the acknowledgement letter for filing of the GRAS notice you submitted to our office, which has been designated as GRAS Notice No. GRN 000904.

Please let me know if you have any questions.

Regards,
Lauren VieBrock

**Lauren VieBrock**
*Consumer Safety Officer/Microbiology Reviewer*

**Center for Food Safety and Applied Nutrition**
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Steptoe

May 13, 2020

Via EMAIL

Ms. Lauren VieBrock
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

**Re: Update to Administrative Information for GRN 904**

Dear Ms. VieBrock,

This letter follows up on your April 22, 2020, letter confirming receipt of the notification we submitted on behalf of Sustainable Bioproducts, Inc., advising the Food and Drug Administration (FDA) of Sustainable Bioproducts, Inc.'s conclusion that its novel food ingredient, fermented microbial protein, is Generally Recognized as Safe (GRAS) for use in a variety of foods. The purpose of this letter is twofold: (1) to inform you that Sustainable Bioproducts, Inc., has legally changed its name and to request that the Notifier for GRAS Notice (GRN) be identified as Nature's Fynd; and (2) to request that the product identified in the GRAS Notice (GRN) be identified in the FDA's online GRAS Notice inventory as "mycelial protein from fermented Fusarium sp." Additional information regarding each of these items follows.

With respect to point 1, as of May 1, 2020, Sustainable Bioproducts, Inc. changed its legal name to "The Fynder Group, Inc." and, in addition, is doing business as Nature's Fynd. The company address has changed to 815 W Pershing Rd, Chicago, IL 60609. Accordingly, we request that you update FDA's internal records to reflect the legal name of the company, and use the "Nature's Fynd" business name as the identified Notifier for GRN 904 on FDA's GRN inventory.[1]

[1] We note that, as an example, GRN 886 identifies the company in the Form 3667 as "Apeel Technology, Inc. ('Doing Business As' Apeel Sciences)" while "Apeel Science" is identified on FDA's website.

Steptoe

As for item 2, in your April 22 letter, you identify the subject of GRN 904 as "fungal protein from cultured Fusarium sp." We anticipate that FDA intends to use this identification for the GRN in its online inventory. As you know, the product was identified in the notification submitted to FDA as "fermented microbial protein." Your letter does not explain why FDA has determined that the product should be identified as "fungal protein from cultured Fusarium sp.," but we request that the product instead be identified as "mycelial protein from fermented Fusarium sp." This revised identification is scientifically appropriate and accurately describes the product. If you have any concerns with this request, we would ask that you contact us to arrange a teleconference to discuss further.

If you agree with our proposed identification, we will provide you with a copy of the GRN with the new company name and product identification for use on the online GRN inventory. We look forward to hearing from you in this regard.

We appreciate your assistance with this matter. Please do not hesitate to contact us with any questions or concerns.

Sincerely yours,

Joan Sylvain Baughan

## Viebrock, Lauren

**From:** Viebrock, Lauren
**Sent:** Friday, May 29, 2020 10:53 AM
**To:** Baughan, Joan
**Cc:** Attwood, Deborah
**Subject:** RE: Acknowledgement of filing of GRAS Notice No. GRN 000904

Dear Ms. Baughan,

This is to confirm receipt of the email and letter regarding GRN 904. We will be in touch as we proceed with our review of the submission. Thank you.

Best,
Lauren

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**From:** Baughan, Joan <jbaughan@Steptoe.com>
**Sent:** Wednesday, May 13, 2020 5:24 PM
**To:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Cc:** Attwood, Deborah <dattwood@steptoe.com>
**Subject:** RE: Acknowledgement of filing of GRAS Notice No. GRN 000904

Dear Lauren,

Thank you very much for providing us with the acknowledgment letter for GRAS Notice (GRN) No. 904. Attached to this message please find a letter that: (1) notifies FDA regarding a name change for the company responsible for GRN No. 904; and (2) requests that FDA revise the description of the substance that is the subject of GRN No. 904.

Please let us know if you have any questions or concerns regarding this letter.

With best regards,

Joan

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**From:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Sent:** Wednesday, April 22, 2020 10:13 PM
**To:** Baughan, Joan <jbaughan@Steptoe.com>
**Subject:** Acknowledgement of filing of GRAS Notice No. GRN 000904

Dear Ms. Baughan,

Attached to this email, please find the acknowledgement letter for filing of the GRAS notice you submitted to our office, which has been designated as GRAS Notice No. GRN 000904.

Please let me know if you have any questions.

Regards,
Lauren VieBrock

**Lauren VieBrock**
*Consumer Safety Officer/Microbiology Reviewer*

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## Viebrock, Lauren

**From:** Baughan, Joan <jbaughan@Steptoe.com>
**Sent:** Wednesday, October 07, 2020 10:16 AM
**To:** Viebrock, Lauren
**Cc:** Attwood, Deborah
**Subject:** RE: GRN 904 Questions
**Attachments:** GRN904_ResponseToFDA_CoverLetter_7Oct2020.pdf; GRN904_NaturesFyndResponseToFDA_7Oct2020.pdf; GRN904_NaturesFyndResponseToFDA_AppendixA_ATCCDepositCertificate_PTA10698.pdf; GRN904_NaturesFyndResponseToFDA_AppendixB_BatchAnalysis.pdf; GRN 904 Questions for notifier.pdf

Dear Lauren,

Thank you for providing us with the Agency's detailed comments on GRAS Notice 904. Our response is attached. Should you have any further questions or comments, please let us know, preferably by telephone or email so that we may reply as quickly as possible.

Kind regards,

Joan

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**From:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Sent:** Tuesday, September 22, 2020 4:14 PM
**To:** Baughan, Joan <jbaughan@Steptoe.com>
**Cc:** Attwood, Deborah <dattwood@steptoe.com>
**Subject:** GRN 904 Questions

Hi Joan and Deborah,

Thank you for the discussion regarding our questions on GRN 904. Please find the discussed questions attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

**Lauren VieBrock**
*Regulatory Review Scientist/Microbiology Reviewer*

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October 7, 2020

Via EMAIL

Ms. Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
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College Park, Maryland 20740

**Re: Response to FDA's Questions Regarding GRAS Notice 904**

Dear Ms. VieBrock,

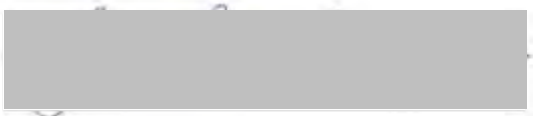
This letter and its enclosures, submitted on behalf of Nature's Fynd, respond to your September 22, 2020 letter regarding Nature's Fynd's notification advising the Food and Drug Administration (FDA) as to its conclusion that Fy Protein™ (Nutritional Fungi Protein) ("Fy Protein") is Generally Recognized as Safe (GRAS) for use as a food ingredient and macronutrient. FDA has designated this notification as GRAS Notice (GRN) 904.

Included with this letter are Nature's Fynd's responses to the questions in your September 22, 2020 letter, with two appendices. As you will see, Nature's Fynd has provided comprehensive responses to FDA's regulatory, chemistry, toxicology, and microbiology questions, supported by the abundant, publicly available scientific literature on Fusarium species of microorganism and other relevant materials. In particular, Nature's Fynd has expanded upon and clarified the relation between *Fusarium strain flavolapis* and *Fusarium venenatum* to further support its conclusion that the publicly available toxicity data on *F. venenatum* can serve as toxicological data for *F. strain flavolapis*, corroborated by the additional toxicity testing conducted with Fy Protein. Additionally, Nature's Fynd presents a compelling discussion supporting its conclusion that the toxicological potential of *F. strain flavolapis* is determined by the potential presence of and exposure to toxic secondary metabolites, namely mycotoxins. The available standard toxicological tests corroborate the conclusion that macroingredients produced by *F. strain flavolapis* and *F. venenatum* present no inherent toxicity and are not pathogenic. Therefore, safety is determined by the mycotoxin content and potential allergenicity of the product. Both of these safety concerns have been fully addressed in GRN 904 and the enclosed response.

Steptoe

We look forward to receiving a letter from the Agency acknowledging receipt of this response. Please contact us by telephone or e-mail if there are any questions regarding this reply so that we can respond right away.

With kind regards,



Joan Sylvain Baughan

Enclosures

# FDA Question Responses

GRN 904

Nature's Fynd
(The Fynder Group, Inc.)

October 7, 2020

# Executive Summary

Nature's Fynd hereby responds to FDA's request for clarifying information concerning regulatory, chemistry, toxicology and microbiology questions in connection with GRAS Notice (GRN) 904. This clarifying information further supports the GRAS determination outlined in our original GRN submission, dated January 14, 2020. That is, fermented microbial protein (FMP) -- now referred to as Fy Protein (Nutritional Fungi Protein) -- is generally recognized as safe (GRAS) for use as a food ingredient and source of macronutrients in meat, dairy, and flour alternatives.

In establishing the safety profile of Fy Protein we have assessed safety, biological, and toxicological information on the source organism, *Fusarium strain (str.) flavolapis* (formerly known as *Fusarium novum. yellowstonensis*). Among other sources, we rely on publicly available data from GRN 91, which assessed fungi-based protein produced by a related member of the Fusarium genus, *F. venenatum* strain PTA-2684. We provide clarifying information as further support to this approach. *F. str. flavolapis* and *F. venenatum* are members of related clades based on generally recognized morphometric and molecular phylogenetic analysis. We have established that these two related species of Fusarium genus present similarly low toxicological risks, and similar toxicological profiles in general, using widely-accepted "read across" approaches consistent with FDA GRAS precedents in the field of novel proteins. Considering together our original GRAS Notice and this additional clarifying information, we reiterate our conclusion that Fy Protein is GRAS for its intended use.

# Regulatory: Question 1 (R1)

*"Please provide information connecting the identity of Fusarium novum. yellowstonensis and the F. venenatum strain PTA-2684 that was used to produce the subject of GRN 91."*

## R1 Response:

*Fusarium strain (str.) flavolapis* (formerly known as *Fusarium novum. yellowstonensis*, further discussed in Microbiology responses M2 and M4 below) and *F. venenatum* are both members of the *Fusarium* genus and members of closely related clades based on generally recognized methods of molecular phylogenetic analysis. For fungal organisms, phylogenetic trees describing relatedness are commonly produced based on a maximum likelihood analysis of complete 18S ribosomal RNA (rRNA) gene and internal transcribed spacer (ITS) DNA sequences (Schoch et al., 2012; Yarza et al., 2018).

The phylogenetic tree in Figure 1 below has been generated from publicly available genetic data on *F. str. flavolapis*, *F. venenatum*, 44 additional *Fusarium* strains, and one outgroup, *Neurospora crassa*, according to the methods described in Watanabe et al. (2011). The sequences used to produce this phylogeny are all publicly available in the National Institute of Biotechnology Information (NCBI) GenBank database[1] under the accessions listed in the tree,

[1] https://www.ncbi.nlm.nih.gov/genbank/

and the genome for *F. str. flavolapis* is also provided in NCBI under BioProject accession PRJNA665233[2].

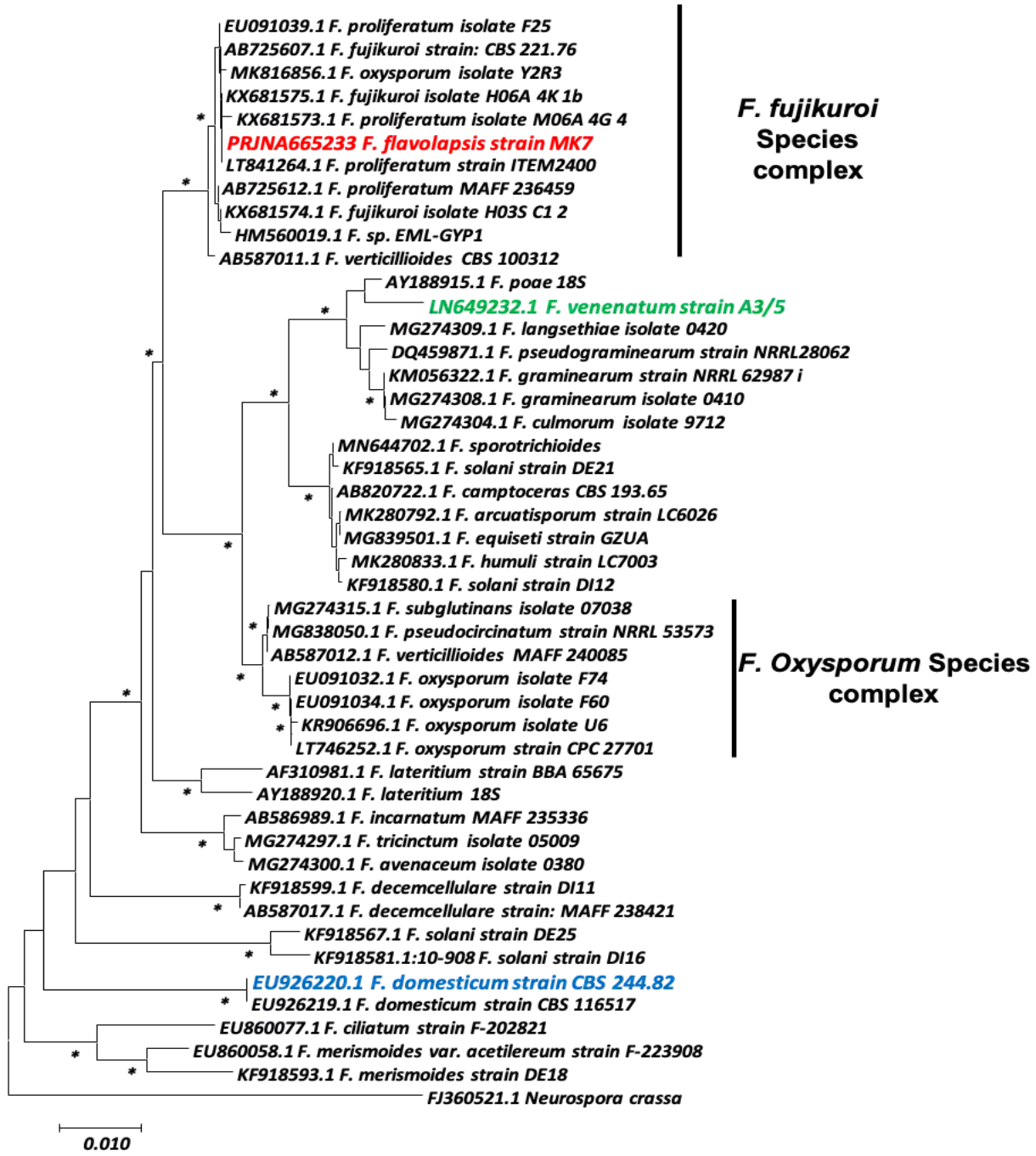
Our phylogenetic analysis places *F. venenatum* PTA-2684 within the *sambucinum* clade with high confidence, and *F. str. flavolapis* within the *fujikuroi* clade with high confidence. *F. venenatum*'s placement is consistent with previously published research (O'Donnell et al., 1998; O'Donnell et al., 2013). F*ujikuroi* and *sambucinum* are sister clades, meaning that the clades share a recent common ancestor (Figure 1, Watanabe et. al, 2011; O'Donnell et al., 2013).

The phylogenetic placement of these two strains is further corroborated by analysis of *beta tubulin* and *elongation factor-1* genes as described in Dubey et al. (2014) and has also been independently verified by igenbio, Inc. (Chicago, IL) using an analysis of 2,965 genes from 23 Fusarium strains, following the protocol outlined in Niehaus et al. (2017). The genomes of the Fusarium strains used for this analysis are similarly all available through NCBI GenBank.

These results can be readily corroborated by the scientific community using publicly available data and generally recognized phylogenetic methods.

[2] https://www.ncbi.nlm.nih.gov/bioproject/PRJNA665233/

**Figure 1**: 18S/ITS DNA sequence phylogenetic tree showing relationship between *Fusarium flavolapis* and other *Fusarium* spp. Including the Quorn strain *F. venenatum* and *F. domesticum*, used in cheese production. Genbank accession numbers are provided before the species name. Branches with high statistical support (>90% bootstrap values (Efron et al., 1996)) are marked with an asterisk(*).



