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APPLICATION TO AMEND THE *AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE* TO ALLOW FOR THE USE OF *LENTINULA EDODES* (SHIITAKE MUSHROOM) MYCELIA AS A PROCESSING AID (STANDARD 1.3.3)

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Application to Amend the *Australia and New Zealand Food Standards Code* to Allow for the Use of *Lentinula edodes* (Shiitake Mushroom) Mycelia as a Processing Aid (Standard 1.3.3)

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Application to Amend the *Australia and New Zealand Food Standards Code* to Allow for the Use of *Lentinula edodes* (Shiitake Mushroom) Mycelia as a Processing Aid (Standard 1.3.3)

A. GENERAL REQUIREMENTS

In accordance with Section 3.1.1 – General Requirements of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), the following general information must be provided:

1. Format of the application
2. Applicant details
3. Purpose of the application
4. Justification for the application
5. Information to support the application
6. Assessment procedure
7. Confidential commercial information
8. Other confidential information
9. Exclusive capturable commercial benefit
10. International and other national standards
11. Statutory declaration
12. Checklist

Each point is addressed in the following subsections.

A.1 Format of the Application

A.1.1 Information Related to Changes to Standard 1.3.3 – Processing Aids

This application for an amendment to Standard 1.3.3 and related Schedules is prepared pursuant to Section 3.3.2 – Processing Aids of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), which requires the following structured format to assess an application for a new processing aid:

A. General information on the application
B. Technical information on the processing aid
C. Information on the safety of the processing aid
D. Information on dietary exposure to the processing aid

The application is presented in this format. At the start of each section (A through D), the information that must be addressed therein is specified in more detail. Additionally, an executive summary for the application has been provided as a separate electronic document with this application. The application has been prepared in English and submitted electronically, as required within the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019).

A.2 Applicant Details

MycoTechnology, Inc. (MycoTechnology) is a manufacturer of mushroom fermentation-based food ingredients. Specifically, MycoTechnology's FermentIQ™ protein, composed of pea and rice protein fermented by *Lentinula edodes* (shiitake mushroom) mycelia, is used as a food ingredient in the United States (U.S.) and in the European Union (EU), and is referred to as fermented pea and rice protein (FPRP) or "FPRP ingredient" throughout the application. The contact details for the person responsible for this application are listed below.

[redacted]

Secondary contact:

[redacted]

In addition, [redacted] at Intertek Health Sciences Inc., assisted in the preparation of this application and will be involved in the submission and stewardship of this application. Her contact details are listed below.

[redacted]

A.3 Purpose of the Application

This application is being submitted to Food Standards Australia New Zealand (FSANZ), and the applicant is seeking approval for *L. edodes* (shiitake) mycelia for use as a processing aid. Specifically, the *L. edodes* mycelia are not genetically modified, and are used for fermentation of pea and rice protein in the manufacture of FermentIQ™ protein ingredient (FPRP). The FPRP ingredient contains ≥75% protein (comprising ≥95% protein concentrates of the raw material inputs)[1] on a dry basis, with an estimated content of shiitake mycelia biomass of <0.1 wt%. This ingredient is used in a wide variety of food and beverage products in the U.S. and the EU for nutritional purposes and in foods needing protein-source properties such as promotion of ease of dry flow, masking of off-flavors, texturing of meat analogues, increase water-holding capacity and gelation, and increase of water solubility. MycoTechnology's FPRP ingredient is not intended for addition to infant formula.

In 2020, MycoTechnology sent an inquiry to FSANZ requesting information from the Advisory Committee on Novel Foods on whether their FPRP produced by fermentation using *L. edodes* would meet the definitions of "non-traditional food" and "novel food" in the Code. The response to this inquiry is included in Appendix A. The Committee concluded that FPRP meets the definition of a "non-traditional food" but is not likely to be considered a novel food for the purposes of the Code. The committee noted further that "*an assessment of public health and safety considerations is not required for Pea and Rice protein fermented by shiitake (FPRP protein) in the context of the novel food provisions in the Code.*" The Committee commented that *L. edodes* is functioning as a processing aid, and since it is not listed as such, an application to amend the Code to include *L. edodes* as a processing aid would be required.

MycoTechnology's *L. edodes* (shiitake) mycelia is considered to meet the definition of a processing aid as defined in Standard 1.3.3 of the *Food Standards Code* ("the Code"), on the basis that it is a "*substance that is used during the course of processing: (a) to perform a technological purpose in the course of processing; and (b) does not perform a technological purpose in the food for sale.*"

As there are currently no specifications for *L. edodes* (shiitake) mycelia included in Schedule 3 of the Code (Identity and Purity), it is the view of MycoTechnology that the following Schedules in the Code will need to be amended to allow for the use of *L. edodes* (shiitake) mycelia as a processing aid in Australia and New Zealand:

- Schedule 3 Identity and Purity
- Schedule 18 Processing Aids

[1] Recipe used in the main fermenter to produce FPRP.

A.4 Justification of the Application

A.4.1 Regulatory Impact Information – Costs and Benefits of Application

a) Impact on the Consumer

The approval of MycoTechnology's *L. edodes* (shiitake) mycelia for use as a processing aid, and subsequent permissibility of MycoTechnology's FPRP ingredient in Australia and New Zealand, will provide consumers with more choices for food products containing non-animal-based protein in the Australia and New Zealand marketplace. Consumers who seek out non-animal alternative proteins will have the option to purchase products that are designed to provide the same nutrition as the conventional (animal-derived) counterpart. Consumers will benefit from having access to a nutritious and flavorful alternative to traditional animal-derived food products, with a substantially reduced ecological impact.

The proposed amendment to the Code will not impose any additional economic costs on consumers. Consumers will retain the choice of purchasing traditional products containing animal-derived proteins (*i.e.,* yogurt), or products containing MycoTechnology's FPRP produced by fermentation using *L. edodes* (shiitake) mycelia.

b) Impact on the Industry

The approval of MycoTechnology's *L. edodes* (shiitake) mycelia, and introduction of food products containing MycoTechnology's FPRP ingredient, is not expected to negatively affect the food industry. Food products containing alternative proteins are already available in Australia and New Zealand (*i.e.,* soy leghemoglobin derived from genetically modified *Pichia pastoris*) (FSANZ, 2023a). These products are targeted to consumers wishing to purchase non-animal proteins for various reasons, including health, as well as ethical, ecological, or religious reasons. The introduction of food products containing FPRP produced by fermentation using *L. edodes* (shiitake) mycelia will promote culinary innovation in Australia and New Zealand and may have a beneficial impact on local retailers (grocery stores, restaurants) who carry such products.

c) Impact on the Government

The approval of MycoTechnology's FPRP produced by fermentation using *L. edodes* (shiitake) mycelia in Australia and New Zealand is not expected to have any impact on government agencies. Increasing dietary consumption of alternative non-animal-derived protein sources may have beneficial effects on the environment and public health.

A.4.2 Impact on International Trade

Food products containing MycoTechnology's FPRP produced by fermentation using *L. edodes* (shiitake) mycelia are currently available in the U.S. and the EU. Approval of MycoTechnology's *L. edodes* processing aid in Australia and New Zealand will promote international trade and reduce technical barriers to trade, while continuing to protect public health and safety.

A.5 Information to Support the Application

Technical information regarding the source of MycoTechnology's *L. edodes* processing aid is presented in Section B of this application. Information to support the safety of the processing aid is presented in Section C. This application is prepared in accordance with the relevant sections within the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), including the following:

- Guideline 3.1 General requirements for applications
- Guideline 3.3.2 Processing aids

Information is provided in this application to enable the objectives specified in Section 18 of the *Food Standards Australia New Zealand Act*, which include:

- The protection of public health and safety;
- The provision of adequate information relating to food to enable consumers to make informed choices; and
- The prevention of misleading or deceptive conduct.

Literature Search Strategy

To identify publications relevant to the safety of *L. edodes* published since the submission of MycoTechnology's EU novel food application in 2020 for their FPRP ingredient produced by fermentation using *L. edodes* mycelia, a comprehensive search of the published scientific literature was conducted for the period spanning January 2020 through 9 May 2022 using the electronic search tool, ProQuest Dialog™ (EFSA, 2022). The databases searched included: Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile®.

As detailed in Section C, no published studies were identified that indicated the potential for allergic, toxic, or adverse health effects related to consumption of *L. edodes*.

A.6 Assessment Procedure

The applicant considers the most appropriate assessment procedure for the application herein is related to *Standard 1.3.3 – Processing Aids* of the *Australia New Zealand Food Standards Code* in order to amend S18-9 to add their *L. edodes* (shiitake) mycelia for use as a processing aid. Considering that MycoTechnology's *L. edodes* is not genetically modified, and that shiitake mushrooms have a long history of use as a food, this application is expected to fall under the General Procedure (Subdivision D of the *Food Standards Australia New Zealand Act*), Cost Category Level 2.

A.7 Confidential Commercial Information (CCI)

The applicant requests that the following specific information related to the manufacture of FPRP be considered confidential commercial information (CCI) and informs FSANZ in writing as follows:

- Details regarding the manufacture of FPRP are considered confidential and are provided in Appendix B.

As such, the applicant requests that the above data and information be considered CCI by FSANZ due to their proprietary nature that is of significant commercial value to the company. Non-confidential descriptions of the CCI are provided in the respective sections of this application.

A.8 Other Confidential Information

No other confidential information is contained within this application.

A.9 Exclusive Capturable Commercial Benefit (ECCB)

The strain of *L. edodes* used by MycoTechnology (strain ID No. WC 1008) is available from the fungi catalog maintained at Pennsylvania State University. Therefore, other manufacturers may benefit from permission to use *L. edodes* as a processing aid in Australia and New Zealand upon approval of this application. Therefore, this application would not confer exclusive capturable commercial benefit (ECCB) in accordance with Section 8 of the *Food Standards Australia New Zealand Act.*

A.10 International and Other National Standards

A.10.1 United States of America

MycoTechnology notified the U.S. Food and Drug Administration (FDA) of the Generally Recognized as Safe (GRAS) status of their pea and rice protein fermented by *L. edodes* (shiitake) mycelia (FPRP), and received a "no objection" letter from the FDA in 2020 (U.S. FDA, 2020). The ingredient is GRAS for use at levels ranging from 1.04 to 33.3% in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, dairy product analogues, fats and oils, grain products and pastas, milk products, plant proteins and products, processed fruits and fruit juices, processed vegetable and vegetable juices, soups and soup mixes, non-baked goods (bars), and confectionaries.

A.10.2 Europe

MycoTechnology's FPRP ingredient received a positive opinion regarding its use as a novel food from the European Food and Safety Authority (EFSA) in 2022 (EFSA, 2022). The ingredient is permitted for use at levels ranging from 1.04 to 100 g/100 g in baked goods, beverages, breakfast cereals, dairy product analogues, confectionaries, salad dressings, processed meat and meat products, flavored milk drinks, meal replacements, yogurt, pasta, meat alternatives, and soups.

A.10.3 India

In May 2022, the Food Safety and Standards Authority of India (FSSAI) approved the marketing of MycoTechnology's FPRP, produced by fermentation using *L. edodes* as described herein, as an ingredient in foods, with no specified limits on the use of the ingredient. The approval letter from FSSAI is provided in Appendix A.

A.10.4 Brazil

MycoTechnology's FPRP, produced by fermentation using *L. edodes* as described herein, is permitted for use in foods in Brazil (per Regulations No. 16/1999 and No. 19/199). The ingredient is permitted for the uses and use levels specified by EFSA (2022). Documentation indicating approval for the use of MycoTechnology's FPRP as a food ingredient in Brazil is provided in Appendix A.

A.11 Statutory Declaration

A signed Statutory Declaration for Australia is provided as Appendix C.

A.12 Checklist

A completed checklist relating to the information required for submission with this application is provided in Appendix C.

B. TECHNICAL INFORMATION ON THE PROCESSING AID

In accordance with Section 3.3.2 – Processing Aids of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), the following technical information is provided:

1. Information on the type of processing aid
2. Information on the identity of the processing aid
3. Information on the chemical and physical properties of the processing aid
4. Manufacturing process
5. Specification for identity and purity
6. Analytical method for detection

Each point is addressed in the following subsections.

B.1 Type of Processing Aid

MycoTechnology utilizes a strain of non-genetically modified *Lentinula edodes* (shiitake) mycelia as a processing aid (specifically, as a fermentation aid) in the manufacture of their blended rice and pea protein concentrate. Submerged fermentation with *L. edodes* mycelia is performed to improve organoleptic qualities of the input pea and rice protein raw materials; however, the input pea and rice protein raw materials are not substantially modified following fermentation. Following submerged fermentation, the rice and pea proteins are concentrated, thermally processed (shiitake mycelia are heat-killed), spray-dried, and packaged to yield the final product. The resulting product is primarily fermented pea and rice protein powder (≥75% protein on a dry-basis), with less than 0.1% of the heat-killed shiitake mycelia remaining in the final product.

Of the major processing aid categories listed in Schedule 18 of the Code, MycoTechnology's *L. edodes* is most appropriately categorized under Schedule 18-9, or Section 1.3.3-11 – Processing aids that perform various technological purposes (FSANZ, 2022, 2023).

B.2 Identity of the Processing Aid

The strain of *L. edodes* used to produce the rice and pea protein concentrate was originally obtained from Pennsylvania State University, ID No. WC 1008,[2] and is not genetically modified. The strain was genotyped (whole genome sequencing) by a third-party laboratory and the cultures were identified as *Lentinula edodes* (100% match) by internal transcribed spacer (ITS) sequencing data (28S DNA) (MycoTechnology, Inc., unpublished data available in Appendix D). Under the conditions used in the manufacture of MycoTechnology's FPRP ingredient (*i.e.,* aqueous culture), *L. edodes* grows as the vegetative form (Tsivileva *et al.*, 2005; Aminuddin *et al.*, 2007; Aminuddin *et al.*, 2013). This vegetative form is identified herein as "shiitake mycelia." Based on this information, MycoTechnology concluded that the organism used as a processing aid in the manufacture of the FPRP ingredient is the *L. edodes* vegetative form (shiitake mycelia). The taxonomic information for the *L. edodes* species is shown in Table B.2-1, and additional information on the confirmation of the species and strain identity of *L. edodes* is provided in Appendices B and D.

[2] https://plantpath.psu.edu/facilities/mushroom/cultures/spawn.

Table B.2-1 Taxonomic Information for *Lentinula edodes*

Kingdom	Fungi
Phylum	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Ompalotaceae
Genus	*Lentinula*
Species	*edodes*
Strain	WC 1008

The *L. edodes* mycelia was chosen as a processing aid based on improvements in digestibility, nutritional value, physical properties, and organoleptic characteristics observed in the fermented compared to unfermented pea and rice protein (Clark *et al.*, 2022). These properties are a result of the production by *L. edodes* of a variety of proteases and phytases, which increase solubility and digestibility of the pea and rice proteins (Clark *et al.*, 2022). The amino acid content and Digestibility Indispensable Amino Acid Scores (DIAAS) for MycoTechnology's FPRP are provided in Section B.5.2.

The expression of various lignin-modifying enzymes by *L. edodes* also contributes to improvements in organoleptic and physical characteristics in fermented compared to unfermented pea and rice proteins (Clark *et al.*, 2022). Thus, *L. edodes* mycelia was chosen as a processing aid based on its improvements to pea and rice proteins during fermentation, and based on the long history of safe dietary consumption of *L. edodes* (shiitake).

B.3 Chemical and Physical Properties of the Processing Aid

B.3.1 Organoleptic Properties

To assess changes in volatile compounds associated with the organoleptic profile of unfermented and fermented pea and rice protein concentrate mixtures, both protein blends were subjected to gas chromatography (GC)–mass spectrometry (MS) and GC-olfactometry and Combined Hedonic Aroma Response Measurement (CHARM) analyses (Appendix E – Compositional Data, November 2020; MycoTechnology Inc.). These results have been described below in Table B.3.1-1.

Table B.3.1-1 Odorant Quantification Before and After Fermentation

CHARM FPRP	CHARM Unfermented Raw Materials	Odor Perception	Odorant
354	289	Green	Hexanal
330	70.5	Mushroom	1-Octen-3-one
276	13	Fatty	2, 6-Decadienal
264	113	Green/fatty	2, 4-Nonadienal
90	9	Buttery	2, 3-Butanedione
62	104	Potato	Methional
31	1	Malt	3-Methyl butanal
20	6	Fresh cut grass/aldehyde	2-Nonenal
18	80	Mustard/cabbage	Methanethiol
8	682	Earthy	Bergamotene-like

Table B.3.1-1 Odorant Quantification Before and After Fermentation

CHARM FPRP	CHARM Unfermented Raw Materials	Odor Perception	Odorant
1	1	Roasted/nutty	2-Methyl-3-furanthiol
1	4	Musty	1, 5-Octadienone
3	9	Cucumber/aldehyde	Nonanal
1	107	Beany	Galbazine

CHARM = Combined Hedonic Aroma Response Measurement.

The results indicate a decrease in the earthy, beany, potato, and mustard off-notes in the fermented protein blend compared to the unfermented blend, while fragrances associated with "fatty" and "musty" were increased. The analysis also indicated an overall change in the relative abundance of volatile compounds in the fermented protein blend as compared to the unfermented one. Several of the volatile compounds, including galbazine, methyl mercaptan, methional, and a sesquiterpene similar to bergamotene (bergamotene-like) were described as imparting unpleasant off-flavors. Specifically, off-note compounds methional, methyl mercaptan, and bergamotene-like compound which are present in the unfermented protein blend were substantially reduced in the fermented protein blend by 40%, 78%, and 99%, respectively. Moreover, the potent beany off-notes associated with galbazine present in the unfermented protein blend were not detected in the fermented sample. The data also show a substantial increase in the levels of oxylipins: 1-octen-3-one; 2,6-decadienal; 2,4-nonadienal; and 2,3 butanedione in the fermented protein blend as compared to the unfermented blend.

To further understand the aroma profile of the fermented and unfermented protein blends, a sensory evaluation was carried out by a trained sensory panel of 11 people. The sensory results (Appendix E) correlates with data from the CHARM analysis, indicating a decrease in pea and rice notes and overall improvement aroma of the fermented blend. Interestingly, the GC-MS data indicate a relative increase in oxylipins in the fermented protein blend; however, this change was not reflected in the sensory profiles provided by the sensory panel. In fact, 2,3 butanedione had a positive impact to the sensory profiling of the fermented protein blend. Altogether, these results indicate an improvement in the organoleptic characteristic in the fermented pea and rice protein concentration blend *versus* the unfermented protein blend.

The improvement in organoleptic qualities of the pea and rice protein concentrates during the fermentation process described in Section B.4 may be due to the secretion of enzymes by the shiitake mycelia during the main fermentation step which act to modify certain volatile organic compounds (VOCs) known to impart unpleasant organoleptic qualities of pea protein concentrates and rice protein concentrates. As discussed below, MycoTechnology concluded that shiitake mycelia are in lag phase during the main fermentation step (see Section B.4), but the literature shows that even during lag phase, shiitake mycelia remain metabolically active, due to adaptation of the organism to a change in media (Cavallazzi *et al.*, 2005). Shiitake mycelia are known to secrete a number of fungal enzymes, such as pectinases, cellulases, amylases, laccases, laminarinases, and xylanases (Mata *et al.,* 2016). In particular, it is known that shiitake mycelia constitutively express laccases (Matjuškova *et al.,* 2017), and expression of laccases in shiitake mycelia may be upregulated or stimulated by the presence of lignin-derived phenols and or polymeric lignin materials (Matjuškova *et al.*, 2017; Agrawal *et al.,* 2018). Copper-containing laccases have the ability to oxidize a wide range of aromatic and non-aromatic compounds which include substituted phenols, some inorganic ions, and variety of non-phenolic compounds (Agrawal *et al.*, 2018). Laccase is currently used in the food industry for a variety of functional applications including improvement of food sensory parameters (Osma *et al.*, 2010). For example, Schroeder *et al*. (2008) demonstrated that laccase treatment of apple juice degraded

the levels of certain phenolic compounds, guaiacol and 2,6-dibromophenol, responsible for off-flavors in apple juice. Sensory panelists did not detect a difference between apple juices spiked with guaiacol and 2,6-dibromophenol compared to non-spiked juice after continuous enzymatic treatment with laccase (Schroeder *et al.*, 2008). These results are corroborated by the sensory testing observations reported by MycoTechnology for the pea and rice protein concentrates fermented with shiitake mycelia.

From this information, MycoTechnology concludes that confirmed improvements to the organoleptic qualities of the rice and pea protein concentrate relative to the protein input are likely due to the action of secreted enzymes (*e.g.,* laccase) from the shiitake mycelia processing aid to modify molecules that confer unpleasant organoleptic qualities to pea and rice protein concentrates.

B.3.2 Enzyme Expression

As described previously, the reduction in certain odorant compounds in FPRP is believed to be the result of fungal enzyme activity. To capture the totality of the fungal enzymes expressed and secreted during FPRP fermentation, the gene expression profile of the shiitake mycelium in the main fermenter medium was evaluated by RNAseq analysis to identify potential enzymes imparting functionality in the FPRP fermentation process (Rapaport *et al.*, 2013). The secretion of each individual protein was determined by the presence of a signal peptide (SP) at the N-terminus of the corresponding protein sequence (Auclair *et al.,* 2012). Proteins tagged with an SP sequence are destined to the secretory pathway and most likely secreted into the fermentation medium. The presence of an SP was predicted *in silico* based on protein sequence.

Approximately half of the secreted enzymes are carbohydrate-active enzymes, which are enzymes involved in either synthesis or breakdown of saccharides. These enzymes are most likely involved in the breakdown of maltodextrin, carrot root powder, and mango puree (included as raw materials in the fermentation of FPRP), and are predicted to not play a role in changing the organoleptic profile. Although proteinases are predicted to be expressed and secreted, little change in the whole protein composition was observed between the sterile raw material and FPRP samples. Approximately 7% of the secreted enzymes corresponded to the category of phenol oxidases, including laccases and tyrosinases, which have been previously linked to changes in organoleptic characteristics of food substrates (Schroeder *et al.,* 2008; VTT Technical Research Centre of Finland, 2008). MycoTechnology believes these enzymes are the reason for organoleptic improvement in the FPRP ingredient. The enzymatic activity of these enzymes in the main fermenter and the final FPRP ingredient was measured using the laccase assay as described by Ratcliffe *et al.* (1994), and the tyrosinase assay as described by Srinivasan *et al.* (1995). These results are outlined in Table B.3.2-1, and show that the enzyme activity is measurable in the main fermenter, but not in the final FPRP ingredient.