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Dubey, S. C., Priyanka, K., & Singh, V. (2014). Phylogenetic relationship between different race representative populations of Fusarium oxysporum f. Sp. Ciceris in respect of translation elongation factor-1 $\alpha$, $\beta$ -tubulin, and internal transcribed spacer region genes. *Archives of Microbiology*, *196*(6), 445–452. https://doi.org/10.1007/s00203-014-0976-0

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Niehaus, E.-M., Kim, H.-K., Münsterkötter, M., Janevska, S., Arndt, B., Kalinina, S. A., Houterman, P. M., Ahn, I.-P., Alberti, I., Tonti, S., Kim, D.-W., Sieber, C. M. K., Humpf, H.-U., Yun, S.-H., Güldener, U., & Tudzynski, B. (2017). Comparative genomics of geographically distant Fusarium fujikuroi isolates revealed two distinct pathotypes correlating with secondary metabolite profiles. *PLOS Pathogens*, *13*(10), e1006670. https://doi.org/10.1371/journal.ppat.1006670

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O'Donnell, K., Rooney, A. P., Proctor, R. H., Brown, D. W., McCormick, S. P., Ward, T. J., Frandsen, R. J. N., Lysøe, E., Rehner, S. A., Aoki, T., Robert, V. A. R. G., Crous, P. W., Groenewald, J. Z., Kang, S., & Geiser, D. M. (2013). Phylogenetic analyses of RPB1 and RPB2 support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genetics and Biology*, *52*, 20–31. https://doi.org/10.1016/j.fgb.2012.12.004

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova, E., Voigt, K., Crous, P. W., Miller, A. N., Wingfield, M. J., Aime, M. C., An, K.-D., Bai, F.-Y., Barreto, R. W., Begerow, D., … Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (Its) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241–6246. https://doi.org/10.1073/pnas.1117018109

Watanabe, M., Yonezawa, T., Lee, K., Kumagai, S., Sugita-Konishi, Y., Goto, K., & Hara-Kudo, Y. (2011). Molecular phylogeny of the higher and lower taxonomy of the Fusarium genus and differences in the evolutionary histories of multiple genes. *BMC Evolutionary Biology*, *11*(1), 322. https://doi.org/10.1186/1471-2148-11-322

Yarza, P., Yilmaz, P., Panzer, K., Glöckner, F. O., & Reich, M. (2017). A phylogenetic framework for the kingdom Fungi based on 18S rRNA gene sequences. *Marine Genomics*, *36*, 33–39. https://doi.org/10.1016/j.margen.2017.05.009

# Regulatory: Question 2 (R2)

*"Please clarify what information the GRAS panel was provided."*

## R2 Response:

In order to reach the generally recognized as safe consensus for Fy Protein, the GRAS panelists were provided with the following:

- Proposed GRAS Notification
- University of Nebraska Food Allergy Research and Resource Program (FARRP) Allergenicity assessment reports (attachments 2-5 of GRN 904)
- Copies of GRAS Notices 91, 608, and 609
- Calculations and referenced literature for the mycotoxin safety assessment

# Regulatory: Question 3 (R3)

*"Please confirm that Nature's Fynd 'concluded that food produced from F. novum yellowstonensis is unlikely to represent any additional risk of food allergy beyond what is currently available in the human food supply.' On page 36 of the notice, the conclusion is attributed to FARRP."*

## R3 Response:

Nature's Fynd confirms that it has concluded that food containing Fy Protein produced from *Fusarium str. flavolapis* is unlikely to represent any additional risk of food allergy beyond what is currently available in the human food supply.

# Chemistry: Question 1 (C1)

*"We noted some discrepancies between Tables 11 and 12 of your notice that list the foods and use levels intended for Fusarium species mycelium and those used for estimation of dietary exposure. The exposure table includes food categories and their estimated intakes that were presented in GRN 000609. Please clarify any differences in the food categories intended for the use of Fusarium species mycelium and that any additional uses are accounted for in your estimate of dietary exposure.*

*For example, food categories listed in Table 11 of the notice but not in Table 12 include: chicken nuggets, lunch meats, frozen or shelf stable prepared meals, cakes, brownies, cookies, doughnuts, pancakes, waffles, tortillas, crackers, non-dairy creamer, non-dairy cheese, and mayonnaise. Conversely, ready-to-eat breakfast cereals are included in Table 12 but not in Table 11."*

## C1 Response:

As noted in our original GRAS Notice, Table 11 outlines anticipated use levels of Fy Protein in a variety of foods in which we would expect Fy Protein may be used. The first column (Column 1) of Table 11 identifies the general food categories within which food products containing Fy Protein may fall and the second column (Column 2) of Table 11 provides specific examples of foods within those categories in which Fy Protein may be used. The third and fourth columns (Columns 3 and 4, respectively) of Table 11 note the corresponding range of anticipated use level of Fy Protein in the enumerated food, e.g., 2.5-23.3 dry wt %.

Table 12 then outlines the Estimated Daily Intake (EDI) of Fy Protein utilizing examples -- pulled from Table 11 -- of widely consumed foods in which Fy Protein may be used. For example, soy patties are an example of a widely consumed meat analogue, and that specific example was chosen for use in the exposure assessment in Table 12. For purposes of performing an EDI calculation for "meat patty with soy protein" in Table 12, we used the Fy

Protein maximum use level of 23.3 dry wt% noted in Table 11 for “plant protein products, including meat/poultry analogs” was used. We followed this approach in calculating EDIs for the additional foods enumerated in Table 12. All of the foods’ EDI calculations in Table 12 fall within the broad categories identified in Table 11. Thus, we can confirm that all currently anticipated uses of Fy Protein are accounted for in the estimate of dietary exposure.

# Chemistry: Question 2 (C2)

*“The method of manufacture described in your notice is described as a solid-state fermentation, in which the mycelia are grown until the medium is consumed and form a biomat, and a heat treatment step to stop growth.*

*The identity of the components used in the fermentation medium are vaguely described. For example, the ingredients include 'trace metals' with a citation to Hutner et al. 1950. Based on the description of the method of manufacture, the media components may be completely consumed by the mycelium. Please elaborate on the identity of metals used in the manufacturing process and their potential presence in the final product.*

*In the case of previously notified mycoprotein ingredients, the RNA content of the fungal biomass produced by fermentation is reduced by heat treatment steps in which RNA is degraded to mononucleotides that then diffuse out of the cells and are separated from the biomass by centrifugation (Wiebe, M.G., Appl. Microbiol. Biotechnol. (2002) 58:421–427 DOI: 10.1007/s00253-002-0931-x). Although the Fusarium mycelium that is subject of your notice is subject to a specified limit of 2% RNA and batch records demonstrate lower levels, please discuss if there are steps in the production of the that degrade RNA or remove its degradation products.”*

## C2 Response:

### Method of Manufacture

To clarify, as indicated on page 10 of GRN 904, the method of manufacture is a “surface fermentation” that occurs on the surface of a liquid medium. This is not a “solid-state” fermentation, which occurs on the surface of a solid-state media (Cerda et al., 2019).

### Trace Metals

FDA’s Compliance Program Guidance Manual 7304.019 Toxic Elements in Food and Foodware, and Radionuclides in Food -- Import and Domestic -- identifies lead, cadmium, arsenic, and mercury (as methylmercury) as metals of concern (FDA, 2008). FDA’s Toxic Element Working Group also identifies aluminum, antimony, barium, beryllium, silver, strontium, nickel, thallium, uranium, and vanadium as metals that are harmful to health (FDA, 2020).

Nature’s Fynd confirms that no compounds containing these metals are intentionally used in the “trace metals” component of the fermentation medium or in the manufacturing process of Fy Protein. Nature’s Fynd also confirms that cobalt salts are not used in the trace metals mixture; the absence of cobalt salts is a modification to the trace elements described in Hutner et al. (1950).

Fy Protein is subject to heavy metal (arsenic, lead, cadmium, mercury) specifications of <0.1 ppm. Heavy metals are tested for in every batch of Fy Protein, and results have been consistently <0.1 ppm. Similar heavy metals specifications of <0.1 ppm have been set for other protein replacement products (see, e.g., GRN 91 (mycoprotein), GRN 608 (pea protein), GRN 609 (Rice protein)).

### RNA Content

Natively, Fy Protein has <2% RNA, as included in the GRAS Notice specifications. The levels of RNA have been measured at harvest, after post-processing, and freezing to confirm that levels are not above 2%. As presented in GRN 904 -Table 3: Specifications and Batch Analysis Summary (dry weight), the RNA content is 0.01% average for the three example non-consecutive batches. These measurements were made with a sensitive fluorescence-based assay widely used in academic research and Industry (Invitrogen, 2016). As such, we do not implement specific steps in our production process to actively degrade RNA or remove its degradation products.

References:


Cerda, A., Artola, A., Barrena, R., Font, X., Gea, T., & Sánchez, A. (2019). Innovative production of bioproducts from organic waste through solid-state fermentation. *Frontiers in Sustainable Food Systems*, 3, 63. https://doi.org/10.3389/fsufs.2019.00063

invitrogen. (2016). *Technical Note: Comparison of fluorescence-based quantitation with UV absorbance measurements*. ThermoFisher Scientific.

*Media for Chlamydomonas: Hutner's trace elements*. (n.d.). Retrieved October 5, 2020, from https://www.chlamycollection.org/trace.html

United States Food and Drug Administration (FDA): Center for Food Safety and Applied Nutrition. (2020). *Metals and your food.* https://www.fda.gov/food/chemicals-metals-pesticides-food/metals-and-your-food

United States Food and Drug Administration (FDA) (2008). *Food and Drug Administration Compliance Program Guidance Manual 7304.019, Chapter 04: Pesticide and Chemical Contaminants*. https://www.fda.gov/media/142504/download


## Chemistry: Question 3 (C3)

*"In your notice, the sources of some of the methods of analysis for specification parameters are not disclosed (for example, methods for determination of fumonisins and RNA). Please provide clarifying information that all methods of analysis are validated for the respective analytes and conditions."*

### C3 Response:

Nature's Fynd confirms that all methods of analysis utilized to confirm compliance with the established specifications are validated for the respective analytes and conditions. GRN 904

lists the methods of analysis for the majority of the specifications, and included below are the instruments used and the validated methods for all of the specifications.

**Table 1:** Full list of specifications, instruments, and references for methods of analysis.

| Specification | Instrument | Reference |
|---|---|---|
| Water content | Vacuum oven | AOAC 925.09 |
| Protein | Combustion | AOAC 990.03; AOAC 992.15 |
| Total sugars | HPLC | AOAC 982.14, (Mod) |
| Total dietary fiber | HPLC | AOAC 2009.01; AOAC 2011.25 |
| Total fat | GC-MS | AOAC 996.06 mod |
| Ash | Oven | AOAC 942.05 |
| RNA | Qubit RNA Assay | Invitrogen Technical Note |
| Carbohydrates | Calculation | CFR 21 101.9 |
| Glycerol | GC-MS | Shen et al 2013 |
| Total aerobic bacteria | Plate culture | U.S.P. Ch. 61 |
| Total yeast | Plate culture | U.S.P. Ch. 61 |
| Total mold | Plate culture | U.S.P. Ch. 61 |
| E. coli | Tube culture | U.S.P. Ch. 62 |
| Salmonella | PCR | AOAC 2003.09 |
| Fumonisin | US-Multitoxin LC-MS/MS | Sulyok et. al 2006 |
| Mycotoxin Screen | US-Multitoxin LC-MS/MS | Sulyok et. al 2006 |
| Mycotoxin Quantitative | LC-MS/MS | Han et al. 2014 |
| Mycotoxin Qualitative | LC-MS/MS | Sulyok et. al 2007 |
| Nitrate | Ion Chromatography | EN 12014-2 |
| Heavy Metals | ICP-MS | J. AOAC vol. 90 (2007) (Mod) |

## References


Han, Z., Tangni, E. K., Huybrechts, B., Munaut, F., Scauflaire, J., Wu, A., & Callebaut, A. (2014). Screening survey of co-production of fusaric acid, fusarin C, and fumonisins B1, B2 and B3 by Fusarium strains grown in maize grains. *Mycotoxin Research*, *30*(4), 231–240. https://doi.org/10.1007/s12550-014-0207-1

invitrogen. (2016). *Technical Note: Comparison of fluorescence-based quantitation with UV absorbance measurements*. ThermoFisher Scientific.

Shen, Y., & Xu, Z. (2013). An improved GC–MS method in determining glycerol in different types of biological samples. *Journal of Chromatography B*, 930, 36–40. https://doi.org/10.1016/j.jchromb.2013.04.034

Sulyok, M., Berthiller, F., Krska, R., & Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communications in Mass Spectrometry*, 20(18), 2649–2659. https://doi.org/10.1002/rcm.2640

Sulyok, M., Krska, R., & Schuhmacher, R. (2007). A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to

semi-quantitative screening of moldy food samples. *Analytical and Bioanalytical Chemistry*, *389*(5), 1505–1523. https://doi.org/10.1007/s00216-007-1542-2

# Toxicology: Question 1 (T1)

*"You state on Pg. 31 that:*

> *'[s]afety studies, including animal toxicology studies commissioned by the company and referenced in the publicly available literature on similar Fusarium-derived products, have indicated that there are no acute or chronic exposure concerns from consumption of FMP.'*

*It does not appear that safety studies with your article of commerce have been published in a peer-reviewed journal and can only be used to corroborate your GRAS conclusion. Thus, pivotal studies used for your GRAS conclusion would rely on published data from another species of Fusarium, Fusarium venenatum. It is not clear on what scientific basis it was determined that various species of Fusarium would have the same toxicological potential or lack thereof. Please provide a rationale, with appropriate references to justify your reasoning, on how you concluded that published data and information used in GRN91 can substitute for toxicological data for your substance."*

## T1 Response

### Response Overview

Based on generally recognized methods for evaluating toxicity in Fusarium, we have determined that *F. str. flavolapis* and *F. venenatum* grown under controlled conditions possess the same toxicological potential or lack thereof. Therefore, we conclude that the published data and information used in GRN 91 can appropriately serve as toxicological data for Fy Protein from *F. str. flavolapis*.

Our determination that *F. str. flavolapis* and *F. venenatum* possess the same toxicological potential or lack thereof is based on an assessment of similarities from a phylogenetic and compositional standpoint, as well as similarities and differences in their potential and actual production of toxic secondary metabolites. This determination is consistent with generally recognized methods for read-across of biological substances and is additionally based on well-established literature and methods for evaluating the production of toxic secondary metabolites in *Fusarium* as detailed below.

Our determination can readily be verified by the scientific community using generally recognized methods of toxicity assessment for Fusarium and mycotoxins, as well as publicly available genetic data on *F. str. flavolapis* and *F. venenatum*. This conclusion is further corroborated by the results of our studies on *F. str. flavolapis* that include *in vitro* genotoxicity studies and a 14-day *in vivo* study.

### Overarching Basis for Comparing Toxicological Profiles

*Read-across Precedent*

Within FDA GRAS precedent, there is a well-accepted practice of leveraging toxicological data from a prior GRAS Notice where -- as here -- there are clear structural, compositional, and phylogenetic similarities between the Notified substance and the subject of the prior GRAS

Notice. In fact, numerous successful GRAS Notices have incorporated this approach to supporting their GRAS conclusions for novel proteins and other complex biological substances, see e.g., GRN 737 and GRN 811. Thus, it is our view that our approach of referencing pivotal studies and data from GRN 91 to support a GRAS conclusion for *F. str. flavolapis* in our present GRAS Notice aligns with FDA precedent.

Further, in recent successful instances of so-called "read-across" in the GRAS context, FDA has accepted reasoning based, in part, on the generally recognized methodology outlined by Pariza and Johnson (2001). Although originally intended for assessing the safety of modified organisms for enzyme production, the decision-tree approach advanced by Pariza and Johnson can effectively apply more broadly to novel protein production, including the safety of secondary metabolites, as evidenced by GRN 737 for soy leghemoglobin protein derived from *Pichia pastoris* (Jin et al., 2018).

While the decision-tree methodology from 2001 has largely focused on modified strains of the same species lineage, advancements in bioinformatics and "omics" that have occurred over the twenty years since Pariza and Johnson's publication now allow scientists to similarly assess distinct species for their common lineages and shared evolutionary relationships to inform toxicology assessments (Pielaat et al., 2013). Toxicology experts and regulatory agencies in the U.S. and other developed nations are increasingly incorporating these modern approaches, particularly metabolomics (the systematic identification and quantification of small molecule metabolic products in their safety assessments of distinct species; Rivetti et al., 2019; Viant et al., 2019; Sauer et al., 2010).