Table B.3.2-1 Laccase and Tyrosinase Activities in the Main Fermenter and Fermented Pea and Rice Protein in 4 Independent Fermentation Batches

Sample	Laccase (units/mg) ± SEM	Tyrosinase (units/mg) ± SEM
MF Batch 1	8.1 ± 0.7	9.7 ± 0.9
MF Batch 2	4.9 ± 0.5	9.8 ± 0.9
MF Batch 3	5.3 ± 0.6	12.3 ± 0.2
MF Batch 4	6.3 ± 0.5	9.8 ± 0.2
FPRP Batch 100506	ND[a]	ND[b]
FPRP Batch 100507	ND[a]	ND[b]

Table B.3.2-1 Laccase and Tyrosinase Activities in the Main Fermenter and Fermented Pea and Rice Protein in 4 Independent Fermentation Batches

Sample	Laccase (units/mg) ± SEM	Tyrosinase (units/mg) ± SEM
FPRP Batch 100510	ND[a]	ND[b]
FPRP Batch 101034	ND[a]	ND[b]

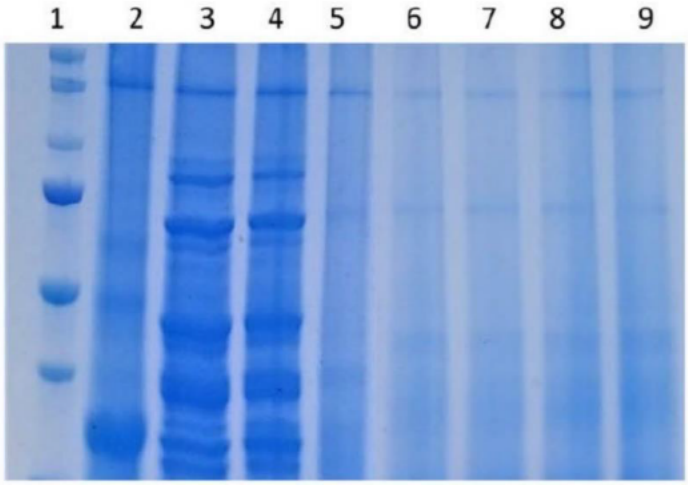
MF = main fermenter; ND = not detected; SEM = standard error of the mean.
[a] Limit of detection of 0.035 units/mg dried biomass.
[b] Limit of detection of 0.09 units/mg dried biomass.

B.3.3 Protein Composition of Fermented Pea and Rice Protein

Introduction of the shiitake mycelia processing aid changes the organoleptic properties, but not the protein composition, of the rice and pea protein materials. This was demonstrated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, the results of which are presented in Figure B.3.3-1. This figure shows the pattern of protein content of the FPRP raw materials before fermentation (Lanes 2 to 4). Importantly, Lane 5 shows the raw materials after sterilization and before fermentation. Comparison of Lanes 4 and 5 to different batches of the FPRP final ingredient (Lanes 6 through 9) shows no major differences in the protein content in these samples. In all FPRP final ingredient samples, sharp and distinguishable protein bands are present, including bands matching those present in the sterile raw material.

Therefore, MycoTechnology concluded the SDS-PAGE analysis in Figure B.3.3-1 indicates that the fermentation process using the shiitake mycelia processing aid does not significantly alter the protein composition of the raw materials beyond the thermal (pre-fermentation sterilization) processes involved in the production of the FPRP ingredient.

Figure B.3.3-1 SDS-PAGE Analysis of Unfermented and Fermented Pea and Rice Protein



FPRP = fermented pea and rice protein; SDS-Page = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
Lane 1: Protein molecular weight markers; Lane 2: Raw rice protein concentrate; Lane 3: Raw pea protein concentrate;
Lane 4: Unfermented FPRP raw material (35 and 65% rice and pea proteins, respectively); Lane 5: Unfermented FPRP sterilized material (35 and 65% rice and pea proteins, respectively); Lane 6: FPRP final product (Batch 100506);
Lane 7: FPRP final product (Batch 100507); Lane 8: FPRP final product (Batch 100510); Lane 9: FPRP final product (Batch 101034).

B.3.4 Impurity Profile

A specification is in place for the shiitake mycelia (an aerobic plate count of <10 colony-forming units (CFU)/g) to ensure that the processing aid is free of contamination (summarized in Section B.5; details provided in Appendix F). Furthermore, specifications for heavy metals, microbials, and mycotoxins have been put in place for the rice and pea protein fermented with the shiitake mycelia. Batch samples of rice and pea protein concentrate are routinely tested to verify compliance with the set chemical and microbiological specification parameters (see Section B.5).

B.4 Non-confidential Summary of Manufacturing Process

MycoTechnology's FPRP is manufactured in accordance with Title 21 *Code of Federal Regulations* (CFR) §117 "*Current Good Manufacturing Practice, Hazard Analysis, And Risk-Based Preventive Controls for Human Food*" (U.S. FDA, 2022).

The process of manufacturing the FPRP involves fermentations of a primary culture shiitake mycelia, followed by fermentation of 65% pea protein and 35% rice protein concentrates. Additional starting materials include maltodextrin, mango puree, carrot powder, and an anti-foam agent. During the main fermentation, the *L. edodes* and pea and rice proteins are slowly stirred for 40 hours at a fixed temperature and pH. The growth of the shiitake biomass between fermentations is quantified by pH monitoring and visual appearance. Parameters that are monitored throughout the manufacturing process include temperature, pH, aerobic plate count, agitation, appearance, and microscopy. Details on the timing of measurement of these parameters and acceptance criteria are presented in Appendix B. The resulting protein material is heat-treated, concentrated, and spray-dried, with the final FPRP containing ≤0.1% of the processing aid (shiitake mycelia). Confidential details of the manufacturing process are provided in Appendix B.

B.5 Specifications and Analytical Data

B.5.1 Raw Materials

The specifications for the raw pea and rice proteins used in the manufacture of FPRP are outlined in Table B.5.1-1. The raw pea protein concentrate is obtained from the mechanically milled and wet fractionated portion of de-hulled yellow peas (*Pisum sativum*), while the raw rice protein concentrate is made after concentrating and filtering hydrolyzed rice (*Oryza sativa*) slurry which has undergone an all-natural enzymatic process. Both these proteins are GRAS for use in foods in the U.S. (pea protein under GRAS Notice [GRN] 608, and rice protein under GRN 609) (U.S. FDA, 2016a,b).

Table B.5.1-1 Pea and Rice Protein Raw Material Specifications

Parameter	Pea Protein Specification	Rice Protein Specification	Methods
Source	[redacted]	[redacted]	NA
Appearance	Off-white	Off-white to light beige	Conforms to standard

Table B.5.1-1 Pea and Rice Protein Raw Material Specifications

Parameter	Pea Protein Specification	Rice Protein Specification	Methods
Odor	Inherent pea odor	Odorless	Sensory
Protein	≥75% (dry basis)	≥75% (dry basis)	AOAC 990.03
Microbiological			
Aerobic plate count	≤10,000 CFU/g	≤10,000 CFU/g	AOAC 966.23
Yeast	≤200 CFU/g	≤200 CFU/g	FDA-BAM, 7th edition
Mold	≤100 CFU/g	≤100 CFU/g	FDA-BAM, 7th edition
Listeria	Negative /25 g	Negative /25 g	AOAC 2016.07
Coliforms	≤10 CFU/g	≤10 CFU/g	AOAC 966.24
Salmonella	Negative /25 g	Negative /25 g	AOAC-R11100201
Escherichia coli	≤10 CFU/g	≤10 CFU/g	AOAC 966.24
Heavy metals			
Mercury	<0.1 ppm	<1 ppm	ICP-MS, FDA EAM 4.7
Cadmium	<0.2 ppm	<0.5 ppm	ICP-MS, FDA EAM 4.7
Arsenic	<0.5 ppm	<1 ppm	ICP-MS, FDA EAM 4.7
Lead	<0.1 ppm	<1 ppm	ICP-MS, FDA EAM 4.7
Mycotoxins			
Mycotoxins	<5 ppb	<5 ppb	HPLC AOAC 991.31

AOAC = Association of Official Analytical Collaboration; CFU = colony-forming units; FDA-BAM = Food and Drug Administration Bacteriological Analytical Manual; FDA EAM = Food and Drug Administration Elemental Analysis Manual; GMO = genetically modified organism; HPLC = high-performance liquid chromatography; ICP-MS = inductively coupled plasma mass spectrometry; NA = not applicable; ppb = parts per billion; ppm = parts per million.

The microbial specifications for the shiitake mycelia (glycerol stock) are outlined in Table B.5.1-2. MycoTechnology has also had the shiitake mycelia (glycerol stock) analyzed for the presence of heavy metals (arsenic, cadmium, lead, and mercury). The analytical report is presented in Appendix F, and demonstrates that the contents of arsenic, cadmium, lead, and mercury of the shiitake mycelia (glycerol stock) are consistently below the limits of detection (10, 5, 5, and 5 ppb, respectively).

Table B.5.1-2 Shiitake Mycelia Raw Material Specifications

Parameter	Shiitake Mycelia Specification	Methods
Species and Strain Identification	*Lentinula edodes* WC1008	Presented in Appendix D
Aerobic plate count	<10 CFU/g	AOAC 966.23
Arsenic	10 ppb	AOAC 2011.19, 993.14, 2015.01 (modified)
Cadmium	5 ppb	
Lead	5 ppb	
Mercury	5 ppb	

AOAC = Association of Official Analytical Collaboration; CFU = colony-forming units; ppb = parts per billion.

The specifications for the final FPRP fermented with shiitake mycelia are outlined in Table B.5.1-3. These product specifications assure the purity and protein content required for the product's technical characteristics as a food ingredient, formulation aid, and texturizer in foods in which protein is used for nutritional purposes and in foods needing protein-source properties such as promotion of ease of dry flow, masking of off-flavors, texturing of meat analogues, increase water-holding capacity and gelation, and increase of water solubility. The specifications were selected to maximize product safety by placing limits on microbial contamination as well as aflatoxins and heavy metals.

Table B.5.1-3 Pea and Rice Protein Fermented with Shiitake Mycelia Specifications

Parameter	Specifications	Methods
Protein	≥75% (dry basis)	AOAC 990.03
Moisture	≤7%	AOAC 985.14
Total fat	≤10%	AOAC 942.05
Ash	≤10%	AOAC 996.06, mod
Carbohydrates	≤15%	Calculation
Microbiological		
Aerobic plate count	<1,000 CFU/g	AOAC 966.23
Coliforms	<10 CFU/g	AOAC 991.14
Yeast and mold	<100 CFU/g	FDA-BAM, 7th edition
Listeria	Negative/25 g	AOAC 2016.07
Salmonella	Negative/25 g	AOAC-R11100201
Escherichia coli	<10 CFU/g	AOAC 966.24
Heavy metals		
Mercury	<0.1 ppm	ICP-MS, FDA EAM 4.7
Cadmium	<0.1 ppm	
Arsenic	<0.1 ppm	
Lead	<0.3 ppm	
Mycotoxins		
Aflatoxin B1	<1 ppb	HPLC AOAC 991.31, mod
Aflatoxin B2	<1 ppb	
Aflatoxin G1	<1 ppb	
Aflatoxin G2	<1 ppb	
Aflatoxin total	<3 ppb	

AOAC = Association of Official Analytical Collaboration; CFU = colony-forming units; FDA-BAM = Food and Drug Administration Bacteriological Analytical Manual; FDA EAM = Food and Drug Administration Elemental Analysis Manual; HPLC = high-performance liquid chromatography; ICP-MS = inductively coupled plasma mass spectrometry; mod = modified; ppb = parts per billion; ppm = parts per million.