As such, and consistent with methods outlined in the decision-tree approach and modern bioinformatics, our assessment of the safety of *F. str. flavolapis* has focused on understanding its phylogenetic and compositional relationship to *F. venenatum*, as well as a comparison of the toxicological potential of *F. str. flavolapis* and *F. venenatum*. The evaluation described in further detail below is grounded in published data on: a) the genetic pathways and regulation of toxic secondary metabolites in *Fusarium*, b) analytical methods for quantifying mycotoxins, and c) established safe exposure limits to mycotoxins.

*Overarching Similarity of F. str. flavolapis and F. venenatum*

Our multi-faceted comparison of *F. str. flavolapis* and *F. venenatum* clearly indicate strong similarities between the two species pertinent to understanding their toxicological potentials and supportive of "read-across" to pivotal toxicology studies on *F. venenatum*. These similarities are summarized as follows:

- Phylogenetics: *F. str. flavolapis and F. venenatum* are both *Fusarium spp.* and members of closely related clades, as described in our response to Regulatory Q1 (R1).
- Composition: *F. str. flavolapis and F. venenatum* have consistent nutritional and protein profiles, as discussed in Table 10: Nutritional Comparison of the subject Notice, GRN 904.
- Intended Use: The products derived from both *spp.* are both intended for use as a replacement for animal-based proteins in the human diet, as discussed in Section III "Estimate of Dietary Exposure" of the subject GRN, though at different levels and different food categories.
- Toxicological Potential: Similarities *and* differences in the potential and actual production of toxic secondary metabolites in *F. str. flavolapis* and *F. venenatum* are well

understood through generally recognized methods of genetic analysis and analytical testing, as discussed in further detail below.

- Toxic Effect: The assessment of toxicological potential is corroborated by a battery of in vitro and in vivo toxicology studies that have been conducted on both test substances, additionally discussed below.

## Genetic and Analytical Basis of Similar Toxicological Profiles

As one of the most widely studied genera of fungi, there is a wealth of publicly available information on Fusarium metabolomics and toxicity to support the general recognition of similar toxicological profiles between *F. str. flavolapis and F. venenatum* when grown in controlled environmental conditions. This determination stems from understanding: 1) toxic secondary metabolites produced by Fusarium species; 2) the genes responsible for producing those secondary metabolites; and 3) analytical measurements of toxin production in these fungi. The focus on toxic secondary metabolites (mycotoxins) to assess the holistic toxicological potential of *Fusarium spp.* is generally recognized in the literature and discussed in further detail below.

*Fusarium: The Subject of Significant Scientific Inquiry*

*Fusarium* is among the most intensely-studied group of fungi, with over 43,000 primary research papers and reviews published over the last century (Web Of Science 9/28/20; Summerell and Leslie, 2011). Genetically diverse *Fusarium* strains inhabit temperate and tropical environments worldwide, are abundant in soils and plant tissues, and have evolved a wide range of morphologies and life history strategies (Leslie and Summerell, 2006; Summerell and Leslie, 2011). The best studied members of this genus are plant pathogens that cause crop diseases, especially among food cereals such as barley and wheat (Nganje et al., 2004), as well as *F. venenatum, which is* used to produce mycoprotein, an ingredient in human food (Wiebe, 2002).

*Mycotoxins: Secondary Metabolites of Concern in Fusarium*

As *Fusarium* can cause hundreds of millions of dollars in damage to crops each year (McMullen et al., 2012; Moretti et al., 2017) and can result in exposure of humans and livestock to toxic fungal secondary metabolites (mycotoxins) with harmful health consequences (Placinta et al., 1999; WHO 2002; Bennett and Klich, 2003; Pestka, 2010; Zain 2011; Kovalsky et al., 2016; Eshelli et al., 2018; WHO 2018a), these food safety issues have made the comprehensive identification and characterization of *Fusarium* mycotoxins the primary focus of research in this genus over the past 60 years (Keller et al., 2005; Summerell and Leslie, 2011; Perincherry et al., 2019).

More specifically, this focus on the evaluation of mycotoxins in the FDA GRAS and novel food context for filamentous fungi is generally recognized based, in part, on the discussion in Pariza and Johnson (2001) of the toxigenic potential of organisms commonly used in food production:

> *"The oral toxins produced by filamentous fungi are small molecular weight organic molecules, usually less than 1000 Da in size (Chu, 2000). These are referred to as mycotoxins.*
>
> *For example, all test material from mold [filamentous fungi] sources should be assayed for mycotoxins that are known to be synthesized by closely related species."*

*Mycotoxins in Fusarium: A Well-defined Domain of Compounds*

While this wealth of research has clearly shown that *the* genus can produce an array of mycotoxins and that closely-related species can produce different subsets of those mycotoxins (Table 2; Marasas et al., 1984; O'Donnell et al., 2018), there actually has been no significant discovery of a new mycotoxin from Fusarium in more than a quarter century, despite keen scientific interest and significant scientific advancements in genomics and analytical technologies.

**Table 2:** Reference table of mycotoxins identified in *Fusarium spp.*

| Mycotoxin | Year Identified | Responsible Pathway | Gene Presence in *F str. flavolapis* | Analytical Detection in Fy Protein | Method |
|---|---|---|---|---|---|
| Aurofusarin | 1937[A] | PKS[P] | No[P] | Not tested | Not tested |
| Beauvericin | 1991[B] | NRPS[Q,R,S] | Yes[R] | 0.08ppm avg | LC-MS/MS[CC] |
| Deoxynivalenol (trichothecene) | 1975[C] | Isoprenoid[T] | No[GG, X] | No | LC-MS/MS[DD] |
| Diacetoxyscirpenol (trichothecene) | 1971[D] | Isoprenoid[GG,X,Y] | No[GG,X] | No | LC-MS/MS[DD] |
| Enniatin (A, A1, B, B1) | 1973[E] | NRPS[R] | No[R] | No | LC-MS/MS[CC] |
| Fumonisin (B1, B2, B3) | 1988[F] | PKS[T,U] | Yes[U] | 0.055ppm avg total FUM | LC-MS/MS[DD] |
| Fusaric acid | 1966[G] | PKS[V, W] | Yes[V] | No | LC-MS/MS[EE] |
| Fusarin C | 1984[H] | NRPS-PKS hybrid, [U,W] | Yes[U,W] | No | LC-MS/MS[CC] |
| Fusaproliferin | 1995[I] | [I] * | * | No | LC-MS/MS[FF] |
| HT-2 Toxin | 1974[J] | Isoprenoid [GG,X,AA] | No[GG,X] | No | LC-MS/MS[DD] |
| Moniliformin | 1974[K] | * [Y,Z] | * | No | LC-MS/MS[EE] |
| Neosolaniol (trichothecene) | 1972[L] | Isoprenoid[GG,X,AA] | No[GG,X] | No | LC-MS/MS[DD] |

| | | | | | |
|---|---|---|---|---|---|
| T-2 Toxin | 1969[M] | Isoprenoid[GG,X,AA] | No[GG,X] | No | LC-MS/MS [DD] |
| Trichothecenes | 1992[N] | Isoprenoid[GG,X,AA] | No[GG,X] | No | LC-MS/MS[DD] |
| Zearalenone | 1970[O] | PKS[BB] | No[BB] | No | LC-MS/MS[DD] |

*Fusaproliferin and moniliformin have been biochemically characterized and associated with Fusarium in the literature, though the genetic pathways are yet to be definitively described. Nonetheless, without analytical standards available, qualitative analytical testing, by LC-MS/MS, confirmed that *F. str. flavolapis* does not have any compounds matching the molecular weight of fusaproliferin or moniliformin (Sewram et al. 1999, Sulyok et al. 2007).

Data from: Ashley et al., (1937)[A,] Gupta et al., (1991)[B] Yoshizawa & Morooka, (1975)[C] Harwig & Stock, (1971)[D] Audhya TK, Russel, (1973)[E] Gelderblom et al., (1988)[F] Narayanan & Shanmugasundaram (1966)[G], Gelderblom et al., (1984)[H,] Ritieni et al.(1995)[I] Ellison & Kotsonis (1974)[J] Springer et al. (1974)[K] Ueno (1972)[L] Marasas, (1969)[M] Smith (1992)[N,] Caldwell et al (1970)[O] Frandsen et al (2006)[P,] Wu et al. (2018)[Q] Liuzzi et al. (2017)[R,] Wang & Xu (2012)[S] Proctor (2003)[T] Wiemann et al.(2013)[U] Brown et al. (2015)[V] Niehaus et al. (2013)[W] King et al. (2018)[X] Bashyal et al. (2019)[Y] Eugenia et al. (2004)[Z,] Wu et al. (2010)[AA] Gaffoor & Trail (2006)[BB] Han et al.(2014)[CC] Sulyok et al. (2006)[DD] Sulyok et al. (2007)[EE] Sewram et al., (1999)[FF] McCormick et al. (2011)[GG]

This chronology strongly indicates that the Fusarium mycotoxins have been discovered, and that the mycotoxic potential of *Fusarium* for food safety is thoroughly characterized. Despite extensive characterization of toxic secondary metabolites in Fusarium and recent advancement in biochemical analysis, no new mycotoxins in Fusarium have been identified in the last twenty-five years (Table 2 and references therein).

*Mycotoxin Genetics: Highly Conserved Controls for Toxin Production in Fusarium*

Detailed genetic and biochemical studies have shown that Fusarium mycotoxins are predominantly produced in just three main biosynthetic pathways that are characterized by their core enzymes: polyketide synthase (PKS) pathways, non-ribosomal protein synthetase (NRPS) pathways, and terpene (isoprenoid) synthesis pathways (Table 1; Keller et al., 2005; Desjardins 2006; Keller 2015; Munkvold 2017).

These core enzymes are well-conserved across fungi and easily identified using protein-protein searches (Wiemann et al., 2013; Niehaus et al., 2016; Niehaus et al., 2017; King et al., 2018; Villafana et al., 2019). Furthermore, the genes required for biosynthesis of a single mycotoxin are frequently clustered in the genome in biosynthetic gene complexes (BGCs; Keller et al., 2005 and references therein; Desjardins 2006; Brown et al., 2015).

Sequence conservation and genomic organization of mycotoxin biosynthesis pathways enables accurate identification, characterization, and even prediction of the potential for a given strain to produce mycotoxins just based on its genome sequence and generally recognized methods of bioinformatics (Keller et al., 2005; Desjardins, 2006; Jestoi, 2008; Antonissen et al., 2014; Niehaus et al., 2016; Gruber-Dorninger et al., 2017; King et al., 2018; Perincherry et al., 2019; Haque et al., 2020).

*Genetic Variation in BGCs: Indicative of Expression, Not Mycotoxin Diversity*

While new mycotoxins have certainly evolved in the history of *Fusarium*, typically via duplication and divergence of BGCs, or by combination of two core pathways (e.g. fusarin C is synthesized by a PKS/NRPS hybrid pathway; Niehaus et al., 2013), lineage or strain-specific BGCs can be identified through genome-wide searches for the core enzyme. This approach has been successfully used to identify BGCs in new genome sequences from strains across the Fusarium genus (Wiemann et al., 2013; King et al., 2018; Villafana et al., 2019).

Importantly though, genetic variation within BGCs has been linked to changes in mycotoxin levels (loss or up/down-regulation), but not new mycotoxins (Suga et al., 2018; Sultana et al., 2019). Thus, while it is very unlikely that a newly characterized Fusarium strain would produce a novel mycotoxin, particularly one of concern for food safety, bioinformatic analyses can also be used to reliably predict the potential for those strains to produce mycotoxins and analytical testing can be conducted to identify their presence. As described below, this is the approach we have taken in the assessment of *F. str. flavolapis and F. venenatum*.

*Metabolite Genetics in F. venenatum and F. str. flavolapis*

Given that the mycotoxic potential of *Fusarium* is well characterized at the biochemical and genomic levels, we can conclude, to a reasonable certainty that *F. str. flavolapis* has a toxicological potential similar to or lower than *F. venenatum*. As detailed in our response to Regulatory question 1, *F. str. flavolapis* is found in the *fujikuroi* group while *F. venenatum* is found in the *sambucinum* group (O'Donnell et al., 2013). Both the *sambucinum* and *fujikuroi* clades contain common plant pathogens that have been intensely studied for their toxicity (*F. graminearum* and *F. proliferatum*, respectively, among others; Figure 1).

Analyses of core biosynthetic enzymes and BGCs in our publicly-available *F. str. flavolapis* genome sequence and gene annotation set show that *F. str. flavolapis* contains BGCs that are known to be required for production of fumonisins B1, B2, and B3; fusaric acid; fusarin C; and beauvericin. *Fusarium str. flavolapis* potential and actual mycotoxin production are consistent with actual mycotoxin production in other *fujikuroi* clade members (Munkvold 2017; O'Donnell et al., 2018). *F. venenatum* contains BGCs to produce fusarin C, beauvericin, aurofusarin, and trichothecenes (King et al., 2018 and independent analyses). Though the genetic machinery to produce mycotoxins can vary between *Fusarium spp.*, we demonstrate that *F. str. flavolapis* and *F. venenatum* have the same toxicological potential or lack thereof when grown under controlled conditions by coupling generally recognized methods of bioinformatics and biochemical analysis with well-established mycotoxin limits.

Beyond mycotoxin genetics, the proteome genetics of *F. str. flavolapis* and *F. venenatum* have been assessed holistically for allergenic potential. As discussed in GRN 904 Attachment 4, the allergenic proteome of *F. str. flavolapis* and *F. venenatum* show high sequence homology. This comparison further supports the overarching basis of similarity between *F. str. flavolapis* and *F. venenatum* pertinent for toxicological potential assessment.

*Analytical Evidence of Genetic Potential for Mycotoxins - F. str. flavolapis*

We assessed actual *F. str. flavolapis* mycotoxin production when grown under controlled conditions by screening for a panel of 25 mycotoxins encompassing all known classes using generally recognized LC-MS/MS methods and detected only fumonisins and beauvericin. As detailed in GRN 904, these mycotoxins were detected at levels corresponding to dietary intakes that are well below the safety thresholds established by expert organizations, namely the

Provisional Maximum Tolerable Daily Intake (PMTDI) for fumonisins, established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the EFSA CONTAM panel review of beauvericin(Table 22 GRN 904). Altogether, these results show actual and potential *F. str. flavolapis* mycotoxin production results in a dietary intake that is well below limits based on regulatory guidelines, satisfying a key requirement for generally recognized safety (Pariza and Johnson, 2001).

*Genetic and Analytical Conclusion of Similar Toxicological Profiles*

As described above, we have followed well-established phylogenetic, genomic, and analytical approaches for determining that *F. str. flavolapis* has the same toxicological potential or lack thereof as *F. venenatum* by demonstrating that:

- Toxicity of *Fusarium spp.* is well understood by the scientific community,
- Toxicological inquiry in Fusarium is focused on the toxic secondary metabolites, mycotoxins,
- Mycotoxins in *Fusarium spp.* are well characterized with a limited domain of compounds,
- The genes that control the type/quantity of mycotoxins in Fusarium are well understood,
- Established genetic methods elucidate the toxicological potential of *F. spp.*, specifically *F. str. flavolapis* and *F. venenatum*,
- The toxicological potential based on genetics is verifiable with established analytical methods, and
- *F. venenatum* and *F. str. flavolapis* produced limited mycotoxins when grown under controlled conditions of the manufacturing method.

The validity of this methodology can be verified by the scientific community leveraging the extensive set of published literature referenced in this discussion and the published genomes of *F. str. flavolapis* and *F. venenatum.*

## *In vitro* & *in vivo* Basis of Similar Toxicological Profiles

While there is no genetic or analytical evidence that *F. str. flavolapis* produces mycotoxins at levels of concern for food safety when Fy Protein is consumed under the intended uses, there also is strong evidence from *in vitro* and *in vivo* studies that further corroborate our conclusion that *F. str. flavolapis* has a toxicological potential similar to or less than that of *F. venenatum*.

*In vitro Basis of Similar Toxicological Profiles*

As noted in GRN 904, Section VI. A. 2.a-b “Genetic Toxicity,” Fy Protein has been tested using a battery of *in vitro* toxicity assays that has been shown to be sufficient to detect carcinogens and *in vitro* toxicity in rodents (Kirkland et al., 2011) and are used to support the safety of human consumption (FDA 2007). These tests would be expected to detect harmful effects from potentially unknown secondary metabolites commonly associated with filamentous fungi (Pariza and Johnson, 2001).

Testing found no significant increase in DNA mutation rate (Ames tests; OECD 471), similar to the results from the Ames test on *F. venenatum* which showed non-mutagenicity (Miller and Dwyer 2001), when both metabolically active and inactive bacterial cells were treated with the highest recommended concentration of *F. str. flavolapis* (5 mg / plate). Additionally, testing found no significant increase in cell death or increase in the number of micronuclei in the cells (GRN 904), using a human lymphocyte micronucleus test (OECD 487) in which human

peripheral lymphocytes were treated with the highest concentration of *F. str. flavolapis* (50 ug/ml).

Together, the Ames test and micronucleus tests show that *F. str. flavolapis* has no mutagenic or cytotoxic effects *in vitro* and F. venenatum has non-mutagenic effects, further supporting the genetic and analytical results detailed above.

*In vivo Basis of Similar Toxicological Profiles*
Finally, the *in vitro* results are further corroborated by the acute 14-day oral toxicity tests in rats fed high amounts of Fy Protein, as reported in GRN904, Section VI. A. 1 "Acute Oral Toxicity Test". The test identified no physical or gross morphological differences between controls and rats fed Fy Protein up to 15% of their diet (GRN 904).

## Conclusion of Similar Toxicological Profiles

Based on the foregoing similarities and assessment of the extensive published and peer-reviewed data on *Fusarium* toxicity and *F. venenatum* toxicity, as well as the data on *F. str. flavolapis* toxicity, we have determined that *F. str. flavolapis* and *F. venenatum* have the same toxicological potential or lack thereof, when grown under controlled conditions, and that the read across to pivotal data cited in GRN 91 for *F. venenatum* offers sound scientific support for our conclusion that the estimated consumption of Fy Protein is safe to a reasonable certainty. .

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## Toxicology: Question 2 (T2)

*“It appears that the genome sequence of your strain is neither published nor publicly available. As such, data and information related to any bioinformatic analyses would be considered as unpublished results. For example, there is no way for the scientific community to independently*

*confirm your conclusion that "[your] strain is predicted to be most closely related to members of the Fusarium fujikuroi species complex" (pg. 6, Attachment 2) is valid. Furthermore, it is not clear how phylogenetic relatedness determined by bioinformatic analyses translate to inference of toxicological and safety data across species. Given genetically close species within Fusarium genus can produce vastly different sets of mycotoxins and secondary metabolites due to small changes in gene clusters and/or environmental growing conditions (King et al., 2018; O'Donnell et al., 1998; Perincherry et al., 2019; Villafana et al., 2019), please provide a rationale as to how the safety of your strain can be surmised based solely on bioinformatic analyses and screening of limited number of mycotoxins and secondary metabolites."*

## T2 Response

### *Fusarium str. flavolapis* Reference Genome and Annotation

The *Fusarium str. flavolapis* reference genome sequence and annotation is publicly available from NCBI under BioProject accession PRJAN665233. We note that NCBI GenBank accession numbers are generally recognized as a means to publicly identify organisms and genes and are broadly utilized in GRAS Notices, such as GRNs 672, 660, and 631.

Though less direct, prior to such publication in GenBank, the identity of *F. str. flavolapis* as a member of the *F. fujikuroi* complex could also have been corroborated by the scientific community using generally recognized genome sequencing techniques on PTA-10698. This ATCC sample has been available to the public since March 2, 2010.

### Phylogenetic Placement of *F. str. flavolapis* within the *Fusarium* Genus

As detailed in the response to Regulatory Question 1, we determined the relationship of *F. str. flavolapis* to other *Fusarium* strains, firmly placing it within the *Fusarium fujikuroi* group (Figure 1).

### Genetic and Analytical Determination of Toxicological Potential in *F. str. flavolapis*

Please see the response to Toxicology Question 1 above, specifically Section 3, for a detailed discussion on leveraging toxicological and safety data from other *Fusarium* to justify safety of *F. str. flavolapis,* as well as the general recognition of these methods.

### Variation in Mycotoxins Among Closely Related Species

*Summary Analysis of Cited References*

In this question, the reviewers assert:

> *"…genetically close species within Fusarium genus can produce vastly different sets of mycotoxins and secondary metabolites due to small changes in gene clusters and/or environmental growing conditions (King et al., 2018; O'Donnell et al., 1998; Perincherry et al., 2019; Villafana et al., 2019)…"*

While aspects of this statement are correct, a closer inspection conveys a different, more general point: *Fusarium spp.* vary in their genomic potential for secondary metabolite production, even between closely related strains (King et al., 2018; O'Donnell et al., 1998; Perincherry et al., 2019; Villafana et al., 2019). This does not imply that the sets of mycotoxins and secondary metabolites are "vastly different" from the range of known and well characterized toxic compounds. Instead, the variation in mycotoxin production between

*Fusarium spp.* reported by O'Donnell et al. (2018), among others, is variation in the particular subsets of known mycotoxins that those closely-related strains produce.

The potential and actual variation in the coding for and production of toxic secondary metabolites in Fusarium can readily be evaluated based on generally recognized bioinformatics and analytical methods, as has been conducted on *F. str. flavolapis*. As such, these sources do not contradict our determination that *F. str. flavolapis* and *F. venenatum* have the same toxicological potential or lack thereof. Rather, the references cited are corroborative of the analysis as discussed in Toxicology Question 1, and instead show that:

1. Each biosynthetic gene complex (BGC) produces a different mycotoxin;
2. New mycotoxins have historically evolved by duplication and divergence of known BGCs;
3. Genetic variation within a BGC can cause differences in mycotoxin expression levels, loss of mycotoxin production, or loss of certain decorations to the core chemical backbone (Proctor et al., 2006; Suga et al., 2018; Sultana et al. 2019); and
4. Genetic variation within a BGC does not demonstrate the production of novel mycotoxins outside of those already discovered

<u>*Detailed Analysis of Cited References*</u>
Both King et al. (2018) and O'Donnell (1998), compare the genomes of *Fusarium venenatum* and *Fusarium graminearum* which produce different but well characterized mycotoxins - Type A trichothecenes (*F. venenatum*) and Type B trichothecenes (*F. graminearum*). Though different, both mycotoxins are produced via a well-understood, common metabolic pathway which relies on shared genes, diverging only late in the biosynthetic pathway (McCormick et al., 2011).

Villafana et al. (2019) shows that the ability to produce trichothecene mycotoxins is constrained by the presence of a single gene and its homologs - t*ri3-14* which is required for the synthesis of all types of trichotenene (King et al., 2018, Villafana et al., 2019). *Tri3-14* homologs are not found in the *F. str. flavolapis* genome, indicating *F. str. flavolapis* is not capable of trichothecene production. Though O'Donnell (1998) points out that *F. venenatum* does not produce zearalenone or Type B trichothecenes as compared to *F. graminearum*, this does not indicate the potential for undetermined mycotoxins among these two species, nor does it indicate the potential in *F. str. flavolapis*. The pathways for zearalenone syntheses are well characterized, requiring two polyketide synthases (Kim et al., 2005, Gaffoor and Trail, 2006). Neither of these polyketide synthases nor their homologs are found in either *F. str. flavolapis* or *F. venenatum*, though they are present in *F. graminearum* based on BLAST searches.

Perincherry et al. (2019) focus their review on the influences of environmental conditions and plant-pathogen interactions on secondary metabolite production, with the former summarizing prior studies relating to temperature, moisture, nitrogen availability, and pH. Citations are provided in which each of these environmental factors may influence Fusarium growth and the associated production of mycotoxins, but not changes in the type of mycotoxin produced. Nature's Fynd produces Fy Protein under carefully controlled and consistent environmental conditions to prevent accumulation of mycotoxins as described earlier in Toxicology Question 1, Analytical Evidence of Genetic Potential for Mycotoxins - *F. str. flavolapis*.

Beyond the references cited, we note that recent studies have documented horizontal gene transfer in Fusarium as a mechanism that can introduce metabolomic diversity among species (Villani et al., 2019, Tralamazza et al., 2019). However, as noted in Toxicology Question 1, this mycotoxin diversity has still been limited to known mycotoxins, which are highly conserved among filamentous fungi and *Fusarium spp.*, and does not suggest the introduction of novel mycotoxins. Nature's Fynd uses industry best practices to assure purity of *Fusarium str. flavolapis* throughout the strain propagation and manufacturing process, thus preventing the possibility of horizontal gene transfer introducing unexpected events.

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# Toxicology: Question 3 (T3)

*"OFAS notes that a reference was found (King et al., 2018) that indicates that within Fusarium sp., Fusarium venenatum (species that was subject of GRN91) is more closely related to Fusarium graminearum than to Fusarium fujikuroi. Given than Fusarium graminearum is genetically closely related to Fusarium venenatum, but with a much more pathogenic phenotype, please explain how genetic similarity of your strain to Fusarium fujikuroi would ensure a safety profile similar to that of Fusarium venenatum, especially in light of the fact that some strain of Fusarium fujikuroi, but not Fusarium venenatum, can produce fumonisin through a functional biosynthetic FUM gene cluster."*

## T3 Response

Please see the response to Toxicology Question 1 for a more detailed discussion on the scientific validity of comparing *F. venenatum* and *F. str. flavolapis*, particularly Section 3. In that response, as well as the response to Regulatory Question 1, we clearly establish that *F. str. flavolapis* is a member of the *Fusarium* genus, and closely-related to agronomically important crop pathogens that have been intensely studied specifically for their phytotoxin and mycotoxin production.

While *F. venenatum* and *F. str. flavolapis* are members of different clades, the sets of mycotoxins that *Fusarium* strains can produce have been well-defined during the past six decades of *Fusarium* mycotoxin research (e.g. Desjardins 2006). *F. venenatum* and *F. str. flavolapis*, as well as *F. graminearum*, produce different sets of mycotoxins and this is not surprising, as the mycotoxin potential and controlling genetic pathways across the *Fusarium spp.* are well understood.

Some mycotoxins are produced by all or most *Fusarium*, while others are only produced by certain clades (O'Donnell et al., 2013). For example, *F. venenatum* and other *sambucinum* clade strains produce trichothecenes but do not produce fumonisins, while *F. str. flavolapis* and other *fujikuroi* clade strains produce fumonisins but do not produce trichothecenes (Desjardins 2006).

However, studies of mycotoxin production by diverse members of the *fujikuroi*, *sambucinum*, and other *Fusarium* clades has helped define the known world of mycotoxins. Differences in mycotoxin production between clades are well-understood at both the genetic (Niehaus et al., 2016) and biochemical (Niehaus et al., 2016; O'Donnell et al., 2018) levels.

As the reviewer notes, *Fusarium str. flavolapis* does carry a functional *FUM* gene cluster, but the analytical studies, presented in GRN 904 Section VI.D. 1-3, show that fumonisins are produced at extremely low levels by *F. str. flavolapis* under controlled production conditions and that the estimated dietary intakes present a very low safety risk as described in GRN 904 Section III.B.1-3 "Estimate of Dietary Exposure to Mycotoxins".

Pathogenicity of *F. str. flavolapis* is discussed in Microbiology Question 3. We additionally note that "pathogenicity" as referenced in the question relates to plant pathogenicity, as opposed to pathogenicity in humans.

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## Toxicology: Question 4 (T4)

*"Due to intraspecies genetic differentiation, Fusarium fujikuroi has 50 phylogenetic species and 13 independent mating populations, as well as two distinct groups, F- and G-groups, designating fumonisin-producer and nonproducer, respectively (Suga et al., 2019 [sic 2018]). Furthermore, fumonisin biosynthethic genes within the FUM gene cluster are highly homologous between both fumonisin producers and non-producers of Fusarium fujikuroi (Sultana et al., 2019), suggesting that subtle natural variations can greatly impact production of mycotoxins and secondary metabolites. Since you state on pg. 41 of your Notice that "[g]enome sequencing and analysis indicated the potential for formation of the mycotoxins fumonisins, beauvericin, fusarin C, and fusaric acid," please discuss whether your strain has the functional FUM gene cluster capable of producing fumonisins under certain growing conditions."*

## T4 Response

Analysis of the *Fusarium str. flavolapis* genome sequence and annotation indicates that this strain has a functional *FUM* gene cluster with the capacity to produce fumonisins. However, LC-MS/MS analyses, described in GRN 904 Section III.B and VI.D show that, under our controlled production conditions, fumonisins are produced at levels resulting in an estimated dietary intake well below the Provisional Maximum Tolerable Daily Intake (PMTDI) for fumonisins, established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2018).

We note that the Suga et al. (2018) and Sultana et al. (2019) references show that small mutations in specific locations of the 16 *FUM* ORFs necessary for fumonisin production lead to non-functionality and are localized in specific groups of *F. fujikuroi spp*. The strains with this mutation lack the ability to produce fumonisin. While *F. str. flavolapis* contains a functional *FUM* operon, actual production levels of fumonisin are well below levels that present any safety concern under our controlled production conditions and testing to confirm compliance with the established specification is conducted on every batch.

Though the cited references suggest that subtle, natural variations can impact the amount of mycotoxins different strains produce, they do not suggest that this variation leads to production of undetermined toxic secondary metabolites. As discussed in the detailed response to T1, *F. str. flavolapis* has been carefully evaluated for its genetic potential to produce toxic secondary metabolites based on generally recognized bioinformatics methods, our controlled manufacturing method for F. str. flavolapis reduces the toxic secondary metabolites produced, and *F. str. flavolapis* has been assessed for its actual production of mycotoxins using generally recognized methods of chemical analysis.

References:


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# Toxicology: Question 5 (T5)

*"The dataset for the GRAS conclusion for GRN91 included a number of published toxicological studies, as well as studies in humans. While clinical studies often do not provide toxicologically relevant data, they do corroborate that the consumption of mycoprotein GRASed in GRN91 is tolerable and do not cause acute adverse effects in humans. Please provide a rationale for why such tolerability studies in humans are not needed for your substance."*

## T5 Response

We have concluded that Fy Protein is tolerable based on its material similarity to mycoprotein from *F. venenatum* (subject of GRN 91), its low-risk nutritional profile, low-risk potential for allergenicity, and low-risk potential for toxicity concerns. Products with substantial similarities and history of consumption have been used to assess the human tolerability of a new ingredient (for example, whey protein was used as a comparison for Beta-lactoglobulin in GRN 863). This collective information does not show a high concern and is evidence of the adequacy of the data for Fy Protein, making a human tolerability study unnecessary.

*Tolerability of Fusarium-derived Foods*

The available clinical studies conducted by the manufacturer of mycoprotein support that food produced from *F. venenatum* is well-tolerated by humans (Turnbull et al., 1990, Miller and Dwyer, 2001). When considering the similarities between mycoprotein and Fy Protein, particularly those pertinent for tolerability assessment as discussed in further detail below, the mycoprotein tolerability profile serves as a scientifically sound reference point for evaluating the tolerability of Fy Protein.

*Low-risk Nutritional Profile for Tolerability*

The substantial similarities between the nutritional profiles of Fy Protein and mycoprotein indicate that human consumption of Fy Protein is unlikely to result in adverse tolerability. As reviewed in GRN 904, Fy Protein is a readily digestible source of protein and is composed of standard macronutrients. Though Fy Protein is slightly higher in fiber than mycoprotein, this difference is in line with Daily Reference Values and Reference Daily Intakes of foods (21 CFR 101.9) and published tolerable upper intake levels from the National Academy of Sciences (Institute of Medicine, 2000). Both of these sources are generally recognized references for allowances and limits when evaluating the tolerability of food ingredients.

*Low-risk Protein Profile for Tolerability*

Beyond nutritional differences, we also assessed protein differences between Fy Protein and mycoprotein and found that the protein profiles are substantially similar for the purposes of assessing tolerability. This determination is discussed in detail in the subject GRAS notice, the FARRP allergenicity reports, and the responses below to Toxicology Questions 6-8.

*Low-risk Toxicity Profile for Tolerability*

Beyond the dimensions discussed above, we also evaluated Fy Protein for toxic secondary metabolites that may impact tolerability due to an acute adverse effect. Based on the discussion in GRN 904 and this response document, we have determined that Fy Protein has the same toxicological profile as mycoprotein, and therefore there is nothing relating to the presence of mycotoxins in Fy Protein that would be understood better by human tolerability studies.