B.5.2 Batch Analysis

Analysis has been conducted on 5 batches of the final FPRP ingredient fermented with shiitake mycelia, and these results are provided below in Table B.5.2-1. The results of these analyses demonstrate conformity with the specifications outlined and demonstrate that the manufacturing process, using shiitake mycelia as a processing aid, results in a consistent product free of contamination. Certificates of analysis for these 5 batches of FPRP are provided in Appendix F.

Table B.5.2-1 Analysis for 5 Non-consecutive Batches of Pea and Rice Protein Fermented with Shiitake Mycelia[a]

Parameter	Specification	Lot Number and Date of Manufacture				
		Lot 101028 05 May to 06 May 2020	Lot 101487 20 Feb to 21 Feb 2021	Lot 101071 07 June to 08 June 2020	Lot 101610 19 Apr 2021	Lot 101702 29 May to 30 May 2021
Protein[b]	≥75% (dry basis)	78.11	78.48[c]	78.47	79.71	78.45
Protein (as-is)	≥75%	76.00	76.13[c]	77.06	77.56	76.25
Moisture	≤7%	2.7	3.00[c]	1.80	2.70	2.80
Ash (% as-is)	≤10%	3.16	2.99	3.38	6.04	6.15
Ash (% DW)	≤10%	3.25	3.08	3.44	6.21	6.33
Total fat (% as-is)	≤10%	7.97	5.17	9.51	6.44	6.65
Total fat (% DW)	≤10%	8.19	5.33	9.68	6.62	6.84
Carbohydrates (% as-is by calculation)	≤15%	10.17	12.74	8.25	7.26	8.15
Carbohydrates (% DW by calculation)	≤15%	10.45	13.13	8.40	7.46	8.38
Microbiological						
Aerobic plate count	<1,000 CFU/g	<10	<10	<10	<10	290
Coliforms	<10 CFU/g	<10	<10	<10	<10	<10
Yeast and mold	<100 CFU/g	<10	<10	<10	<10	<10
Listeria	Negative/25 g	Negative	Negative	Negative	Negative	Negative
Salmonella	Negative/25 g	Negative	Negative	Negative	Negative	Negative
Escherichia coli	<10 CFU/g	<10	<10	<10	<10	<10
Heavy metals						
Mercury	<0.1 ppm	0.00512	<0.005	<0.005	0.00845	0.00535
Cadmium	<0.1 ppm	0.0339	0.0211	0.0348	0.0312	0.0196
Arsenic	<0.1 ppm	0.0218	0.0198	0.0187	0.0284	0.0223
Lead	<0.3 ppm	0.0386	0.0369	0.0435	0.0232	0.0396
Mycotoxins						
Aflatoxin B1	<1 ppb	<0.5	<0.5	<0.5	0.619	<0.5
Aflatoxin B2	<1 ppb	<0.5	<0.5	<0.5	<0.5	<0.5
Aflatoxin G1	<1 ppb	<0.5	<0.5	<0.5	<0.5	<0.5
Aflatoxin G2	<1 ppb	<0.5	<0.5	<0.5	<0.5	<0.5
Aflatoxin total	<3 ppb	<2	<2	<2	2.119	<2
Deoxynivalenol	NA	<100	<100	<100	<100	<100

CFU = colony-forming units; DW = dry weight; NA = not applicable; ppb = parts per billion; ppm = parts per million.

[a] Input = 65% pea protein and 35% rice protein.

[b] % Protein (DW) = % Protein [(as sampled)/(100 - % Moisture)] * 100

[c] There was an error in the analysis performed by Eurofins for total protein content of Lot 101487. Eurofins performed 4 repeat analyses for protein "as is" and 2 repeated analyses for moisture. The values were averaged and protein on a dry weight basis was calculated as above.

A study to determine the digestibility of MycoTechnology's FPRP ingredient was conducted in ileal canulated pigs (9 males) that were fed diets differing only in protein source. Protein sources were either a blend of pea and rice protein (unfermented) or MycoTechnology's FPRP ingredient. The results show that overall digestibility for MycoTechnology's FPRP ingredient is 99.9%, while the overall digestibility for the unfermented blend is 94.59%. The increase in digestibility can be attributed to the benefits of mycelial fermentation in reducing antinutrients such as phytic acid and in increasing the solubility of the protein, as discussed by Clark *et al.* (2022).

The percentage of pea and rice protein in MycoTechnology's FPRP is 65% pea protein and 35% rice protein. DIAAS were calculated based on amino acid digestibility data generated as part of the protein quality study conducted in pigs. DIAAS were calculated as follows: DIAAS % = 100 x [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein) / (mg of the same dietary indispensable amino acid in 1 g of the reference protein)] (FAO, 2013). DIAAS were calculated using the recommended amino acid scoring pattern for a young child (6 months to 3 years of age). The indispensable amino acid reference patterns (expressed as mg amino acid/g protein) are as follows: His, 20; Ile, 32; Leu, 66; Lys, 57; Sulphur amino acid, 27; Aromatic AA, 52; Thr, 31; Trp, 8.5; Val, 43 (FAO, 2013). The amino acid content of 5 lots of FPRP, along with detailed DIAAS calculations, are presented in Table B.5.2-2.

Table B.5.2-2 Amino Acid Profile for 5 Non-consecutive Batches of Pea and Rice Protein Fermented with Shiitake Mycelia

Parameter	Lot Number and Date of Manufacture				
	Lot 101028 05 May to 06 May 2020	Lot 101487 20 Feb to 21 Feb 2021	Lot 101071 07 June to 08 June 2020	Lot 101610 19 Apr 2021	Lot 101702 29 May to 30 May 2021
Amino acid (%)	75.58	75.4	77.08	76.99	77.45
DIAAS[a]	91 (SAA)	96 (LYS)	97 (SAA)	96 (SAA)	99 (LYS)
Tryptophan (%)	0.78	0.83	0.82	0.84	0.83
Cysteine (%)	0.81	0.90	0.89	0.92	0.87
Methionine (%)	1.26	1.32	1.36	1.32	1.41
Alanine (%)	3.71	3.66	3.75	3.67	3.77
Arginine (%)	6.25	6.30	6.35	6.51	6.38
Aspartic acid (%)	8.16	8.04	8.25	8.19	8.29
Glutamic acid (%)	13.35	13.36	13.56	13.74	13.57
Glycine (%)	3.20	3.17	3.24	3.20	3.26
Histidine (%)	1.77	1.81	1.83	1.89	1.84
Isoleucine (%)	3.61	3.61	3.71	3.67	3.71
Leucine (%)	6.48	6.42	6.60	6.54	6.65
Phenylalanine (%)	4.23	4.17	4.30	4.23	4.32
Proline (%)	3.47	3.48	3.54	3.49	3.58
Serine (%)	3.86	3.74	3.87	3.82	3.90
Threonine (%)	2.81	2.73	2.82	2.76	2.84
Lysine (%)	4.39	4.37	4.52	4.61	4.52
Tyrosine (%)	3.15	3.17	3.26	3.23	3.29
Valine (%)	4.29	4.32	4.41	4.36	4.42

Table B.5.2-2 Amino Acid Profile for 5 Non-consecutive Batches of Pea and Rice Protein Fermented with Shiitake Mycelia

Parameter	Lot Number and Date of Manufacture				
	Lot 101028 05 May to 06 May 2020	Lot 101487 20 Feb to 21 Feb 2021	Lot 101071 07 June to 08 June 2020	Lot 101610 19 Apr 2021	Lot 101702 29 May to 30 May 2021

DIAAS = Digestibility Indispensable Amino Acid Score; LYS = lysine; SAA = sulfur amino acids.
[a] DIAAS values were calculated using the recommended amino acid scoring pattern for a young child (6 months to 3 years of age). The indispensable amino acid reference patterns are expressed as mg amino acids/g protein: Histamine, 20; Isoleucine, 32; Leucine, 66; Lysine, 57; Sulfur AA, 27; Aromatic AA, 52; Threonine, 31; Tryptophan, 8.5; Valine, 43 (FAO, 2013).

B.5.3 Nutritional Analysis

The nutritional composition of the shiitake mycelia processing aid was measured and the results are presented below in Table B.5.3-1. The results demonstrate the shitake mycelia processing aid to be composed primarily of carbohydrates, protein, dietary fiber, and triglycerides. The certificate of analysis is provided in Appendix F.

Table B.5.3-1 Nutritional Analysis of Shiitake Mycelia

Parameter	Result	Methods
Carbohydrates (%)	45.14	21 CFR §101– Calculation
Protein (%)	37.38	AOAC 990.03; AOAC 992.15
Total dietary fiber (%)	36.6	AOAC 991.43
Ash (%)	3.86	AOAC 942.05
Moisture (%)	2.6	AOAC 925.09
Calories (kcal/100 g)	429	21 CFR §101– Atwater calculation
Total sugars (%)	0.88	AOAC 982.14, mod
Fructose (%)	0.17	
Glucose (%)	0.71	
Lactose (%)	<0.15	
Maltose (%)	<0.15	
Sucrose (%)	<0.15	
Total fat as triglycerides (%)	11.02	AOAC 996.06, mod
Total trans fatty acid isomers (%)	0.05	
cis-Polyunsaturated fatty acids (%)	3.98	
cis-Monounsaturated fatty acids (%)	5.22	
Total saturated fatty acids (%)	1.29	
Cholesterol (mg/100 g)	0.8	AOAC 994.10, mod
Calories from total fat (kcal/100 g)	99	21 CFR §101– Calculation
Sodium (%)	0.132	AOAC 984.27, mod; 927.02, mod; 985.01, mod; 965.17, mod
Calcium (%)	0.279	
Iron (%)	0.0052	
Potassium (%)	0.289	
Total vitamin D2 and D3 (IU/100 g)	<4.00	Huang *et al.*, 2014 - Rapid Commun. Mass Spectrum 2014, 28
Vitamin D2 (IU/100 g)	<4.00	
Vitamin D3 (IU/100 g)	<4.00	

Table B.5.3-1 Nutritional Analysis of Shiitake Mycelia

Parameter	Result	Methods

AOAC = Association of Official Analytical Collaboration; CFR = *Code of Federal Regulations*; IU = international units; mod = modified.

B.6 Analytical Method for Detection

An internal method is used to demonstrate that there is no viable processing aid (*L. edodes* mycelium) in the final FPRP product. In short, 1-g samples of FPRP were plated in 2 different shiitake solid growth media in triplicates. Blended shiitake mycelium was added as a positive control to ensure that mycelium would grow on the selected media, while sterile water was used as a negative control. Samples were incubated in a 26°C incubator, in the dark and left undisturbed for 7 days. Mycelia growth was then quantified by measuring viable mycelium in the final FPRP product. Results of this analysis are shown below in Table B.6-1 and demonstrate that there was no fungal/mycelia growth in any of the sample plates at the end of the 7-day incubation period. Batch analyses and methodology are available in Appendix G.

Table B.6-1 Mycelia Growth Results

Sample Lot	MYPGA+AKS Rep 1	MYPGA+AKS Rep 2	MYPGA+AKS Rep 3	PDA Rep 1	PDA Rep 2	PDA Rep 3
101646	ND	ND	ND	ND	ND	ND
101355	ND	ND	ND	ND	ND	ND
101602	ND	ND	ND	ND	ND	ND
101905	ND	ND	ND	ND	ND	ND
Negative Control	ND	ND	ND	ND	ND	ND
Positive Control	D	D	D	D	D	D

AKS = ampicillin disodium salt, kanamycin, spectinomycin; D = detected; MYPGA = malt yeast peptone glucose agar medium; ND = not detected; PDA = potato dextrose agar medium.

C. INFORMATION RELATED TO THE SAFETY OF THE PROCESSING AID

In accordance with Section 3.3.2 – Processing Aids of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), the following technical information is provided:

1. General information on the use of the processing aid in other countries
2. Information on the potential toxicity of the processing aid
3. Information on the potential allergenicity of the processing aid
4. Safety assessment reports prepared by international agencies or other national government agencies, if available

Each point is addressed in the following subsections. Since additional information pertaining to the safety of MycoTechnology's processing aid is available, it is included herein for completeness.

C.1 Support for Safety of *Lentinula edodes* (Shiitake)

C.1.1 History of Safe Consumption of Shiitake

The fruiting bodies of *Lentinula edodes*, also known as shiitake mushroom, are a commonly consumed food, particularly in Asia, and are the second most widely produced mushroom in the world (Bisen *et al.*, 2010). In the EU, an ingredient consisting of a sterile, aqueous mycelial extract of *L. edodes* has been permitted for marketing as a novel food since 2011 (EU, 2011).

Fungi have a long history of use in both culinary and medicinal applications, with more than 2 million tons of mushrooms sold annually (Nakamura, 1992; VanderMolen *et al.*, 2017). Although the chemical and nutritional characteristics of mushrooms vary after harvest and processing, mushrooms in general are rich in protein (approximately 2.26% protein), essential amino acids, and fiber (Finimundy *et al.*, 2014). Edible mushrooms are a high-nutritional quality food and have been used as an alternative to dietary protein in countries with high rates of malnutrition (Finimundy *et al.*, 2014).

In a review of the nutritional compounds found in *Lentinus edodes* (Finimundy *et al.*, 2014) it was reported that the dietary fiber present in *L. edodes* consists of soluble and insoluble fractions. Water-soluble β-glucans and proteins are found in the soluble fraction, while the non-soluble fraction contains polyuronide (acidic polysaccharide), hemicellulose, β-glucan chains with heterosaccharide, lignin, and chitin. Other carbohydrates identified in *L. edodes* mycelia include glucose, galactose, xylose, arabinose, mannose, and fructose (Wasser, 2005). *L. edodes* also contains nutritionally significant levels of vitamins B1, B2, B12, C, D, and E. The aromatic components of *L. edodes* include alcohols, ketones, sulfides, alkanes, and fatty acids. The main constituents which are volatile include matsutakeol (1-octen-3-ol) and ethyl, n-amyl ketone, while the characteristic aroma of shiitake was identified as 1,2,3,5,6-pentathiepane (Finimundy *et al.*, 2014).

As discussed further below (Section C.3), the shiitake mycelia used to ferment the raw protein materials are 98% similar in composition to commonly consumed culinary shiitake mushrooms (VanderMolen *et al.*, 2017).

C.1.2 Safety of Consumption of Shiitake Mycelia at Estimated Dietary Exposure from Use as a Processing Aid

MycoTechnology's FPRP has been marketed for several years in the U.S., EU, Brazil, Canada, India, Japan, South Korea, Singapore, Malaysia, Indonesia, Philippines, Thailand, Chile, Ecuador, and Hong Kong, with no reports of product-related adverse events. Estimated dietary exposures to shiitake mycelia from consumption of MycoTechnology's FPRP at estimated mean and high use levels are 29.3 mg/day and 86.3 mg/day, respectively. The safety of this exposure level for shiitake mycelia was evaluated using a weight-of-evidence approach as described by VanderMolen *et al*. (2017) for the safety assessment of mushrooms in dietary supplements by combining analytical data with *in silico* toxicology evaluation.

VanderMolen *et al*. (2017) assessed the safety of 7 fungal raw materials including *L. edodes*, consisting primarily of mycelium. Briefly, this group used ITS barcoding, screening for known fungal metabolites, similarity analysis between culinary mushrooms and mycelia, toxicological literature review, and evaluation of data establishing presence in market. Based on the weight-of-evidence assessment conducted, VanderMolen *et al*. (2017) concluded,

> "[…] *Shiitake and Maitake are commonly eaten as foods, and shiitake, at least, has a wealth of available toxicological data supporting its safe use. The apparent prevalence in the marketplace, the lack of reported adverse events, as determined by the literature review and the very high degree of similarity between their mycelial growths (the raw materials investigated) and the culinary fruiting bodies to which they were compared give confidence that these materials are safe for consumption at doses consistent with dietary intakes of culinary mushrooms*."

In addition, MycoTechnology has conducted an *in silico* analysis for the presence of proteins involved in the synthesis of mycotoxins in the *L. edodes* mycelia. As summarized in Section C.6, the results of this analysis confirm that *L. edodes* does not produce mycotoxins and is not pathogenic or toxigenic.

C.1.3 Comparison Between the Fruiting Body and Mycelium

The life cycle of mushrooms starts with a spore which produces a primary mycelium. When the mycelia originating from 2 spores mate, a secondary mycelium is produced. This mycelium continues to grow vegetatively. When the vegetative mycelium has matured, its cells are capable of a high rate of reproduction which culminates in the formation of the mushroom fruitbody.

The fruitbody represents the last functional change in the mushroom life cycle and the entire mushroom is composed of compressed mycelia (Stamets and Chilton, 1983). The shiitake mushroom is largely made up of bundles of mycelia composing the pileus (cap) and stalk, and having only a small portion of tissue, located underside of the mushroom cap, that differentiates into gills (lamella) to produce spores (basidiospores) for reproduction of the shiitake organism. Thus, the shiitake mushroom itself, aside from gill tissue on undersides of caps producing spores, is physically indistinguishable from its parent mycelia (Stamets and Chilton, 1983; Liu *et al*., 2016). Based on this information, MycoTechnology concludes that the shiitake mycelia and shiitake mushroom compositions are substantially similar on a physical level, and the safety demonstrated for shiitake mushrooms is directly applicable to shiitake mycelia.

Song *et al*. (2018) reported on the differential expression of 11,675 total genes known to be expressed by shiitake mushrooms and identified that 9,595 of these are not differentially expressed between the mycelia and fruit body. There is an approximately 82% identity in expression activity between shiitake mycelia and shiitake fruiting body tissue. While Song *et al*. (2018) reported that gene expression levels differ, the authors attribute the differential expression to overexpression of genes in the mature fruiting body stage related to "DNA replication, recombination, repair, chromatin structure, and the associated dynamics" and the transcripts from the fruiting body are "significantly enriched in 'replication and repair' and 'transcription' pathways for premeiotic replication, karyogamy, or meiosis." The differential expression between the mycelia and fruit body was reported by Song *et al.* (2018) to be primarily related to the reproductive activity related to shiitake fruiting in the fruiting body, which does not occur in the shiitake mycelia. Based on this information, MycoTechnology concludes that the differences in gene expression between shiitake mycelia and mature fruiting body tissues are of little to no consequence with respect to the safety of consumption of shiitake mycelia.

C.2 Absorption, Distribution, Metabolism, and Excretion (ADME)

As described above in Section C.1.1, it has been established that the shiitake mycelia used to ferment the proteins are 98% similar in composition to commonly consumed culinary shiitake mushrooms (VanderMolen *et al*., 2017). The nutrient components of FPRP are expected to be digested and utilized according to well-documented metabolic pathways. Furthermore, the absence of live shiitake mycelia or fungal enzymes in the final product is achieved through multiple heat-treatment steps and thermal deactivation following fermentation. Therefore, there is no expectation of metabolites that are different from those already produced *via* consumption of these common food ingredients, and a study of absorption, distribution, metabolism, and excretion was not determined to be necessary to support the safety of the processing aid.

C.3 Toxicological Data

A comprehensive search of the scientific literature through October 2019 was conducted using the U.S. National Library of Medicine (NLM) PubMed and TOXLINE databases. A comprehensive search was performed on 18 January 2019 with no date limitations or filters applied to the results. Search terms to identify relevant literature on the mycelia included "lentinula edodes," "shiitake mushroom," and "mycelium" / "mycelia." Search terms to identify relevant literature on the fruiting body included "lentinula edodes," "shiitake mushroom," and the additional keywords (PubMed search only) "safety" / "toxicity" / "carcinogenicity" / "genotoxicity" / "adverse effect" / "tolerability"/ "consumption" / "allergen" / "allergy." A supplemental literature search was performed on 11 October 2019 which utilized the search terms described above as well as a date filter of 2018 through 2019.

To identify further literature published since the initial search, a comprehensive literature search was conducted using the electronic search tool ProQuest Dialog™. The search was conducted in May 2022 using databases including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, Toxicology Abstracts, and Toxfile®; the full search strategy and results are provided in Appendix H.

Relevant literature regarding the safety of dietary consumption of shiitake mycelia is discussed below.

C.3.1 Repeated-Dose Toxicity Studies

Yoshioka *et al*. (2010) assessed the safety of an aqueous suspension of an extract powder of *L. edodes* mycelia (LEM) when administered to Wistar rats (10 animals/sex/group) *via* gavage at doses of 0 or 2,000 mg/kg body weight/day for 28 days. Cultured LEM together with the solid medium were extracted with hot water (temperature not reported) and the LEM extract was prepared by filtration, concentration, sterilization, and lyophilization of the raw extract. Although an LEM extract would not contain insoluble portions of shiitake mycelia cells, and is therefore not identical in composition to the shiitake mycelia present in FPRP, both the LEM extract and shiitake mycelia present in the rice and pea protein concentrate will contain the same water-soluble components. Thus, the Yoshioka *et al*. (2010) LEM extract repeat-dose toxicity data is useful to evaluate safety of the low levels of shiitake mycelia present in the FPRP.

Yoshioka *et al*. (2010) did not report any unscheduled deaths or clinical signs of toxicity. Body weight and food consumption were slightly decreased compared to the control groups, particularly for males. Although there were statistically significant differences from control groups, at the study termination male body weights were 8% (associated with slightly decreased food consumption) and female body weights were 5% less than the sex-specific control groups, respectively. These minor differences are not considered to be adverse. No differences in hematological parameters were reported. Serum biochemistry analysis indicated statistically significantly differences in several parameters compared to controls, including increased phosphorus in both sexes. However, all values were reported as being within the laboratory's historical control ranges. Although females had slightly increased organ weights relative to body weight (thyroid gland, kidneys, adrenals, uterus/ovaries) as did males (thyroid gland, adrenals), these differences were minor and without histopathological correlates. There were no pathological alterations in any examined tissues or organs. Based on these results, Yoshioka *et al.* (2010) concluded that the no-observed-adverse-effect level (NOAEL) of LEM extract was 2,000 mg/kg body weight/day. Based on a direct comparison of dose on a body weight basis, this NOAEL is 1,429 times higher than the 95th to 98th percentile estimated consumption of FPRP in adults in the EU.