*Minimal Incremental Confidence*

FDA's Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food (hereinafter, "Redbook") states that clinical studies with human subjects for macroadditives "may be useful for increasing confidence in the safety of the product for human consumption" (FDA, 1993a) and that a major objective in such testing is to assess aspects of safety that "cannot be addressed adequately by non-human studies or by existing data on population exposure."(FDA,1993b). In this case, there are decades of data supporting human tolerance of Fusarium-based protein (Finnegan, 2019).

*Conclusion*

As discussed extensively in this response, the public literature corroborates Nature's Fynd's conclusion that it is scientifically supportable to utilize the existing safety data for mycoprotein as evidence of the tolerability of Fy Protein. Based on the aforementioned points, Nature's Fynd concludes that clinical studies in humans would be unnecessary, and that evidence of safety and tolerance in humans is well-understood and documented.

References:


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# Toxicology: Question 6 (T6)

*"As you noted on Pg. 36 of your Notice, Codex Alimentarius guidance for evaluating allergy risks of proteins were developed for genetically engineered crops in which only a single or a handful of recombinant proteins are ectopically expressed. Given that it is stated (in Attachment 2) that there are approximately 14,239 annotated genes in your organism, please explain your statement:*

> *'Though Fermented microbial protein (FMP) is not a GE product … the evaluation procedure was considered appropriate and relevant for a new food ingredient" (pg 36 of Notice)'*

*by providing appropriately peer-reviewed documentations to support the general acceptance of this approach by the scientific community."*

## T6 Response

The Codex Alimentarius Commission developed recommended methods for the analysis of potential allergenic proteins derived from biotechnology (Codex 2009). This assessment uses a

multi-factorial approach that includes: a) assessing the source of the protein and the sequence homology of the protein(s) to known allergens in public, peer reviewed databases; b) evaluating resistance to pepsin and intestinal fluids (Thomas et al 2004); and c) if there is a high suspicion of major allergenicity, serum screening is recommended. To note, sequence homology is based on >35% identity over 80 amino acids and one of the most comprehensive databases used is AllergenOnline version 18B, with 2,089 peer reviewed entries.

Though the Codex Alimentarius approach was originally developed to evaluate the allergenicity of limited sets of novel proteins in genetically engineered foods, the same method of analysis is generally recognized in the food industry and among regulators for use in the evaluation of novel foods with broader sets of proteins (EFSA 2016; Fernandez 2020). Further, this approach has also been utilized in GRAS Notices that have received "No Questions" letters from FDA, such as GRNs 684 and 831.

In summary, the results support a low-likelihood of allergic response to Fy Protein based on generally recognized methods of assessment.

References:


Codex Alimentarius Commission, FAO, & World Health Organization (Eds.). (2009). *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* (2. ed). Food and Agriculture Organization [u.a.].

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# Toxicology: Question 7 (T7)

*"While in vitro digestibility studies in simulated gastric fluid and simulated intestinal fluid have been used previously for allergenicity risk assessment of individual proteins, we are not aware from the literature that such assays are established and accepted tools to evaluate the allergenicity potential of complex mixtures of proteins. In fact, it would seem more plausible to test individual potentially allergenic candidate proteins identified from bioinformatic analyses in these types of assays. Please provide either references that indicate that these in vitro digestibility assays are useful tools to predict allergenicity of complex mixture of proteins and/or why testing individual candidate proteins is not a more feasible approach to assessing allergenic potential of your substance."*

## T7 Response

Please see the response and references in T6 discussing the general recognition of *in vitro* digestibility studies for evaluating the allergenic risk of proteins. We note that there are few standardized protocols to assess the digestibility of multiple proteins in whole foods using simulated gastric fluid and a number of challenges are presented when attempting to test individual candidate proteins, because fungal protein isolates are not stable through the isolation and purification process (Hoff, 2003). Additionally, isolating proteins is not representative of the consumption of Fy Protein under the intended use.

References:

Hoff, M., Ballmer-Weber, B. K., Niggemann, B., Cistero-Bahima, A., San Miguel-Moncín, M., Conti, A., Haustein, D., & Vieths, S. (2003). Molecular cloning and immunological characterisation of potential allergens from the mould Fusarium culmorum. *Molecular Immunology*, *39*(15), 965–975. https://doi.org/10.1016/S0161-5890(03)00026-9

# Toxicology: Question 8 (T8)

*"Please explain why a 10→20% gradient gel (10% at the top; 20% at the bottom) was used instead of 4→20%, or similar range distribution. This is because there is no need for a stacking gel when setting up the gradient gel. A wider gradient of the resolving gel should provide better resolution."*

## T8 Response

The *in vitro* digestion assay followed is a generally recognized simulated gastric fluid method presented by Thomas et al. (2004) and is the same gradient gel utilized in other GRAS Notices, (GRN 737 and GRN 773). Polyacrylamide gels with higher concentrations have smaller pore sizes, providing higher resolution for small proteins. The smallest proteins and protein fragments after rapid digestion in our sample are 5-10kDa. A 4-20% gradient would not provide further resolution for proteins less than 5kDa.

References:

Impossible Foods, Inc. (2017, October). *GRAS Notification for Soy Leghemoglobin Protein Preparation derived from Pichia Pastoris* [GRN No. 737]. https://www.fda.gov/media/124351/download

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# Microbiology: Question 1 (M1)

*"Please confirm the ATCC identification information provided for Fusarium oxysporum MK7."*

## M1 Response

The ATCC Certificate is included with this response as Appendix A. As indicated in GRN 904, the prior designation of the *Fusarium spp.* was *Fusarium oxysporum MK7*. The current designation is *Fusarium str. flavolapis*.

# Microbiology: Question 2 (M2)

*"Please identify the strain name of the Fusarium novum. yellowstonensis species."*

## M2 Response

The strain is *Fusarium strain flavolapis.*

As discussed in our July 30, 2019 correspondence to FDA, Nature's Fynd continues to formally establish a binomial name for the microorganism that is the subject of GRN No. 904. The microorganism is currently referred to as *Fusarium strain flavolapis*. "Strain" serves the same purpose that "novum" previously served, i.e., as a placeholder to indicate a future new species designation, and does not indicate that there are multiple strains collected with distinct genetics.

# Microbiology: Question 3 (M3)

*"Please provide evidence and confirm that the strain is non-pathogenic and non-toxigenic."*

## M3 Response

### Overview

We confirm that *Fusarium str. flavolapis* is non-pathogenic and non-toxigenic to consumers when used as intended.

*Pathogenicity Summary*
*Fusarium spp.* are ubiquitously distributed in the environment and humans are regularly exposed to members of the genus without developing infections, with Fusarium being found frequently in the normal gastrointestinal mycobiome of healthy vegetarians (Hallen-Adams, 2017). Invasive Fusarium infections are rare and opportunistic, largely developing in patients with trauma or injury, or in severely immunocompromised individuals (Nucci and Anaissie, 2007; Batista et al, 2020). The Fusarium genus encompasses over 100 species with phylogenetic diversity and variable pathogenicity, and invasive infections are seldom associated with members of the *Fusarium fujikuroi* species complex (O'Donnell et al., 2007; O'Donnell et al., 2015). Furthermore, viable hyphae or conidia are required to initiate infections (Nucci and Anaissie, 2007); in our manufacturing process for Fy Protein as described in GRN 904 and consistent with generally recognized methods of deactivation (U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition, 2018), renders the organism non-viable. As such, *F. str. flavolapis* does not pose significant pathogenic risk to consumers.

*Toxigenicity Summary*
The toxigenic potential of the organism has been evaluated in detail in GRN 904 and is further discussed in the "Toxicology" section of this response document.

## Exposure to *Fusarium spp. and F. str. flavolapis* Pose Limited Pathogenic Risk

*Prevalence of Fusarium spp. in the Environment*
*Fusarium spp.* grow on a wide range of substrates and are ubiquitous in the soil or on organic matter (Nelson et al, 1994). Additionally, *Fusarium spp.* are found globally in water structure biofilms (Nucci and Anaissie, 2007). *Fusarium spp.* have also been isolated in the plumbing systems of 80% of buildings studied in the US (Short et al., 2011), and are present in the gastrointestinal microbiome of 88% of healthy study participants reporting a vegetarian diet (Hallen-Adams and Suhr, 2017; Cohen et al., 1969).

*Low Risk of Fusarium spp. Infection*
Although *Fusarium spp.* are widely distributed in nature, man-made environments, and even in healthy gastrointestinal tracts, *Fusarium spp.* rarely cause significant human disease. As *Fusarium spp.* are well known plant-pathogens commonly associated with important agricultural commodities, studies on human pathogenicity of the genus, and even individual species, have been well examined and documented (Zhang et al, 2006). Fusarium infections have a very low incidence overall despite the widespread environmental distribution of *Fusarium spp.* The Centers for Disease Control and Prevention assessed the incidence of fungal infections in the United States in 2017, and did not specifically list Fusarium infections due to the low number of cases, classifying these infections with other "uncommon and opportunistic mycoses" and "other unspecified mycoses" (Centers for Disease Control and Prevention, 2018).

Healthy individuals can rarely develop superficial infections such as onychomycosis (toenail fungus) due to certain *Fusarium spp.*, and keratitis (a superficial infection of the cornea of the eye) has been described in soft contact lens wearers using contact lens solutions heavily contaminated by *Fusarium spp.* or with ocular trauma, the main predisposing factor in 40-60%

of patients (O'Donnell, et al., 2007; Batista et al, 2020). Outside of these infections, *Fusarium spp.* are of limited pathogenicity in otherwise healthy individuals.

Invasive Fusarium infections are rare and opportunistic, developing almost exclusively in individuals who are severely immunocompromised due to prolonged neutropenia after intensive chemotherapy, exposure to high-dose corticosteroids or immunosuppressant drugs that impair T-lymphocyte immunity, or skin breakdown due to severe trauma or burn injuries (Nucci and Anaissie, 2002; Nucci and Anaissie, 2007; Nucci et al., 2004; Batista et al., 2020). The vast majority of these infections are acquired via inhalation of airborne conidia or direct inoculation of organisms through the skin (Nelson et al, 1994; Nucci and Anaissie, 2007).

While the Fusarium genus encompasses over 100 distinct species with phylogenetic diversity and a spectrum of pathogenicity, only a subset are known to be pathogenic to humans. The *Fusarium solani* species complex, *Fusarium oxysporum* species complex, *Fusarium proliferatum*, and *Fusarium verticillioides* cause over 90% of both superficial and invasive Fusarium infections (Alastuey-Izquierdo et al., 2008; Batista et al., 2020; Hof, 2020). Other than the notable exceptions of *Fusarium proliferatum* and *Fusarium verticillioides*, the 45 members of the *Fusarium fujikuroi* species complex seldom infect humans, rarely causing superficial infections (onychomycosis or keratitis) in heavily exposed individuals or invasive infections even in highly immunocompromised patients (O'Donnell et al., 2007; O'Donnell et al., 2015). *Fusarium str. flavolapis* is not known to cause human infections.

Furthermore, viable hyphae or conidia are required to initiate infections in humans (Nucci and Anaissie, 2007), and when manufactured as described in GRN904, the organism is rendered non-viable. This method is consistent with generally recognized methods of deactivation (U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition, 2018). See response below to quality, manufacturing, and specifications ensuring *Fusarium str. flavolapis* is non-viable. Given the limited intrinsic virulence of *Fusarium str. flavolapis* and the lack of viable hyphae or condia in the finished food product, we believe *Fusarium str. flavolapis* is non-pathogenic to consumers when used as intended.

*Pathogenicity Conclusion*
We conclude that Fy Protein is non-pathogenic to consumers when used as intended. Fusarium is ubiquitously distributed in plants, soil, and water and is often a component of the normal human gastrointestinal mycobiome. Fusarium rarely infects humans, with invasive infections seldom developing even in highly immunocompromised individuals. The vast majority of human infections are caused by members of the *F. solani and F. oxysporum* species complexes, and by *Fusarium proliferatum*, and *Fusarium verticillioides*. *Fusarium str. flavolapis* is not known to cause infections in humans, and the organism is deactivated prior to consumption in finished food products, rendering it non-viable and unable to cause infections. On these bases, we conclude that *Fusarium str. flavolapis*, in the form present in the finished food product, does not pose a pathogenic risk to consumers.

References:

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# Microbiology: Question 4 (M4)

*"Please provide a discussion of the scientific literature available on the organism, including for its previous designation Fusarium oxysporum MK7."*

## M4 Response

The genome of *Fusarium strain flavolapis* has been made publicly available to the scientific community through NCBI BioProject accession PRJNA665233.

*Fusarium strain flavolapis* was isolated in August 2008, from a sample collected from Yellowstone National Park on 7/28/2008 under YNP Scientific Research and Collection Permit, Study # YELL-05068, Permit # YELL-2008-SCI-5068. The isolate was referred to as "strain MK7" by discoverer of the strain, graduate student Mark Kozubal. The strain was identified by microscopy of macroconidia to be a member of the Fusarium genus.

18S rRNA sequencing was done on the isolate in December 2008 and the sequence was closest to *Fusarium oxysporum* at the time of the basic local alignment search tool (BLAST) search using the Genbank database. After the 18S sequencing, the strain was referred to as *F. oxysporum* strain MK7, *F. oxysporum* MK7, or *F. oxysporum* str. MK7. An invention disclosure was completed 6/2/2009 by Montana State University and referred to the strain as *Fusarium oxysporum* strain MK7. The strain was deposited in the American Type Culture Collection under accession deposit number ATCC PTA-10698 as *Fusarium oxysporum* strain MK7 on March 2, 2010.

Since that time, the strain's genome has been fully sequenced along with a large number of *Fusarium spp.* and members of the *F. fujikuroi* complex, allowing for more accurate phylogenetic placement. Phylogenetic analysis using 18S sequences with the ITS region (18S/ITS) are now more commonly used for phylogenetic placement of fungi (Schoch et al., 2012; Yarza et al., 2017) and placed the strain within the *F. fujikuroi* Species Complex (Figure 1). This placement is discussed in further detail in the response to Regulatory Question 1, utilizing the robust scientific literature on Fusarium species in general and the *F. fujikuroi* clade in particular.

For a period of time, the strain was referred to as *Fusarium novum yellowstonensis*, *Fusarium yellowstonensis* or *Fusarium yellowstonensis strain MK7*. However, the name was changed in 2020 to *Fusarium strain flavolapis* to clearly identify the strain and not confuse the consumer nor scientific community, as there are numerous other microorganisms with the species name yellowstonensis (eg, *S. yellowstonensis*, *C. yellowstonensis*, *M. yellowstonensis*, *P. yellowstonensis*, etc.).

This discussion, as well as the NCBI BioProject accession PRJNA665233 reference, should serve to resolve the identity of the *Fusarium spp.* in GRN 904.

References:

Niehaus, E.-M., Kim, H.-K., Münsterkötter, M., Janevska, S., Arndt, B., Kalinina, S. A., Houterman, P. M., Ahn, I.-P., Alberti, I., Tonti, S., Kim, D.-W., Sieber, C. M. K., Humpf, H.-U., Yun, S.-H., Güldener, U., & Tudzynski, B. (2017). Comparative genomics of geographically distant Fusarium fujikuroi isolates revealed two distinct pathotypes correlating with secondary metabolite profiles. *PLOS Pathogens*, *13*(10), e1006670. https://doi.org/10.1371/journal.ppat.1006670

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Fungal Barcoding Consortium, Fungal Barcoding Consortium Author List, Bolchacova, E., Voigt, K., Crous, P. W., Miller, A. N., Wingfield, M. J., Aime, M. C., An, K.-D., Bai, F.-Y., Barreto, R. W., Begerow, D., … Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (Its) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, *109*(16), 6241–6246. https://doi.org/10.1073/pnas.1117018109

Yarza, P., Yilmaz, P., Panzer, K., Glöckner, F. O., & Reich, M. (2017). A phylogenetic framework for the kingdom Fungi based on 18S rRNA gene sequences. *Marine Genomics*, *36*, 33–39. https://doi.org/10.1016/j.margen.2017.05.009

# Microbiology: Question 5 (M5)

*"Please confirm that the manufacturer continuously monitors the fermentation process for contaminants and quality control procedures are taken upon observation of contamination."*

## M5 Response

Nature's Fynd has developed and adopted a strict quality program and process that includes a Food Safety Plan (FSP), Hazard Analysis, and Preventive Controls review and Good Manufacturing Process (GMP) consistent with the requirements of the Federal Food, Drug and Cosmetic Act and the regulations promulgated thereunder. The quality program continuously monitors the process from media screening through freezing and shipment including nutrient and microbiological specifications. Procedures and processes have been developed to test and identify contaminations and hold, traceability, recall, and disposal procedures if a contamination or out of specification product is detected.