Female mice (*Mus musculus* NIH/S; n = 6/group) were given diets providing 0, 3, 6, or 9 g dry *L. edodes*/kg body weight/day (fresh mushroom equivalents of 0, 19.4, 41.9, and 61.4 g/kg body weight/day, respectively) for 5 days (Nieminen *et al*., 2009). Based on a direct body weight conversion, these doses are equivalent to approximately 1,164, 2,514, or 3,684 g fresh shiitake mushrooms/day for a 60-kg human. Food and water consumption, plasma clinical chemistry, and liver and muscle histopathology were evaluated. There were statistically significant, dose-related decreases in high-density lipoprotein (HDL)/total cholesterol (mid- and high-dose groups), and statistically significant, dose-related increases in total protein (low-, mid- and high-dose groups), creatinine kinase (high-dose group), and bilirubin (low-, mid- and high-dose groups). However, the small group sizes limit the power of the statistical analyses conducted in this study to detect significant differences between groups and definitive interpretation of statistical significance and dose-dependency. Furthermore, the very high doses administered in this study limit the relevance of the results to human consumption of mycelia in FPRP at doses approximately 2,000 times lower than the lowest dose in this study. No adverse histopathological findings were reported.

In a study reported by Grotto *et al.* (2016), male Wistar rats (6/group) were administered powdered *L. edodes* reconstituted in water by gavage at doses of 0, 100, 400, or 800 mg/kg body weight/day for 30 days. Reductions in hemoglobin concentration and leukocytes were reported at 400 and 800 mg/kg body weight/day compared to controls; based on these effects, the authors concluded that the safe daily intake of *L. edodes* is 100 mg/kg body weight/day. Based on a direct body weight dose conversion, this dose is approximately 71 times higher than the estimated human consumption of 1.4 mg/kg body weight/day.

C.3.2 Mutagenicity/Genotoxicity

Miyaji *et al*. (2004) assessed the *in vitro* genotoxic and anti-genotoxic effects of aqueous extracts of shiitake mushroom using the Comet assay with Hep-2 cells at test concentrations of 0, 0.5, 1.0, or 1.5 mg/mL and 3 temperatures (4°C, 22°C, or 60°C). The authors reported a "low level" of genotoxic activity at all aqueous extract test concentrations prepared at 22 ± 2 and 60°C and 2 concentrations (1.0 and 1.5 mg/mL) of extract prepared at 4°C. As cytotoxicity data was not reported for this test and the validation status of the performing laboratory is unknown, the results of this study are considered to be of limited reliability. The International Workshop on Genotoxicity Testing (IWGT) has repeatedly concluded that cytotoxicity could be a confounder of Comet assay results, adding that comet assay results are more reliable if obtained in laboratories with demonstrated proficiency (Speit *et al*., 2015). It is also noted that a standardized and validated regulatory testing guideline for the *in vitro* Comet assay is not available and the Organisation for Economic Co-operation and Development (OECD) Test Guideline for the *in vivo* mammalian alkaline Comet assay did not exist until recently (*i.e.,* OECD Test Guideline 489 (*In Vivo mammalian alkaline comet assay*); adopted 29 July 2016 – OECD, 2016). Therefore, Miyaji *et al*. (2004) predated a standardized and validated Comet assay protocol. Furthermore, the Comet assay is not among the standard battery of *in vitro* tests for genetic toxicity assessment of food ingredients or pharmaceuticals recommended by regulatory authorities. In summary, MycoTechnology concludes that this study is of limited relevance to the current safety assessment of shiitake mycelia in the final ingredient.

The same investigators studied the possible anti-genotoxic effects of shiitake mushroom extracts *via* modulation of micronuclei induction after treatment with alkylating agents *in vitro* or *in vivo* (Alves de Lima *et al*., 2001; Miyaji *et al*., 2004). However, these studies are of limited relevance to the current safety assessment and are not discussed further.

In a bacterial reverse mutation assay performed prior to standardized testing guidelines or Good Laboratory Practice (GLP) regulations, a crude ethanol extract of *L. edodes* was reported to have mutagenic activity to tester strains TA100 and TA1535, which are sensitive to base-pair substitutions (von Wright *et al*., 1982). As neither statistical analysis, cytotoxicity, or compositional data were reported, the results of this study are of limited reliability. Additionally, the crude ethanol extract used as the test article is not representative of the LEM used as a processing aid in the production of the FPRP. In consideration of the information above, MycoTechnology concludes that a genotoxic hazard is not likely based on the long history of dietary consumption of *L. edodes* and the low dietary exposure to heat-inactivated shiitake mycelia in the FPRP ingredient.

C.3.3 Reproductive and Developmental Toxicity

Frizo *et al*. (2014) tested the effects of reconstituted *L. edodes* powder (containing 0.53 g β-glucan/100 g mushroom) in a rat (strain not reported) developmental toxicity model. The test compound was administered by gavage at doses of 0 (saline control), 400, or 800 mg/kg body weight/day from Gestational Day (GD) 1 through 20. As this study was available as an abstract only, details of the methods and quantitative results were not available. The fetuses were removed by cesarian section on GD 21. Maternal kidney and liver toxicity were assessed and oxidative stress was determined by measurement of glutathione (GSH). The corpora lutea, implantations, resorptions, and live and dead fetuses were counted. The placentae and fetuses were weighed. External and visceral morphology examinations of fetuses were performed following fixation with Boudin solution. Skeletal evaluations were performed following diaphonization and staining with alizarin red-S. No adverse effects were reported with respect to maternal plasma urea, creatinine, aspartate aminotransferase, or alanine aminotransferase, although a reduction in "glutathione plasma ratio" was reported. It was noted that these effects indicated a lack of maternal toxicity. Increased post-implantation loss, reduced fetal body weight, and fetal "external measurements" were reported, although the statistical or biological significance of these effects and their comparison with controls or historical control ranges were not reported. Given the comparatively high doses used in this study, the history of dietary consumption of shiitake mushrooms, and the lack of availability of study details, the relevance of this study to the current safety assessment is questionable.

C.3.4 Chronic Toxicity and Carcinogenicity

The genotoxicity and subchronic toxicity studies of *L. edodes* discussed above do not raise concern for chronic toxicity or carcinogenicity under the intended use of LEM in the production of the FPRP ingredient as described herein; therefore, a chronic toxicity/carcinogenicity study was not determined to be necessary to support the safety of the processing aid.

C.3.5 Human Studies

Yoshioka *et al*. (2009) evaluated the safety of foods containing an extract of cultured LEM in healthy adult volunteers. The study was published in Japanese with a limited English abstract; therefore details of the methods and results are difficult to discern. Yoshioka *et al.* (2009) evaluated a lyophilized hot water extract of LEM material which was provided to subjects in "granular food." Although an LEM extract would not contain insoluble portions of shiitake mycelia cells, and is therefore not identical in composition to the shiitake mycelia present in the FPRP ingredient, both the LEM extract and shiitake mycelia present in the final FPRP ingredient would likely contain the same water-soluble components. Thus, clinical data pertaining to LEM extract is useful to evaluate the safety of the low levels of shiitake mycelia present in the FPRP fermented with shiitake mycelia.

Eleven healthy adult subjects (8 males and 3 females, ages 33.4 ± 9.4 years) consumed the test foods, providing 5,400 mg LEM extract/day, for 4 weeks. Yoshioka *et al.* (2009) reported no adverse effects resulting from consumption of 5,400 mg LEM extract/day with respect to physical and clinical examinations, except for mild loose stools in 1 subject. The authors concluded that these results demonstrate that LEM is safe at doses up to 5,400 mg/day. The results of this study support the safety of FPRP produced using LEM as a processing aid at estimated consumption levels of up to 86.3 mg/day (1.4 mg/kg body weight/day for a 60-kg adult).

Additional human studies addressing the safety of shiitake mycelial extract are summarized in Appendix I (Okuno and Uno, 2011; Yamaguchi *et al.*, 2011; Nagashima *et al.*, 2013; Suzuki *et al.*, 2013; Choi *et al.*, 2014; Dai *et al.*, 2015; Nagashima *et al.*, 2017). Although these studies were performed to assess the possible therapeutic effects of shiitake mycelial extract on quality of life and immune function, no compound-related adverse events were reported. Therefore, these studies support a conclusion of safety for shiitake mycelia used as a processing aid.

C.4 Safety of Fungal Enzymes

The use of fungal enzymes to modify and improve food products is well established. *L. edodes* is also known to secrete a number of fungal enzymes, such as pectinase (GRN 089) , cellulase (GRNs 292, 479, 584, and 891), amylase (GRNs 022, 024, 079, 126, 594, 617, 664, and 1011), laminarinase (*beta*-glucanase; GRNs 149, 479, 482, 535, and 592), and xylanase (GRNs 054, 195, 479, 482, 567, 589, 675, and 1055 - U.S. FDA, 2001, 2006, 2014a,b, 2015a,b, 2017, 2023), all of which have received no questions from the U.S. FDA regarding conclusions that the enzymes are GRAS for use in foods (Soares *et al.,* 2012; Mata *et al.*, 2016; U.S. FDA, 2018, 2019). Although the source of these enzymes in the GRAS notices submitted to the U.S. FDA is *Aspergillus* (a genus of filamentous fungus closely related to the filamentous fungus genus *Lentinula*), the lack of questions from the U.S. FDA regarding the use of these enzymes in foods provides support for the safety of the same enzymes produced by *L. edodes*. Another group of enzymes, laccases, is known to be constitutively expressed by shiitake mycelia, and their expression may be upregulated or stimulated by the presence of lignin-derived phenols and or polymeric lignin materials (Matjuškova *et al.*, 2017). MycoTechnology concludes that there are no safety concerns associated with these enzymes commonly used for food processing that may be present during the manufacturing process for the FPRP ingredient.

Additionally, the conditions under which the concentration and spray-drying steps of the manufacturing process are conducted (as described in Appendix B) are consistent with conditions that are known to denature and deactivate enzymes (*i.e.,* the fermentation process is terminated by heat treatment followed by an evaporator/concentration step; a thermal deactivation step is carried out at 80°C for 1 minute, followed by spray drying for 1 to 3 minutes [air inlet 250°C; powder outlet 75°C]). Testing for residual laccase enzyme activity in the rice and pea protein concentrate after termination of fermentation was performed according to published methodology[3] and confirmed that no residual laccase enzyme activity was present (MycoTechnology, Inc.; see Appendix E). Based on this information, MycoTechnology concludes that any enzymes secreted by the shiitake mycelia when used as a processing aid are inactivated during the manufacturing of the final FPRP ingredient.

[3] https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-laccase.html.

C.5 Allergenicity

As noted previously, a minimal amount of protein (<0.05%) in the final FPRP ingredient may be contributed by the shiitake mycelium (based on the presence of <0.1% shiitake biomass in the final product). Any shiitake-derived biomass remaining in the final FPRP product, as well as any enzymes secreted by the mycelia during the fermentation process, are expected to be thermally denatured, as described in Appendix B. Unpublished and published clinical trials (summarized below) have shown transient effects such as eosinophilia associated with consumption of shiitake mushroom powder at intake levels at least 46-fold higher than the high-level (95th to 98th percentile) daily estimated intake of 86.3 mg shiitake mycelium/day from consumption of the FPRP ingredient. Shiitake-related dermatitis has also been reported following consumption of fresh or undercooked shiitake mushrooms; however, this is not considered to be relevant to exposure to the minimal amount of shiitake mycelium in the FPRP ingredient due to the high temperatures reached during the manufacturing process. MycoTechnology notes that their FPRP product has been on the market in various countries for years (the U.S., EU, Brazil, Canada, India, Japan, South Korea, Singapore, Malaysia, Indonesia, Philippines, Thailand, Chile, Ecuador, and Hong Kong), with no cases of allergenicity reported.