# Microbiology: Question 6 (M6)

*"Please provide the sample sizes used for analysis for your microbial specifications and provide the raw data for the batch analyses."*

## M6 Response

Sample sizes of 700 grams were used for nutritional, microbiological, and heavy metals testing for each of the three individual batches of homogenized Fy Protein. Aliquots of the homogenized Fy Protein sample were used for the following microbiological tests:

Total aerobic bacteria - Reported as CFU/gram
Mold and yeast - Reported as CFU/gram
E. coli - Reported as CFU or Not Detected/10 grams
Salmonella - Reported as CFU or Not Detected/25 grams

Please see batch analysis in Appendix B.

# Microbiology: Question 7 (M7)

*"Please provide information on whether absence of viable Fusarium is a specification."*

## M7 Response

Nature's Fynd includes the absence of viable Fusarium as a product specification. Thermal deactivation is employed in the manufacturing process, applying moist heat at >55°C for not less than three minutes, a regimen generally recognized to achieve >12 log reduction in viability for Fusarium (Busschaert et al, 1978). All production batches are tested for the presence of mold and yeast with a product specification of <100 CFU/g. If there is presence of mold, the sample is speciated using PCR/DNA sequencing to confirm it is not *Fusarium strain flavolapis*.

References

Busschaert, S. C., Good, R. C., & Szabocsik, J. (1978). Evaluation of thermal disinfection procedures for hydrophilic contact lenses. *Applied and Environmental Microbiology*, *35*(3), 618–621. https://doi.org/10.1128/AEM.35.3.618-621.1978

## Appendix A

American Type Culture Collection Deposit Certificate

*See Attachment*

## Appendix B

Three non-consecutive batch records of Fy Protein (1091, 1099, 1112)

*See Attachment*

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organization dedicated to biological
standards and biodiversity

IP, Licensing and Services
10801 University Boulevard
Manassas, Virginia 20110-2209 USA
Telephone: (800) 638-6597
Facsimile: (703) 334-2932
Internet: http:.//www.atcc.org

**BUDAPEST RESTRICTED CERTIFICATE OF DEPOSIT**
**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF**
**THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE**
**INTERNATIONAL FORM**
**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3**
**AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2**

The American Type Culture Collection (ATCC®) has received your deposit of seeds/strain(s)/strain(s) in connection with the filing of an application for patent. The following information is provided to fulfill Patent Office requirements.

Rebecca W. Mahurin, Ph.D.
Montana State University
Technology Transfer Office
PO Box 172460
Bozeman, MT 59717-2460

Deposited on Behalf of: **National Park Service, Yellowstone National Park catalog number YELL189801**

Date of Receipt of seeds/strain(s) by the ATCC®: **March 2, 2010**

| Identification Reference by Depositor: | ATCC ®Patent Deposit Designation: | Quantity Received: |
|---|---|---|
| **Fusarium oxysporum: MK7** | **PTA-10698** | **25 vials** |

The ATCC® understands that:

1. The deposit of these seeds/strain(s) does not grant ATCC® a license, either express or implied, to infringe the patent, and our release of these seeds/strain(s) to others does not grant them a license, either express or implied, to infringe the patent.

2. If the deposit should die or be destroyed during the effective term of the patent, it shall be your responsibility to replace it with viable material. It is also your responsibility to supply a sufficient quantity for distribution for the deposit term. ATCC® will distribute and maintain the material for 30 years or 5 years following the most recent request for the deposit, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

Prior to the issuance of a U.S. Patent, the ATCC® agrees in consideration for a one-time service charge, not to distribute these seeds/strain(s) or any information relating thereto or to their deposit except as instructed by the depositor or relevant patent office. After relevant patent issues we are responsible to release the seeds/strain(s) and they will be made available for distribution to the public without any restrictions. We will inform you of requests for the seeds/strain(s) for 30 years from date of deposit.

A global bioscience nonprofit
organization dedicated to biological
standards and biodiversity

IP, Licensing and Services
10801 University Boulevard
Manassas, Virginia 20110-2209 USA
Telephone: (800) 638-6597
Facsimile: (703) 334-2932
Internet: http:.//www.atcc.org

The deposit was tested **March 11, 2010** and on that date, the seeds/strain(s) were viable
International Depository Authority: American Type Culture Collection (ATCC®), Manassas, VA, USA

Signature of person having authority to represent ATCC®:

**March 12, 2010**

ATCC® Patent Depository — Date

cc: Erich Veitenheimer

Ref: Docket or Case No: MONT-114/00US

Eurofins Scientific Inc. (Des Moines)

2200 Rittenhouse Street Suite 150
Des Moines, IA 50321
+1 515 265 1461
ENACClientServices@EurofinsUS.com

The Fynder Group, Inc.

Mark Kozubal
960 Technology Blvd.
Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats

**Received On:** 01Aug2019
**Reported On:** 02Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010420 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1091 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD252 - Protein - Combustion** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 990.03; AOAC 992.15 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Protein | 11.50 % |
| Nitrogen - Combustion | 1.84 % |
| Protein Factor | 6.25 |

| **QD250 - Ash** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 942.05 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 07Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Ash | 0.72 % |

| **QD038 - Carbohydrates, Calculated** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | CFR 21-calc. | ISO/IEC 17025:2017<br>A2LA 2927.01 | 07Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Carbohydrates, Calculated | 10.51 % |

| **QD148 - Moisture by Vacuum Oven** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 925.09 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 05Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Moisture and Volatiles - Vacuum Oven | 76.0 % |

| **QD059 - Fat by Acid Hydrolysis** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 954.02 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|

The Fynder Group, Inc.

Mark Kozubal
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# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 02Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010420 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1091 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD059 - Fat by Acid Hydrolysis** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 954.02 | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Crude Fat By Acid Hydrolysis | 1.48 % |

| **QD06T - Cadmium (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Cadmium (Cd) | <0.010 mg/kg |

| **QD06S - Lead (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Lead (Pb) | 0.011 mg/kg |

| **QD06R - Mercury (Mwd-ICP-MS, Most Matrices)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Mercury (Hg) | <0.010 mg/kg |

| **QD06Q - Arsenic (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Arsenic (As) | <0.010 mg/kg |

| **QA012 - Nitrate (Ion Chromatography)** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | Internal Method based on EN 12014-2 | 13Aug2019 | 3 |

| **Parameter** | **Result** |
|---|---|
| Nitrate (as NO3) | <10 mg/kg |

| **QD01T - Total dietary fiber HPLC (Includes LMWSDF)** | **Reference** | **Completed** |
|---|---|---|
| | AOAC 2009.01 & AOAC 2011.25 | 21Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Low molecular wgt soluble dietary fiber(LMWSDF) | <0.10 % |

The Fynder Group, Inc.

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# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 02Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010420 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1091 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

**QD01T - Total dietary fiber HPLC (Includes LMWSDF)** | **Reference** AOAC 2009.01 & AOAC 2011.25 | **Completed** 21Aug2019

| Parameter | Result |
|---|---|
| High molecular wgt soluble dietary fiber (HMWSDF) | 0.83 % |
| Insoluble dietary fiber (IDF) | 6.77 % |
| Soluble Dietary Fiber (LMWSDF + HMWSDF) | 0.83 % |
| Total dietary fiber (IDF + HMWSDF + LMWSDF) Corrected Result | 7.60 % |

**QQ129 - Sugar Profile (AOAC, Most Matrices)** | **Reference** AOAC 982.14, mod. | **Accreditation** ISO/IEC 17025:2017 A2LA 2927.01 | **Completed** 08Aug2019

| Parameter | Result |
|---|---|
| Fructose | <0.15 % |
| Glucose | <0.15 % |
| Sucrose | <0.15 % |
| Maltose | <0.15 % |
| Lactose | <0.15 % |
| Total sugars | <0.35 % |

**QA16L - Glycerol (Food, GC-MS)** | **Reference** ECAL Internal | **Completed** 13Aug2019 | **Sub** 3

| Parameter | Result |
|---|---|
| Glycerol | <1.00 % (w/w) |

**QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** AOAC 996.06 mod. | **Accreditation** ISO/IEC 17025:2017 A2LA 2927.01 | **Completed** 09Aug2019

| Parameter | Result |
|---|---|
| Fatty Acid Profile | Reported as Fatty Acids |
| C4:0 (Butyric Acid) | <0.02 % |
| C6:0 (Caproic acid) | <0.02 % |
| C8:0 (Caprylic acid) | <0.02 % |
| C10:0 (Capric acid) | <0.02 % |
| C11:0 (Undecanoic acid) | <0.02 % |
| C12:0 (Lauric Acid) | <0.02 % |
| C14:0 (Myristic acid) | <0.02 % |

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# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 02Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010420 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1091 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 996.06 mod. | ISO/IEC 17025:2017<br>A2LA 2927.01 | 09Aug2019 |

| Parameter | Result |
|---|---|
| C14:1 (Myristoleic acid) | <0.02 % |
| C15:0 (Pentadecanoic acid) | <0.02 % |
| C15:1 (Pentadecenoic acid) | <0.02 % |
| C16:0 (Palmitic Acid) | 0.21 % |
| C16:1 Omega 7 | <0.04 % |
| C16:1 Total (Palmitoleic Acid + isomers) | <0.04 % |
| C16:2 (Hexadecadienoic Acid) | <0.02 % |
| C16:3 (Hexadecatrienoic Acid) | <0.02 % |
| C 16:4 (Hexadecatetraenoic Acid) | <0.02 % |
| C17:0 (Margaric Acid) | <0.02 % |
| C17:1 (Heptadecenoic Acid) | <0.02 % |
| C18:0 (Stearic Acid) | 0.07 % |
| C18:1 (Vaccenic acid) | <0.03 % |
| C18:1 Omega 9 (Oleic Acid) | 0.16 % |
| C18:1, Total (Oleic Acid + isomers) | 0.16 % |
| C18:2 Omega 6 (Linoleic Acid) | 0.60 % |
| C18:2, Total (Linoleic Acid + isomers) | 0.63 % |
| C18:3 Omega 3 (Alpha Linolenic Acid) | 0.09 % |
| C18:3 Omega 6 (Gamma Linolenic Acid) | <0.02 % |
| C18:3, Total (Linolenic Acid + isomers) | 0.09 % |
| C18:4 Omega 3 (Octadecatetraenoic Acid) | <0.02 % |
| C18:4 Total (Octadecatetraenoic Acid) | <0.02 % |
| C20:0 (Arachidic Acid) | <0.02 % |
| C20:1 Omega 9 (Gondoic Acid) | <0.02 % |
| C20:1 Total (Gondoic Acid + isomers) | <0.02 % |
| C20:2 Omega 6 | <0.02 % |
| C20:2 Total (Eicosadienoic Acid) | <0.02 % |
| C20:3 Omega 3 | <0.02 % |
| C20:3 Omega 6 | <0.02 % |
| C20:3, Total (Eicosatrienoic Acid) | <0.02 % |
| C20:4 Omega 3 | <0.02 % |
| C20:4 Omega 6 (Arachidonic Acid) | <0.02 % |
| C20:4, Total (Eicosatetraenoic Acid) | <0.02 % |
| C20:5 Omega 3 (Eicosapentaenoic Acid) | <0.02 % |
| C21:5 Omega 3 (Heneicosapentaenoic Acid) | <0.02 % |

The Fynder Group, Inc.

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Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 02Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010420 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1091 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

**QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W**
**Reference:** AOAC 996.06 mod.
**Accreditation:** ISO/IEC 17025:2017 A2LA 2927.01
**Completed:** 09Aug2019

| Parameter | Result |
|---|---|
| C22:0 (Behenic Acid) | <0.02 % |
| C22:1 Omega 9 (Erucic Acid) | <0.02 % |
| C22:1 Total (Erucic Acid + isomers) | <0.02 % |
| C22:2 Docosadienoic Omega 6 | <0.02 % |
| C22:3 Docosatrienoic, Omega 3 | <0.02 % |
| C22:4 Docosatetraenoic Omega 6 | <0.02 % |
| C22:5 Docosapentaenoic Omega 3 | <0.02 % |
| C22:5 Docosapentaenoic Omega 6 | <0.02 % |
| C22:5 Total (Docosapentaenoic Acid) | <0.02 % |
| C22:6 Docosahexaenoic Omega 3 | <0.02 % |
| C24:0 (Lignoceric Acid) | <0.02 % |
| C24:1 Omega 9 (Nervonic Acid) | <0.02 % |
| C24:1 Total (Nervonic Acid + isomers) | <0.02 % |
| Total Omega 3 Isomers | 0.10 % |
| Total Omega 5 Isomers | <0.05 % |
| Total Omega 6 Isomers | 0.61 % |
| Total Omega 7 Isomers | <0.05 % |
| Total Omega 9 Isomers | 0.16 % |
| Total Monounsaturated Fatty Acids | 0.18 % |
| Total Polyunsaturated Fatty Acids | 0.72 % |
| Total Saturated Fatty Acids | 0.31 % |
| Total Trans Fatty Acids | <0.02 % |
| Total Fat as Triglycerides | 1.27 % |
| Total Fatty Acids | 1.21 % |

**UM7MY - Total Aerobic Microbial Count - USP Chapter <61>**
**Reference:** U.S. Pharmacopeia Chapter 61
**Completed:** 09Aug2019
**Sub:** 2

| Parameter | Result |
|---|---|
| Total Aerobic Microbial Count | 2,500 cfu/g |

**UMAEK - Salmonella - AOAC 2003.09**
**Reference:** AOAC 2003.09
**Accreditation:** ISO/IEC 17025:2017 A2LA 3329.01
**Completed:** 09Aug2019
**Sub:** 2

| Parameter | Result |
|---|---|
| Salmonella spp. | Not Detected /25 g |

The Fynder Group, Inc.

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Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 02Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010420 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1091 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **UMIZH - Confirmation Escherichia Coli - USP Chapter <62>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 62 | 02Oct2020 | 2 |

| **Parameter** | **Result** |
|---|---|
| E. coli | Not Detected /10 g |

| **UMR5L - Moulds - USP Chapter <61>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 61 | 09Aug2019 | 2 |

| **Parameter** | **Result** |
|---|---|
| Mold | < 10 cfu/g |
| **Parameter** | **Result** |
| Yeast | < 10 cfu/g |
| **Parameter** | **Result** |
| Yeast & Moulds | < 10 cfu/g |

**Comments:**
Analytical Report set to display only tests for UM7MY: Total Aerobic Microbial Count, UMR5L : Yeast and Mold, UMIZH : Test for absence of Escherichia coli, UMAEK : Salmonella, QD252 : Protein - combustion, QD148 : Moisture by vacuum oven, QQ129 : Sugar Profile, QA16L : Glycerol, QD01T : Total Dietary Fiber HPLC, QD038 : Carbohydrates, calculated, QD059 : Fat by Acid Hydrolysis, QD05C : Fatty Acids - Full, QD250 : AshQD06T : Cadmium, QD06S : Lead, QD06R : Mercury, QD06Q : Arsenic, QA012 : Nitrate - Ion chromatography, per client request.

Subcontracting partners:

1 - Eurofins GeneScan, LA
2 - Eurofins Microbiology Laboratories (Des Moines), IA
3 - Eurofins Central Analytical Laboratories, LA

Respectfully Submitted,

ACCREDITED

David Gross
Support Services Manager

The Fynder Group, Inc.

Mark Kozubal
960 Technology Blvd.
Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-125245-03
Report Supersedes AR-19-QD-125245-02

**Client Code:** [redacted]
**PO Number:** Mats

**Received On:** 01Aug2019
**Reported On:** 02Oct2020

Eurofins Scientific Inc. (Des Moines)

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The Fynder Group, Inc.

Mark Kozubal
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Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats

**Received On:** 01Aug2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010422 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1099 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD252 - Protein - Combustion** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 990.03; AOAC 992.15 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Protein | 11.75 % |
| Nitrogen - Combustion | 1.88 % |
| Protein Factor | 6.25 |

| **QD250 - Ash** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 942.05 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 07Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Ash | 0.67 % |

| **QD038 - Carbohydrates, Calculated** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | CFR 21-calc. | ISO/IEC 17025:2017<br>A2LA 2927.01 | 07Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Carbohydrates, Calculated | 9.97 % |

| **QD148 - Moisture by Vacuum Oven** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 925.09 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 05Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Moisture and Volatiles - Vacuum Oven | 76.5 % |

| **QD059 - Fat by Acid Hydrolysis** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 954.02 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|

The Fynder Group, Inc.

Mark Kozubal
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Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010422 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1099 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD059 - Fat by Acid Hydrolysis** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 954.02 | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Crude Fat By Acid Hydrolysis | 1.63 % |

| **QD06T - Cadmium (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Cadmium (Cd) | <0.010 mg/kg |

| **QD06S - Lead (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Lead (Pb) | <0.010 mg/kg |

| **QD06R - Mercury (Mwd-ICP-MS, Most Matrices)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Mercury (Hg) | <0.010 mg/kg |

| **QD06Q - Arsenic (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Arsenic (As) | <0.010 mg/kg |

| **QA012 - Nitrate (Ion Chromatography)** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | Internal Method based on EN 12014-2 | 13Aug2019 | 3 |

| **Parameter** | **Result** |
|---|---|
| Nitrate (as NO3) | <10 mg/kg |

| **QD01T - Total dietary fiber HPLC (Includes LMWSDF)** | **Reference** | **Completed** |
|---|---|---|
| | AOAC 2009.01 & AOAC 2011.25 | 19Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Low molecular wgt soluble dietary fiber(LMWSDF) | 0.11 % |

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# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010422 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1099 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

**QD01T - Total dietary fiber HPLC (Includes LMWSDF)** | **Reference** AOAC 2009.01 & AOAC 2011.25 | **Completed** 19Aug2019

| Parameter | Result |
|---|---|
| High molecular wgt soluble dietary fiber (HMWSDF) | 0.60 % |
| Insoluble dietary fiber (IDF) | 6.40 % |
| Soluble Dietary Fiber (LMWSDF + HMWSDF) | 0.71 % |
| Total dietary fiber (IDF + HMWSDF + LMWSDF) | 7.11 % |

**QQ129 - Sugar Profile (AOAC, Most Matrices)** | **Reference** AOAC 982.14, mod. | **Accreditation** ISO/IEC 17025:2017 A2LA 2927.01 | **Completed** 08Aug2019

| Parameter | Result |
|---|---|
| Fructose | <0.15 % |
| Glucose | <0.15 % |
| Sucrose | <0.15 % |
| Maltose | <0.15 % |
| Lactose | <0.15 % |
| Total sugars | <0.35 % |

**QA16L - Glycerol (Food, GC-MS)** | **Reference** ECAL Internal | **Completed** 13Aug2019 | **Sub** 3

| Parameter | Result |
|---|---|
| Glycerol | <1.00 % (w/w) |

**QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** AOAC 996.06 mod. | **Accreditation** ISO/IEC 17025:2017 A2LA 2927.01 | **Completed** 09Aug2019

| Parameter | Result |
|---|---|
| Fatty Acid Profile | Reported as Fatty Acids |
| C4:0 (Butyric Acid) | <0.02 % |
| C6:0 (Caproic acid) | <0.02 % |
| C8:0 (Caprylic acid) | <0.02 % |
| C10:0 (Capric acid) | <0.02 % |
| C11:0 (Undecanoic acid) | <0.02 % |
| C12:0 (Lauric Acid) | <0.02 % |
| C14:0 (Myristic acid) | <0.02 % |
| C14:1 (Myristoleic acid) | <0.02 % |

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# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010422 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1099 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 996.06 mod. | ISO/IEC 17025:2017<br>A2LA 2927.01 | 09Aug2019 |

| Parameter | Result |
|---|---|
| C15:0 (Pentadecanoic acid) | <0.02 % |
| C15:1 (Pentadecenoic acid) | <0.02 % |
| C16:0 (Palmitic Acid) | 0.16 % |
| C16:1 Omega 7 | <0.04 % |
| C16:1 Total (Palmitoleic Acid + isomers) | <0.04 % |
| C16:2 (Hexadecadienoic Acid) | <0.02 % |
| C16:3 (Hexadecatrienoic Acid) | <0.02 % |
| C 16:4 (Hexadecatetraenoic Acid) | <0.02 % |
| C17:0 (Margaric Acid) | <0.02 % |
| C17:1 (Heptadecenoic Acid) | <0.02 % |
| C18:0 (Stearic Acid) | 0.05 % |
| C18:1 (Vaccenic acid) | <0.03 % |
| C18:1 Omega 9 (Oleic Acid) | 0.13 % |
| C18:1, Total (Oleic Acid + isomers) | 0.13 % |
| C18:2 Omega 6 (Linoleic Acid) | 0.58 % |
| C18:2, Total (Linoleic Acid + isomers) | 0.59 % |
| C18:3 Omega 3 (Alpha Linolenic Acid) | 0.08 % |
| C18:3 Omega 6 (Gamma Linolenic Acid) | <0.02 % |
| C18:3, Total (Linolenic Acid + isomers) | 0.09 % |
| C18:4 Omega 3 (Octadecatetraenoic Acid) | <0.02 % |
| C18:4 Total (Octadecatetraenoic Acid) | <0.02 % |
| C20:0 (Arachidic Acid) | <0.02 % |
| C20:1 Omega 9 (Gondoic Acid) | <0.02 % |
| C20:1 Total (Gondoic Acid + isomers) | <0.02 % |
| C20:2 Omega 6 | <0.02 % |
| C20:2 Total (Eicosadienoic Acid) | <0.02 % |
| C20:3 Omega 3 | <0.02 % |
| C20:3 Omega 6 | <0.02 % |
| C20:3, Total (Eicosatrienoic Acid) | <0.02 % |
| C20:4 Omega 3 | <0.02 % |
| C20:4 Omega 6 (Arachidonic Acid) | <0.02 % |
| C20:4, Total (Eicosatetraenoic Acid) | <0.02 % |
| C20:5 Omega 3 (Eicosapentaenoic Acid) | <0.02 % |
| C21:5 Omega 3 (Heneicosapentaenoic Acid) | <0.02 % |
| C22:0 (Behenic Acid) | <0.02 % |

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# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010422 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1099 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

**QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W**
**Reference:** AOAC 996.06 mod.
**Accreditation:** ISO/IEC 17025:2017 A2LA 2927.01
**Completed:** 09Aug2019

| Parameter | Result |
|---|---|
| C22:1 Omega 9 (Erucic Acid) | <0.02 % |
| C22:1 Total (Erucic Acid + isomers) | <0.02 % |
| C22:2 Docosadienoic Omega 6 | <0.02 % |
| C22:3 Docosatrienoic, Omega 3 | <0.02 % |
| C22:4 Docosatetraenoic Omega 6 | <0.02 % |
| C22:5 Docosapentaenoic Omega 3 | <0.02 % |
| C22:5 Docosapentaenoic Omega 6 | <0.02 % |
| C22:5 Total (Docosapentaenoic Acid) | <0.02 % |
| C22:6 Docosahexaenoic Omega 3 | <0.02 % |
| C24:0 (Lignoceric Acid) | <0.02 % |
| C24:1 Omega 9 (Nervonic Acid) | <0.02 % |
| C24:1 Total (Nervonic Acid + isomers) | <0.02 % |
| Total Omega 3 Isomers | 0.09 % |
| Total Omega 5 Isomers | <0.05 % |
| Total Omega 6 Isomers | 0.59 % |
| Total Omega 7 Isomers | <0.05 % |
| Total Omega 9 Isomers | 0.13 % |
| Total Monounsaturated Fatty Acids | 0.14 % |
| Total Polyunsaturated Fatty Acids | 0.68 % |
| Total Saturated Fatty Acids | 0.24 % |
| Total Trans Fatty Acids | <0.02 % |
| Total Fat as Triglycerides | 1.11 % |
| Total Fatty Acids | 1.06 % |

**UM7MY - Total Aerobic Microbial Count - USP Chapter <61>**
**Reference:** U.S. Pharmacopeia Chapter 61
**Completed:** 09Aug2019
**Sub:** 2

| Parameter | Result |
|---|---|
| Total Aerobic Microbial Count | 4,100 cfu/g |

**UMAEK - Salmonella - AOAC 2003.09**
**Reference:** AOAC 2003.09
**Accreditation:** ISO/IEC 17025:2017 A2LA 3329.01
**Completed:** 09Aug2019
**Sub:** 2

| Parameter | Result |
|---|---|
| Salmonella spp. | Not Detected /25 g |

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# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010422 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1099 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **UMIZH - Confirmation Escherichia Coli - USP Chapter <62>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 62 | 01Oct2020 | 2 |

| **Parameter** | **Result** |
|---|---|
| E. coli | Not Detected /10 g |

| **UMR5L - Moulds - USP Chapter <61>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 61 | 09Aug2019 | 2 |

| **Parameter** | **Result** |
|---|---|
| Mold | < 10 cfu/g |

| **Parameter** | **Result** |
|---|---|
| Yeast | < 10 cfu/g |

| **Parameter** | **Result** |
|---|---|
| Yeast & Moulds | < 10 cfu/g |

**Comments:**

Analyttcal Reportt sett tto display only ttestts fior UMY: Tottal Aerobic Microbial Counţt UMR5L : Yeastt and Mold UMIZH : Testt fior absence ofi Escherichia colj UMAEK : Salmonella, QD252 : Prottein- combustton, QD148 : Moistture by vacuum oven QQ129 : Sugar Profile, QA16L : Glycerol, QD01T : Tottal Diettary Fiber HPLC QD038 : Carbohydratteş calculatted QD059 : Fatt by Acid Hydrolysis, QD05C : Fatty Acids - Full, QD250 : AshQD06T : Cadmium, QD06S : Lead, QD06R : Mercury, QD06Q : Arsenic, QA012 : Nittratte Ion chromattography per clientt requestt

Subcontracting partners:

1 - Eurofins GeneScan, LA
2 - Eurofins Microbiology Laboratories (Des Moines), IA
3 - Eurofins Central Analytical Laboratories, LA

Respectfully Submitted,

ACCREDITED

David Gross
Support Services Manager

The Fynder Group, Inc.

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960 Technology Blvd.
Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-127008-02
Report Supersedes AR-19-QD-127008-01

**Client Code:** [redacted]
**PO Number:** Mats

**Received On:** 01Aug2019
**Reported On:** 01Oct2020

Eurofins Scientific Inc. (Des Moines)

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# ANALYTICAL REPORT

AR-19-QD-127010-02
Report Supersedes AR-19-QD-127010-01

**Client Code:** [redacted]
**PO Number:** Mats

**Received On:** 01Aug2019
**Reported On:** 06Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010424 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1112 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD252 - Protein - Combustion** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 990.03; AOAC 992.15 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 02Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Protein | 11.69 % |
| Nitrogen - Combustion | 1.87 % |
| Protein Factor | 6.25 |

| **QD250 - Ash** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 942.05 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 02Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Ash | 0.68 % |

| **QD038 - Carbohydrates, Calculated** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | CFR 21-calc. | ISO/IEC 17025:2017<br>A2LA 2927.01 | 07Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Carbohydrates, Calculated | 9.33 % |

| **QD148 - Moisture by Vacuum Oven** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 925.09 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 03Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Moisture and Volatiles - Vacuum Oven | 77.2 % |

| **QD059 - Fat by Acid Hydrolysis** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 954.02 | ISO/IEC 17025:2017<br>A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|

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# ANALYTICAL REPORT

AR-19-QD-127010-02
Report Supersedes AR-19-QD-127010-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 06Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010424 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1112 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD059 - Fat by Acid Hydrolysis** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 954.02 | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Crude Fat By Acid Hydrolysis | 0.81 % |

| **QD06T - Cadmium (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Cadmium (Cd) | <0.010 mg/kg |

| **QD06S - Lead (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Lead (Pb) | <0.010 mg/kg |

| **QD06R - Mercury (Mwd-ICP-MS, Most Matrices)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Mercury (Hg) | <0.010 mg/kg |

| **QD06Q - Arsenic (Mwd-ICP-MS)** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | J. AOAC vol. 90 (2007) 844-856 (Mod) | ISO/IEC 17025:2017 A2LA 2927.01 | 06Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Arsenic (As) | <0.010 mg/kg |

| **QA012 - Nitrate (Ion Chromatography)** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | Internal Method based on EN 12014-2 | 12Aug2019 | 2 |

| **Parameter** | **Result** |
|---|---|
| Nitrate (as NO3) | 18 mg/kg |

| **QD01T - Total dietary fiber HPLC (Includes LMWSDF)** | **Reference** | **Completed** |
|---|---|---|
| | AOAC 2009.01 & AOAC 2011.25 | 19Aug2019 |

| **Parameter** | **Result** |
|---|---|
| Low molecular wgt soluble dietary fiber(LMWSDF) | <0.10 % |

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# ANALYTICAL REPORT

AR-19-QD-127010-02
Report Supersedes AR-19-QD-127010-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 06Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010424 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1112 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

**QD01T - Total dietary fiber HPLC (Includes LMWSDF)** | **Reference** AOAC 2009.01 & AOAC 2011.25 | **Completed** 19Aug2019

| Parameter | Result |
|---|---|
| High molecular wgt soluble dietary fiber (HMWSDF) | 0.55 % |
| Insoluble dietary fiber (IDF) | 6.65 % |
| Soluble Dietary Fiber (LMWSDF + HMWSDF) | 0.55 % |
| Total dietary fiber (IDF + HMWSDF + LMWSDF) | 7.20 % |

**QQ129 - Sugar Profile (AOAC, Most Matrices)** | **Reference** AOAC 982.14, mod. | **Accreditation** ISO/IEC 17025:2017 A2LA 2927.01 | **Completed** 06Aug2019

| Parameter | Result |
|---|---|
| Fructose | <0.15 % |
| Glucose | <0.15 % |
| Sucrose | <0.15 % |
| Maltose | <0.15 % |
| Lactose | 0.49 % |
| Total sugars | 0.49 % |

**QA16L - Glycerol (Food, GC-MS)** | **Reference** ECAL Internal | **Completed** 12Aug2019 | **Sub** 2

| Parameter | Result |
|---|---|
| Glycerol | <1.00 % (w/w) |

**QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** AOAC 996.06 mod. | **Accreditation** ISO/IEC 17025:2017 A2LA 2927.01 | **Completed** 09Aug2019

| Parameter | Result |
|---|---|
| Fatty Acid Profile | Reported as Fatty Acids |
| C4:0 (Butyric Acid) | <0.02 % |
| C6:0 (Caproic acid) | <0.02 % |
| C8:0 (Caprylic acid) | <0.02 % |
| C10:0 (Capric acid) | <0.02 % |
| C11:0 (Undecanoic acid) | <0.02 % |
| C12:0 (Lauric Acid) | <0.02 % |
| C14:0 (Myristic acid) | <0.02 % |
| C14:1 (Myristoleic acid) | <0.02 % |

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# ANALYTICAL REPORT

AR-19-QD-127010-02
Report Supersedes AR-19-QD-127010-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 06Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010424 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1112 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 996.06 mod. | ISO/IEC 17025:2017 A2LA 2927.01 | 09Aug2019 |

| Parameter | Result |
|---|---|
| C15:0 (Pentadecanoic acid) | <0.02 % |
| C15:1 (Pentadecenoic acid) | <0.02 % |
| C16:0 (Palmitic Acid) | 0.17 % |
| C16:1 Omega 7 | <0.04 % |
| C16:1 Total (Palmitoleic Acid + isomers) | <0.04 % |
| C16:2 (Hexadecadienoic Acid) | <0.02 % |
| C16:3 (Hexadecatrienoic Acid) | <0.02 % |
| C 16:4 (Hexadecatetraenoic Acid) | <0.02 % |
| C17:0 (Margaric Acid) | <0.02 % |
| C17:1 (Heptadecenoic Acid) | <0.02 % |
| C18:0 (Stearic Acid) | 0.05 % |
| C18:1 (Vaccenic acid) | <0.03 % |
| C18:1 Omega 9 (Oleic Acid) | 0.12 % |
| C18:1, Total (Oleic Acid + isomers) | 0.13 % |
| C18:2 Omega 6 (Linoleic Acid) | 0.56 % |
| C18:2, Total (Linoleic Acid + isomers) | 0.57 % |
| C18:3 Omega 3 (Alpha Linolenic Acid) | 0.09 % |
| C18:3 Omega 6 (Gamma Linolenic Acid) | <0.02 % |
| C18:3, Total (Linolenic Acid + isomers) | 0.10 % |
| C18:4 Omega 3 (Octadecatetraenoic Acid) | <0.02 % |
| C18:4 Total (Octadecatetraenoic Acid) | <0.02 % |
| C20:0 (Arachidic Acid) | <0.02 % |
| C20:1 Omega 9 (Gondoic Acid) | <0.02 % |
| C20:1 Total (Gondoic Acid + isomers) | <0.02 % |
| C20:2 Omega 6 | <0.02 % |
| C20:2 Total (Eicosadienoic Acid) | <0.02 % |
| C20:3 Omega 3 | <0.02 % |
| C20:3 Omega 6 | <0.02 % |
| C20:3, Total (Eicosatrienoic Acid) | <0.02 % |
| C20:4 Omega 3 | <0.02 % |
| C20:4 Omega 6 (Arachidonic Acid) | <0.02 % |
| C20:4, Total (Eicosatetraenoic Acid) | <0.02 % |
| C20:5 Omega 3 (Eicosapentaenoic Acid) | <0.02 % |
| C21:5 Omega 3 (Heneicosapentaenoic Acid) | <0.02 % |
| C22:0 (Behenic Acid) | <0.02 % |