During a 10-week clinical study of the potential cholesterol-lowering effect of shiitake mushroom powder (4 g/day)[4], 17 of 49 subjects (no demographic details provided) in the treatment arm withdrew from the trial because of either rash (7 subjects) or abdominal discomfort (10 subjects). Two subjects had marked peripheral eosinophilia at the time they stopped ingesting the study product. However, their eosinophilia resolved after discontinuation. The cause for the findings was unknown; however, food allergy was considered unlikely because the reaction developed after prolonged exposure (Levy *et al*., 1998). In the absence of additional information, it is difficult to interpret the findings from this limited study report.

Levy *et al.* (1998) reported on the effect of ingestion of shiitake mushroom powder on the induction of eosinophilia, changes in eosinophil-active cytokines, eosinophil proteins in blood and stool, or gastrointestinal symptoms. This open-label study was conducted in 10 healthy subjects (9 males and 1 female; average age 40.6 years; range, 31 to 63 years). Exclusion criteria included a history of allergy to mushrooms, disease associated with significant eosinophilia, and gastrointestinal disease. Additional exclusion criteria included baseline blood eosinophil counts greater than 500/mm^3, abnormal serum immunoglobin E (IgE) levels, use of prescription medication (except oral contraceptives), and pregnancy. The subjects consumed 4 g shiitake mushroom powder/day for 10 weeks (trial 1), and the same protocol was repeated in these subjects after 3 to 6 months (trial 2). The investigators defined responders as subjects having peak eosinophil counts ≥4 times their average baseline counts. Each trial had 4 responders, and trial 2 had 1 new and 3 repeat responders. Responders had increased blood eosinophils, serum major basic protein, stool eosinophil-derived neurotoxin, and factors that enhanced eosinophil viability. Anti-shiitake IgE was not detected, but anti-shiitake IgG was increased in 2 responders. Gastrointestinal symptoms coincided with eosinophilia in 2 subjects. Gastrointestinal symptoms (*e.g.,* abdominal cramping, more frequent and loose stools) and eosinophilia resolved after discontinuing shiitake ingestion. The authors stated that eosinophilic response to shiitake does not appear to be a typical allergic reaction because of the inability to detect anti-shiitake IgE and by the delayed and gradual time-course of the response. However, the authors concluded, "*the response is likely immune-mediated because it is associated with cytokines that enhance eosinophil viability and elevations in anti-shiitake IgG in two of the five responders*."

[4] Jacobson D, Hill JO (1994) [Personal Communication]. University of Colorado. Summarized by Levy *et al.*, 1998.

Although the results of these unpublished and published clinical trials reported by Levy *et al*. (1998) show transient effects associated with consumption of shiitake mushroom powder at a dose of 4 g/day for 10 weeks, this intake level is at least 46-fold higher than the high-level (95th to 98th percentile) daily estimated intake of 86.3 mg shiitake mycelium from FPRP. Therefore, MycoTechnology concludes that similar adverse effects resulting from consumption of FPRP are highly unlikely.

The potential for shiitake mushroom-induced dermatitis in some consumers is well documented in the literature (select case reports are summarized in Appendix I) (Nakamura, 1992; Hanada and Hashimoto, 1998; Levy *et al.*, 1998; Kopp *et al.*, 2009; Czarnecka *et al*., 2014; Johnson *et al*., 2016; Fang *et al*., 2017; Nagarajan *et al*., 2017; Didona *et al*., 2018; Forward *et al*., 2018; Maher *et al*., 2018; Santos *et al*., 2018; Ribeiro *et al*., 2019). Nguyen *et al*. (2017) discussed the clinical features of shiitake dermatitis in a recent systematic review of the literature. Nguyen's review identified 50 reported patient cases (38 males, 12 females; mean age: 44.58 years) of this "rare" cutaneous reaction, and identified that "the majority" of cases were reported after consumption of raw mushrooms (93% of cases were associated with raw, lightly cooked, or undercooked mushrooms (Nguyen *et al*., 2017). The authors reported that shiitake dermatitis "is self-limiting, resolving in approximately 12.5 d without treatment." The authors postulated that a heat-labile *beta*-glucan in the cell walls of shiitake mushrooms (lentinan) may be responsible for the dermatitis observed in sensitive individuals.

Experiments conducted by another investigator have shown a presumed association between lentinan exposure and dermatitis by demonstrating a cutaneous response to the consumption of shiitake mushrooms cooked at 100°C but not to those cooked at 150°C (Nguyen *et al*., 2017). Since lentinan is expected to degrade at 150°C, heat processing of shiitake mycelia would likely minimize shiitake dermatitis from consumption of the FPRP fermented with shiitake mycelia. As noted in Section C.4, the manufacturing process of the FPRP fermented with shiitake mycelia involves thermal deactivation (80°C for 1 minute) and high-temperature concentration and spray-drying steps (air inlet 250°C; powder outlet 75°C), which are sufficient to denature and deactivate enzymes and lentinan. MycoTechnology concludes that the rarely reported cutaneous shiitake dermatitis is very unlikely to occur from consumption of FPRP fermented with shiitake mycelia due to heat-treatment steps during the manufacturing process, and the low dietary exposure to shiitake mycelia in the final product (86.3 mg/day at the high level [95th to 98th percentile] of intake).

C.6 Pathogenicity and Toxigenicity

As shown in Section C.1.3, the shiitake mycelium is compositionally equivalent to the shiitake fruiting body (*i.e.,* shiitake mushrooms). The very long history of consumption of shiitake mushrooms supports that this species does not produce toxicologically significant amounts of mycotoxins or toxic metabolites.

MycoTechnology has conducted an *in silico* analysis for the presence of proteins involved in the synthesis of mycotoxins. The results of this analysis (shown in Appendix F) confirm that *L. edodes* does not express proteins that match significantly with 12 known mycotoxin-producing proteins. MycoTechnology considers that, given the results of the *in silico* analysis and the long history of dietary consumption of shiitake mushrooms, no further testing is required to demonstrate the species' lack of pathogenicity or toxigenicity.

C.7 Authoritative Reviews/Approvals – the United States, the European Union, Brazil, and India

MycoTechnology received a "no objection" letter from the FDA in 2020 regarding the GRAS status of their FPRP ingredient, for use at levels ranging from 1.04 to 33.3% in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, dairy product analogues, fats and oils, grain products and pastas, milk products, plant proteins and products, processed fruits and fruit juices, processed vegetable and vegetable juices, soups and soup mixes, non-baked goods (bars), and confectionaries (U.S. FDA, 2020).

MycoTechnology has also received a positive opinion from EFSA in 2022 (EFSA, 2022) regarding the use of their FPRP ingredient as a novel food ingredient. The ingredient is intended for use at levels ranging from 1.04 to 93.3 g/100 g in baked goods, beverages, breakfast cereals, dairy product analogues, confectionaries, salad dressings, processed meat and meat products, flavored milk drinks, meal replacements, yogurt, pasta, meat alternatives, and soups. The European Commission has authorized the FPRP as a novel food (EU 2023/6 – EU, 2023). MycoTechnology's FPRP ingredient is also permitted for use in India and Brazil for uses and at use levels similar to those permitted in the EU.

Although the subject of the evaluations conducted by EFSA, the U.S. FDA, FSSAI, and the Brazilian Health Regulatory Agency (ANVISA) was MycoTechnology's FPRP, the safety and suitability of the *L. edodes* mycelium as a processing aid were considered as part of these evaluations. Therefore, the lack of questions from the U.S. FDA and the positive opinions from EFSA, FSSAI, and ANVISA regarding the use of MycoTechnology's FPRP as a food ingredient support the safety of the *L. edodes* processing aid used to produce the FPRP ingredient.

In addition, MycoTechnology's FPRP is marketed in the following countries based on in-country registration after regulatory suitability was assessed: Canada, Japan, South Korea, Singapore, Malaysia, Indonesia, the Philippines, Thailand, Chile, Ecuador, and Hong Kong.

C.8 Summary

Overall, the safety of shiitake mycelia for its intended conditions of use as a processing aid in the manufacturing of MycoTechnology's FPRP ingredient is supported based on the following:

1. A long history of safe dietary consumption of shiitake mushrooms;

2. The approval of a mycelial extract of *L. edodes* as a novel food ingredient in the EU (since 2011) and the lack of questions from the U.S. FDA (2020);

3. A high degree of compositional equivalence between the processing aid (shiitake mycelia) and the shiitake fruiting body;

4. A lack of toxicologically relevant, compound-related adverse effects reported at doses relevant to human exposure in well-designed toxicological studies conducted with the shiitake fruiting body; and

5. The approval by EFSA and the lack of questions from the U.S. FDA for the FPRP ingredient manufactured using the *L. edodes* processing aid that is the subject of this application.

D. INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID

In accordance with Section 3.3.2 – Processing Aids of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), the following technical information is provided:

1. A list of foods or food groups likely to contain the processing aid or its metabolites
2. The levels of residues of the processing aid or its metabolites for each food or food group
3. For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption
4. The percentage of the food group in which the processing aid is likely to be found or the percentage of the market likely to use the processing aid
5. Information relating to the levels of residues in foods in other countries
6. For foods where consumption has changed in recent years, information on likely current food consumption

Each point is addressed in the following subsections.

D.1 Proposed Use and Use Level

Shiitake mycelia is intended for use as a processing aid in the manufacturing of FPRP. In accordance with Section 3.3.2 – F of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), information pertaining to the intended uses and use levels of FPRP in the EU are provided herein. MycoTechnology confirms that the FPRP ingredient is intended to be used under the same conditions of use in Australia and New Zealand as those permitted in the EU. The intended use levels and the food categories to which the FPRP ingredient will be added are summarized in Table D.1-1 below. Use levels for the ingredient range from 1.04 to 40 g/100 g, with the processing aid comprising ≤0.1% of the final ingredient. MycoTechnology's shiitake mycelia will be used exclusively as a processing aid in the manufacture of FPRP fermented with shiitake mycelia.