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# ANALYTICAL REPORT

AR-19-QD-127010-02
Report Supersedes AR-19-QD-127010-01

**Client Code:** [redacted]
**PO Number:** Mats
**Received On:** 01Aug2019
**Reported On:** 06Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-08010424 | **Sample Registration Date:** | 01Aug2019 |
| **Client Sample Code:** | 1112 | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | Mat | **Sample Reference:** | |

| **QD05C - Fatty Acids-Full Omega 9,6&3 & Trans %W/W** | **Reference** | **Accreditation** | **Completed** |
|---|---|---|---|
| | AOAC 996.06 mod. | ISO/IEC 17025:2017 A2LA 2927.01 | 09Aug2019 |

| Parameter | Result |
|---|---|
| C22:1 Omega 9 (Erucic Acid) | <0.02 % |
| C22:1 Total (Erucic Acid + isomers) | <0.02 % |
| C22:2 Docosadienoic Omega 6 | <0.02 % |
| C22:3 Docosatrienoic, Omega 3 | <0.02 % |
| C22:4 Docosatetraenoic Omega 6 | <0.02 % |
| C22:5 Docosapentaenoic Omega 3 | <0.02 % |
| C22:5 Docosapentaenoic Omega 6 | <0.02 % |
| C22:5 Total (Docosapentaenoic Acid) | <0.02 % |
| C22:6 Docosahexaenoic Omega 3 | <0.02 % |
| C24:0 (Lignoceric Acid) | <0.02 % |
| C24:1 Omega 9 (Nervonic Acid) | <0.02 % |
| C24:1 Total (Nervonic Acid + isomers) | <0.02 % |
| Total Omega 3 Isomers | 0.10 % |
| Total Omega 5 Isomers | <0.05 % |
| Total Omega 6 Isomers | 0.56 % |
| Total Omega 7 Isomers | <0.05 % |
| Total Omega 9 Isomers | 0.13 % |
| Total Monounsaturated Fatty Acids | 0.13 % |
| Total Polyunsaturated Fatty Acids | 0.67 % |
| Total Saturated Fatty Acids | 0.25 % |
| Total Trans Fatty Acids | <0.02 % |
| Total Fat as Triglycerides | 1.10 % |
| Total Fatty Acids | 1.05 % |

**Comments:**

Analyttcal Reportt sett tto display only ttestts fio2QD: Prottein- combustton, QD148 : Moistture by vacuum oven, QQ129 : Sugar Profile, QA16L : Glycerol, QD01T : Tottal Diettary Fiber HPLC, QD038 : Carbohydrattes, calculatted, QD059 : Fatt by Acid Hydrolysis, QD05C : Fatty Acids - Full, QD250 : AshQD06T : Cadmium, QD06S : Lead, QD06R : Mercury, QD06Q : Arsenic, QA012 : Nittratte Ion chromattography per clientt requestt

Subcontracting partners:

1 - Eurofins GeneScan, LA
2 - Eurofins Central Analytical Laboratories, LA

The Fynder Group, Inc.

Mark Kozubal
960 Technology Blvd.
Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-127010-02
Report Supersedes AR-19-QD-127010-01

**Client Code:** [redacted]
**PO Number:** Mats

**Received On:** 01Aug2019
**Reported On:** 06Oct2020

Respectfully Submitted,

David Gross
Support Services Manager

Eurofins Scientific Inc. (Des Moines)

2200 Rittenhouse Street Suite 150
Des Moines, IA 50321
+1 515 265 1461
ENACClientServices@EurofinsUS.com

The Fynder Group, Inc.

Mark Kozubal
960 Technology Blvd.
Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-102631-02
Report Supersedes AR-19-QD-102631-01

**Client Code:** [redacted]
**PO Number:** Micro

**Received On:** 03Jul2019
**Reported On:** 01Oct2020

| | | | |
|---|---|---|---|
| **Eurofins Sample Code:** | 464-2019-07030733 | **Sample Registration Date:** | 03Jul2019 |
| **Client Sample Code:** | 1112 S3 Mat | **Condition Upon Receipt:** | acceptable, non-perishable |
| **Sample Description:** | 1112 S3 Mat.32321 | **Sample Reference:** | |

| **UM7MY - Total Aerobic Microbial Count - USP Chapter <61>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 61 | 11Jul2019 | 1 |

| **Parameter** | **Result** |
|---|---|
| Total Aerobic Microbial Count | 2,000 cfu/g |

| **UMAEK - Salmonella - AOAC 2003.09** | **Reference** | **Accreditation** | **Completed** | **Sub** |
|---|---|---|---|---|
| | AOAC 2003.09 | ISO/IEC 17025:2017<br>A2LA 3329.01 | 11Jul2019 | 1 |

| **Parameter** | **Result** |
|---|---|
| Salmonella spp. | Not Detected /25 g |

| **UMIZH - Confirmation Escherichia Coli - USP Chapter <62>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 62 | 01Oct2020 | 1 |

| **Parameter** | **Result** |
|---|---|
| E. coli | Not Detected /10 g |

| **UMR5L - Moulds - USP Chapter <61>** | **Reference** | **Completed** | **Sub** |
|---|---|---|---|
| | U.S. Pharmacopeia Chapter 61 | 11Jul2019 | 1 |

| **Parameter** | **Result** |
|---|---|
| Mold | <10 cfu/g |
| **Parameter** | **Result** |
| Yeast | <10 cfu/g |
| **Parameter** | **Result** |
| Yeast & Moulds | <10 cfu/g |

**Comments:**
Analytcal Report set to display only tests for UM 7MY: Total Aerobic Microbial Count, UMR5L : Yeast and Mold, UMIZH: Test for absence of Escherichia coli, UMAEK : Salmonella, per client request.

The Fynder Group, Inc.

Mark Kozubal
960 Technology Blvd.
Bozeman, MT 59718

# ANALYTICAL REPORT

AR-19-QD-102631-02
Report Supersedes AR-19-QD-102631-01

**Client Code:** [redacted]
**PO Number:** Micro

**Received On:** 03Jul2019
**Reported On:** 01Oct2020

Subcontracting partners:
1 - Eurofins Microbiology Laboratories (Des Moines), IA

Respectfully Submitted,

David Gross
Support Services Manager

## Viebrock, Lauren

**From:** Baughan, Joan <jbaughan@Steptoe.com>
**Sent:** Friday, December 11, 2020 10:10 AM
**To:** Viebrock, Lauren
**Cc:** Attwood, Deborah
**Subject:** RE: GRN 000904
**Attachments:** FDA_NF name change_signed-c2.pdf

Dear Lauren,

Thank you very much for the feedback. We understand that FDA will not replace the version of GRAS Notice 904 currently posted on the online inventory of GRAS Notifications with an updated version, and confirm that Nature's Fynd wishes to have FDA continue its evaluation of GRN 904. We appreciate that FDA updated the Inventory listing details page for GRN 904 with the company's new name and address, and that FDA's response letter to GRN 904 will reflect these changes as well as the change in the substance name.

As per your request, we are attaching a letter on Nature's Fynd letterhead, signed by the company's Director of Regulatory Affairs, indicating the name change of the company and its current mailing address. The letter also informs FDA of the status of the binomial name determination for the microorganism and the intended common or usual name for the finished ingredient (we understand that the Office of Food Additive Safety (OFAS) does not make any determination as to the appropriate common or usual name for a substance under its review). We understand and appreciate that this letter will be reflected on the details page for the online listing of GRN 904.

Finally, as we are already into the holiday season and considering that FDA's 90-day extension for its review of GRN 904 is scheduled to conclude shortly thereafter, on January 17, 2021, we wanted to confirm that Nature's Fynd will be fully available to respond to any questions from FDA during this period and that FDA should not hesitate to contact us if we can provide any further information to help the agency complete its review.

We wish you the very best for the holidays and thank you for your continued assistance.

Kind regards,

Joan

**Joan Baughan**
Partner
jbaughan@Steptoe.com

Steptoe

815 W Pershing Road,
Suite #4, Chicago, IL 60609

www.NaturesFynd.com

December 8, 2020

**Via email**

Ms. Lauren VieBrock
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

**Re: Update to Administrative information for GRN 904**

Dear Ms. VieBrock,

This letter follows up on your December 3, 2020 email requesting that we provide you with a letter officially notifying the agency of administrative changes to: (1) the Company name and updated address; (2) the scientific name of the organism referenced in GRN 904; and (3) the ingredient name referenced in GRN 904.

First, on May 1, 2020, Sustainable Bioproducts, Inc. changed its legal name to "The Fynder Group, Inc." The Company is now doing business as "Nature's Fynd." The Company address has changed to:

**815 W Pershing Rd, Suite #4**
**Chicago, IL 60609**

Accordingly, we request that you update FDA's internal records to reflect the legal name of the Company, and use the "Nature's Fynd" business name as the identified Notifier for GRN 904 on FDA's GRN inventory.

815 W Pershing Road,
Suite #4, Chicago, IL 60609

www.NaturesFynd.com

Second, Nature's Fynd continues in the process of establishing a binomial name for the microorganism that is the subject of GRAS No. 904.[1] As you know, GRN No. 904 utilized the name *Fusarium novum. yellowstonensis* to indicate that the microorganism is of the Fusarium genus but a novel species identified in Yellowstone National Park. You may be aware that it can take some time to establish the appropriate taxonomic classification of a novel microorganism to finalize the species name. Through this process, Nature's Fynd has determined that the prevalence of species using *yellowstonensis* in the name presents a risk of scientific and consumer confusion. *Flavolapis*, by contrast, would be a unique species name. Because the species designation process is not yet complete, however, the microorganism currently is being referred to as *Fusarium strain flavolapis*, where "strain" serves the same purpose that "novum" previously served, i.e., as a placeholder to indicate a future new species designation. Because *Fusarium flavolapis* is unique, there will be no need to include a strain designation once the species has been established.

Finally, Nature's Fynd plans to use the name "Fy Protein™ (Nutritional Fungi Protein)" for the ingredient made from fermented *Fusarium strain flavolapis*, replacing "Fermented Microbial Protein" in GRN 904 submitted on January 14, 2020. Nature's Fynd considers this name to more fully and accurately describe the ingredient.

We appreciate your assistance with this matter. Please do not hesitate to contact us with any questions or concerns.

**Sincerely,**

**Brian Furey**
Director of Regulatory Affairs
Nature's Fynd

[1] As indicated in GRN 904, *Fusarium flavolapis*, identified as "*Fusarium oxysporum*: MK7," was deposited with the ATCC (American Type Culture Collection) on March 2, 2010 and assigned reference no. PTA-10698. This name reflected the information available to Nature's Fynd at the time regarding the classification of the novel microorganism, but the ATCC reference number remains applicable to *Fusarium flavolapis*.

## Viebrock, Lauren

**From:** Baughan, Joan <jbaughan@Steptoe.com>
**Sent:** Friday, February 19, 2021 2:43 PM
**To:** Viebrock, Lauren
**Subject:** [EXTERNAL] RE: GRN 904 Question
**Attachments:** CoverLetter_ResponseToFDA16Feb2021Question-c2.pdf; GRN904 _ResponseToFDA16Feb2021Question-Final-c2.pdf



Dear Lauren,

Thank you for providing us with the Agency's additional question on GRAS Notice 904. Our response is attached. Should you have any further questions or comments, please let us know, preferably by telephone or email so that we may reply as quickly as possible.

Kind regards,

Joan

**Joan Baughan**
Partner
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Steptoe

+1 202 429 6417 direct
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Steptoe & Johnson LLP
1330 Connecticut Avenue, NW
Washington, DC 20036
www.steptoe.com



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**From:** Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
**Sent:** Tuesday, February 16, 2021 2:45 PM
**To:** Baughan, Joan <jbaughan@Steptoe.com>
**Subject:** GRN 904 Question

Dear Ms. Baughan,

During our review of GRAS Notice No. 000904, we noted an additional question that need to be addressed. Please find the question attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

**Lauren VieBrock**
*Regulatory Review Scientist/Microbiology Reviewer*

**Center for Food Safety and Applied Nutrition**
**Office of Food Additive Safety**
**U.S. Food and Drug Administration**
Tel: 301-796-7454
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Steptoe

February 19, 2021

Via EMAIL

Ms. Lauren VieBrock
Regulatory Review Scientist/Microbiology Reviewer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
5001 Campus Drive
College Park, Maryland 20740

Re: **Response to FDA's February 16, 2021 Question Regarding GRAS Notice 904**

Dear Ms. Viebrock,

The purpose of this letter and its enclosure, submitted on behalf of Nature's Fynd, is to respond to your February 16, 2021, correspondence regarding Nature's Fynd's Generally Recognized as Safe (GRAS) Notice GRN 904, which advises the Food and Drug Administration (FDA) as to Nature's Fynd's conclusion that Fy Protein™ (Nutritional Fungi Protein) ("Fy Protein") is GRAS for use as a food ingredient and macronutrient.

Your February 16, 2021, correspondence asked Nature's Fynd to provide narrative to support its conclusion in GRN 904 that it is very unlikely that usual intakes of Fy Protein™ would result in adverse gastrointestinal (GI) effects. As described in the attached response, Nature's Fynd concludes that Fy Protein™ is well-tolerated upon consumption, as demonstrated by the absence of major GI effects from a significant number of Fy Protein™ tastings and supported by the low rate of GI effects for intakes of the analogous product in GRN 91.

We look forward to receiving a letter from the Agency acknowledging receipt of this response. Please contact us by telephone or e-mail if there are any questions regarding this reply so that we can respond right away.

With kind regards,

Joan Sylvain Baughan

Enclosure

# FDA Question Response

GRN 904

Nature's Fynd
(The Fynder Group, Inc.)

February 19, 2021

## Question

*"On page 41 of GRN 904, you discuss reports of adverse gastrointestinal effects from consumption of a similar product. Please provide narrative to elaborate on your conclusion that it is very unlikely that usual intakes of your ingredient would result in adverse gastrointestinal effects."*

We confirm that it is very unlikely that usual intake of Fy Protein, as indicated in our GRAS Notice (GRN 904), would result in adverse gastrointestinal (GI) effects. As discussed in detail in GRN 904 and our October 7, 2020 response to your questions, the general recognition of safety of Fy Protein is supported by the history of consumption of mycoprotein (the similar product identified on page 41 of GRN 904) because, in brief, both products are from the same genus of Fusarium and contain similar nutritional compositions with high levels of protein (~45% dry wt) and fiber (25-35% dry wt).

The referenced reports on adverse gastrointestinal effects from consumption of the similar product, mycoprotein, are self-reported and incredibly rare. There is a long history of safe consumption of mycoprotein and significant evidence that mycoprotein is well tolerated.

Nature's Fynd has completed several hundred tastings and consumption trials of fungi-based protein from fermented *Fusarium strain flavolapis* (a/k/a Fusarium sp. mycelia), the subject of GRN 904. The tastings ranged from informal research and development evaluations to formal third-party marketing and multi-day culinary events. There have been no reported instances of major GI issues, such as diarrhea or vomiting, and the only anecdotal indications of minor GI discomfort, such as bloating, have been consistent with those experienced by individuals with known fiber intolerance that then consume large amounts of fiber.

Based upon the absence of major GI effects from a significant number of tastings and supported by the low rate of GI effects for intakes of the analogous product in GRN 91, Nature's Fynd concludes that usual intakes of Fy Protein are very unlikely to result in adverse GI effects and that the product is well-tolerated upon consumption.