Table D.1-1 European Union Food Categories and Maximum Use Levels of the Rice and Pea Protein Concentrate Fermented with Shiitake Mycelia Intended by the Applicant

EU Food Category	Proposed Food Uses	Proposed Maximum Use Level (g ingredient/100 g)[a]
Unflavoured fermented milk products, heat-treated after fermentation (1.3)	Yogurt	5
Flavoured fermented milk products including heat-treated products (1.4)	Yogurt	5

Table D.1-1 European Union Food Categories and Maximum Use Levels of the Rice and Pea Protein Concentrate Fermented with Shiitake Mycelia Intended by the Applicant

EU Food Category	Proposed Food Uses	Proposed Maximum Use Level (g ingredient/100 g)[a]
Dairy analogues, including beverage whiteners (1.8)	Non-dairy frozen desserts; Other dairy imitates (*e.g.*, non-dairy cheese, cream, coffee creamer); Soy/nut plant-based beverages	11
Cocoa and Chocolate products as covered by Directive 2000/36/EC (5.1)	Chocolate-coated confectionary	7
Breakfast cereals and cereal bars (6.3)	Health bars and grain-based bars; Health bars and grain-based bars containing fruit and vegetables; Ready-to-eat breakfast cereals	33
Fresh pasta (6.4.1)	Fresh pasta	15
Dry pasta (6.4.2)	Dry pasta	15
Fresh pre-cooked pasta (6.4.3)	Fresh pre-cooked pasta	15
Noodles (6.5)	Noodles	15
Bread and rolls (7.1)	Bread and rolls	5
Non-heat-treated processed meat (8.3.1)	Processed meat and meat products (*e.g.*, lasagna, meatloaf, sausage, meat patties, ham)	14
Heat-treated processed meat (8.3.2)	Processed meat and meat products (*e.g.*, lasagna, meatloaf, sausage, meat patties, ham)	14
Seasonings and condiments including salad dressings (12.2.2)	Salad dressings	26
Soups and broths (12.5)	Prepared soups, dry soup mixes, and condensed soups (use levels given for soup as consumed)	3.3
Meat alternatives (12.9)	Meat alternatives	40
Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet) (13.3)	Non-milk based individual meal replacements for weight reduction	11
	Milk-based individual meal replacements for weight reduction	1.04
Fruit juices as defined by Directive 2001/112/EC and vegetable juices and mixed fruit and vegetable juices, multivitamin juices and other (mixed) fruit and vegetable juices or nectars (14.1.2)	Fruit juices	1.04
	Vegetable juices	20
Fruit nectars as defined by Directive 2001/112/EC and vegetable nectars and similar products (14.1.3)	Fruit nectar	1.04
Flavoured drinks (14.1.4)	Flavored milk drinks	1.04
	Ready-to-mix beverage powder	33
	Smoothies	20

EC = European Commission; EU = European Union.
[a] Maximum use levels for the ingredient are calculated based on a purity of 75%. Most calculations are mass into volume assuming water.

D.2 Estimated Intake from Proposed Food Uses

D.2.1 Methodology

As mentioned in Section D.1, shitake mycelia will be present at levels of not more than 0.1% in the final FPRP ingredient. Therefore, to determine the estimated intake of the processing aid, the estimated intake of the final ingredient was also determined. This determination is presented below in Sections D.2.2 and D.2.3.

D.2.2 Anticipated Intake of the Rice and Pea Protein Concentrate

The anticipated intake of the FPRP ingredient was estimated based on food consumption data from the EFSA Comprehensive Food Consumption database, for which summary statistics have been published by EFSA and are available at https://www.efsa.europa.eu/en/food- consumption/comprehensive-database. A report of this exposure assessment is included as Appendix J.

At the request of EFSA, Competent Authorities in European countries submitted data on the level of food consumption by the individual consumer, based on the most recent national dietary survey conducted in their country. Food consumption data (a minimum of 2 non-consecutive days of data) for the following countries were collated in the current version of the Comprehensive Database: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Spain, Sweden, and the United Kingdom (UK). Each of these dietary surveys is composed of separate population groups.[5]

Due to the aggregated nature of this survey, data are reported by food category rather than for individual intakes and, as such, represent a highly conservative or worst-case scenario for the estimated intake of the ingredient. Also, as the FPRP ingredient is intended to be used as an alternative product to those protein concentrates already available at similar inclusion levels, MycoTechnology anticipates that the intended uses of FPRP will not result in an increase in the overall consumption of protein.

A summary of estimated intakes of FPRP using food intake data from the EFSA Comprehensive Database is presented below in Tables D.2.2-1 and D.2.2-2. The estimated daily intake of FPRP for each population group from each EU dietary survey is available in Appendix J.

Based upon the maximum proposed use levels for the final ingredient and conservatively assuming concurrent consumption of all proposed food categories, the mean intakes of rice and pea protein concentrate in the total population ranged from 0.8 to 7.4 g/day in infants to 13.4 to 30.6 g/day in adolescents. On a body weight basis, mean intakes ranged from 0.2 to 0.3 g/kg body weight/day in the elderly and very elderly, to 0.5 to 1.4 g/kg body weight/day in toddlers.

[5] https://www.efsa.europa.eu/en/microstrategy/food-consumption-survey.

High-level intakes ranged from 6.3 to 43.4 g/day in infants to 31.9 to 86.3 g/day in adults. On a body weight basis, intakes ranged from 0.4 to 0.7 g/kg body weight/day in the very elderly to 0.8 to 5.9 g/kg body weight/day in infants. High-level intake was calculated in DaDiet software using the High Exposures from Summary Statistics (HESS) model based on total population intake, and corresponds to an intake between the 96th and 98th percentiles for infants, between the 95th and 97th percentiles for adolescents, elderly, and the very elderly, and between the 95th and 98th percentiles for toddlers, children, and adults. HESS is a model built into DaDiet derived from mathematical principles. The use of the HESS model for deriving high-level estimates of exposure is described in the publication by Dempsey (2018) and is cited in a recent novel food application reviewed by EFSA (2020). Depending on the number of food groups of interest, an individual can be a high consumer of a specific number of food groups. Unlike other methods which use the consumer-only 95th percentile as the high-intake value for a high consumer, the HESS method uses a percentile of the total population intake as the high-intake value. This percentile depends on the number of groups of interest, and the consumption probability of the groups. This method accepts presence probability and concentration as inputs. The HESS model was used in DaDiet to derive the results of mean intake and high-level intake of the FPRP ingredient from its proposed uses using data from the Comprehensive Database.

Table D.2.2-1 Estimated Intake of the Rice and Pea Protein Concentrate from Maximum Proposed Food Uses (g/day)

Population	Ages	Number of Surveys	Mean Intake (g/day)		High-level (>95th Percentile)[a] Intake (g/day)	
			Lowest[b]	Highest[b]	Lowest[b]	Highest[b]
Infants	Up to 12 months	9	0.8	7.4	6.3	43.4
Toddlers	12 to 35 months	12	5.3	19.4	18.7	70.9
Children	3 to 9 years	23	12.1	26.7	30.6	73.0
Adolescents	10 to 17 years	21	13.4	30.6	30.1	83.6
Adults	18 to 64 years	22	15.5	29.3	31.9	86.3
Elderly	65 to 74 years	17	11.7	23.2	31.7	74.9
Very Elderly	≥75 years	8	10.6	21.9	30.2	54.3

EU = European Union; HESS = High Exposures from Summary Statistics.
[a] High-level intake was calculated in DaDiet software using the HESS model based on total population intake and corresponds to an intake between the 96th and 98th percentiles for infants, between the 95th and 97th percentiles for adolescents, elderly, and the very elderly, and between the 95th and 98th percentiles for toddlers, children, and adults.
[b] Intakes are assessed for all EU dietary surveys available in the food comprehensive database. The lowest and the highest averages observed among all EU surveys are reported in these columns.

Table D.2.2-2 Estimated Intake of the Rice and Pea Protein Concentrate from Maximum Proposed Food Uses (g/kg bw/day)

Population	Ages	Number of Surveys	Mean Intake (g/kg bw/day)		High-level (>95th Percentile)[a] Intake (g/kg bw/day)	
			Lowest[b]	Highest[b]	Lowest[b]	Highest[b]
Infants	Up to 12 months	9	0.1	0.9	0.8	5.9
Toddlers	12 to 35 months	12	0.5	1.4	1.9	5.1
Children	3 to 9 years	23	0.6	1.3	1.3	3.3
Adolescents	10 to 17 years	21	0.3	0.7	0.6	1.7

Table D.2.2-2 Estimated Intake of the Rice and Pea Protein Concentrate from Maximum Proposed Food Uses (g/kg bw/day)

Population	Ages	Number of Surveys	Mean Intake (g/kg bw/day)		High-level (>95th Percentile)[a] Intake (g/kg bw/day)	
			Lowest[b]	Highest[b]	Lowest[b]	Highest[b]
Adults	18 to 64 years	22	0.2	0.4	0.4	1.3
Elderly	65 to 74 years	17	0.2	0.3	0.4	0.9
Very Elderly	≥75 years	8	0.2	0.3	0.4	0.7

bw = body weight; EU = European Union; HESS = High Exposures from Summary Statistics.
[a] High-level intake was calculated in DaDiet software using the HESS model based on total population intake and corresponds to an intake between the 96th and 98th percentiles for infants, between the 95th and 97th percentiles for adolescents, elderly, and the very elderly, and between the 95th and 98th percentiles for toddlers, children, and adults.
[b] Intakes are assessed for all EU dietary surveys available in the food comprehensive database. The lowest and the highest averages observed among all EU surveys are reported in these columns.

D.2.3 Anticipated Intake of the Shiitake Mycelia as a Processing Aid

At a maximum concentration of <0.1% w/w shiitake mycelia in the final FPRP ingredient, and assuming the worst-case estimated high-level intake (95th to 98th percentile) of FPRP of 86.3 g/person/day for adults (Table D.2.2-1 and Table D.2.2-2), MycoTechnology concludes that the corresponding high-level intake of shiitake mycelia is 86.3 mg/day. At <0.1% w/w shiitake mycelia in FPRP, and assuming a more realistic-case estimated mean intake of the ingredient of 29.3 g/person/day for adults (Table D.2.2-1 and Table D.2.2-2), MycoTechnology concludes that the corresponding level of intake of shiitake mycelia is 29.3 mg/day. At a mean body weight of 60 kg, this would result in an intake of approximately 1.4 mg shiitake mycelia/kg body weight/day at the 95th to 98th percentile level of estimated daily consumption of the FPRP ingredient or 0.48 mg shiitake mycelia/kg body weight/day at the mean level of estimated daily consumption for adults.

There are no undesirable substances expected from dietary exposure to FPRP or shiitake mycelia based on the history of dietary consumption of shiitake, MycoTechnology's manufacturing conditions, and the proposed uses of the final ingredient.

D.3 Global Use of Shiitake Mycelia

Shiitake mycelia as a processing aid is currently used only in the manufacturing of MycoTechnology's FPRP ingredient, which is permitted for use as an ingredient in various foods and beverages in the U.S. and the EU. In the U.S., the ingredient is GRAS for use at levels ranging from 1.04 to 33.3% in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, dairy product analogues, fats and oils, grain products and pastas, milk products, plant proteins and products, processed fruits and fruit juices, processed vegetable and vegetable juices, soups and soup mixes, non-baked goods (bars), and confectionaries (U.S. FDA, 2020). In the EU, the ingredient is permitted for use at levels ranging from 1.04 to 40 g/100 g in baked goods, beverages, breakfast cereals, dairy product analogues, confectionary, salad dressings, processed meat and meat products, flavored milk drinks, meal replacements, yogurt, pasta, meat alternatives, and soups. Notably, FPRP is intended for use in foods and beverages in Australia and New Zealand under identical conditions of use as those permitted in the EU. In the U.S. and the EU, the FPRP ingredient contains no more than 0.1% of the shiitake mycelia processing aid.

D.4 For Foods Where Consumption has Changed in Recent Years, Information on Likely Current Food Consumption

According to a recent report published by the Commonwealth Scientific and Industrial Research Organisation (CSIRO), the plant-derived protein sector is growing exponentially in Australia (CSIRO Futures, 2022). It was estimated in this report that the market for plant-based proteins in Australia is expected to grow from 140 million to 3.0 billion annually by the year 2030, with alternative protein sources (*i.e.,* plant-based, fermentation, and insect) expected to outpace growth in traditional meat sectors by a substantial margin. The use of MycoTechnology's shiitake mycelia processing aid, and by extension, the FPRP product, will contribute to CSIRO's objective of Australia becoming "a global leader in high quality, value-added protein," and will allow for manufacturers of food products and consumers to have a wider variety of nutritious plant-based protein dietary choices.

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