APPLICATION TO AMEND THE AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE TO ALLOW FOR THE USE OF SOY LEGHEMOGLOBIN

PREPARED FOR:
Standards Management Officer
Food Standards Australia New Zealand
Ground Floor
Boeing House
55 Blackall Street
Barton Act 2600
Australia

SUBMITTED BY:
Impossible Foods Inc.
400 Saginaw Drive
Redwood City, CA
94063 USA

DATE:
12 July 2019

Application to Amend the Australia and New Zealand Food Standards Code to Allow for the Use of Soy Leghemoglobin

TABLE OF CONTENTS

List of Appendices

List of Figures and Tables

Application to Amend the Australia and New Zealand Food Standards Code to Allow for the Use of Soy Leghemoglobin

GENERAL REQUIREMENTS

This section is completed in accordance with Chapter 3.1 (General Requirements for Applications) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019).

1. Applicant Details

Contact Information

Applicant: Impossible Foods Inc.
Name of Contact Person:
Mailing address: 400 Saginaw Drive
Redwood City, CA
94063 USA
Telephone:
Email address:

Nature of Applicant's Business

Impossible Foods Inc. (Impossible Foods) started in 2011 with the goal of producing delicious and sustainable plant-based alternatives to meat, fish, and dairy foods. The first product to be commercialised by the company was the Impossible™ Burger, a ground beef analogue product containing soy leghemoglobin derived from *Pichia pastoris* (*P. pastoris*). Impossible Foods developed soy leghemoglobin as a heme-containing ingredient that will impart meat-like characteristics to meat analogue products, such as meatballs, sausage, or as fillings in buns and dumplings.

2. Purpose of the Application

Impossible Foods is submitting this application to amend the Australia New Zealand Food Standards Code (referred to hereafter as the Code) to allow for the use of soy leghemoglobin derived from *P. pastoris* as a component in meat analogue products (such as the Impossible™ Burger) that will be marketed in Australia/New Zealand. Soy leghemoglobin is intended for addition to meat analogue products to provide the nutrition (*i.e.*, source of iron), flavour, and aroma of their traditional animal-derived counterpart.

Soy leghemoglobin is naturally present in the root nodules of the soybean plant (*Glycine max*) (O'Brian *et al*., 1987). As detailed in this application, the *P. pastoris* production strain contains modifications allowing it to express soy leghemoglobin. Following fermentation, the cells are lysed, and the soy leghemoglobin is concentrated by physical means. The soy leghemoglobin is delivered in a liquid preparation (LegH Prep) that is standardised to contain up to 9% soy leghemoglobin on a wet weight basis and a soy leghemoglobin protein purity of at least 65%. The remainder of the protein fraction in the LegH Prep is accounted for by residual proteins from the *P. pastoris* production strain. Small amounts of residual *Pichia* DNA (about 300 mg/L) may also be present in the LegH Prep.

Impossible Foods intends to use soy leghemoglobin only as a component within their meat analogue products, at levels delivering not more than 0.8% soy leghemoglobin. The meat analogue products containing soy leghemoglobin will be marketed in retail outlets such as grocery stores and restaurants in Australia/New Zealand. The soy leghemoglobin derived from *P. pastoris* will not be made available for purchase and use by other food manufacturers, nor will it be sold directly to consumers.

The soy leghemoglobin produced by Impossible Foods is considered to meet the definition of a "non-traditional food", as defined in Standard 1.5.1 of the Code, on the basis that it is a "*substance, where that substance, or the source from which it is derived, does not have a history of human consumption as a food in Australia or New Zealand*". It is also considered to meet the definition of a novel food as defined in the Code, given that it is a non-traditional food requiring an assessment of the public health and safety considerations. Furthermore, since the soy leghemoglobin is produced by fermentation with genetically modified *P. pastoris*, and that residual protein and DNA from the source organism remain in the final ingredient, consideration needs to be given to the provisions within Standard 1.5.2 for "food produced using gene technology", which are defined to mean "*a food which has been derived or developed from an organism which has been modified by gene technology*", with "gene technology" defined further as "*recombinant DNA techniques that alter the heritable genetic material of living cells or organisms*". It is the view of Impossible Foods that the following Schedules in the Code will need to be amended to allow for the use of soy leghemoglobin derived from *P. pastoris* in the meat analogue products that will be marketed in Australia/New Zealand:

- Schedule 3 Identity and purity
- Schedule 25 Permitted novel foods
- Schedule 26 Food produced using gene technology

In addition to soy leghemoglobin, the meat analogue products marketed by Impossible Foods may contain various vitamins and minerals. As such, the provisions in Schedule 17 of the Code pertaining to vitamins and minerals in "analogues derived from legumes", including "analogues of meat, where no less than 12% of the energy value of the food is derived from protein, and the food contains 5 g protein per serve of the food" may also require amendments to allow for the presence of these substances in meat analogue products containing soy leghemoglobin.

3. Justification for the Application

3.1 Regulatory Impact Information

3.1.1 Costs and Benefits of Application

a) Impact on the Consumer

The approval of soy leghemoglobin produced by Impossible Foods, and the introduction of meat analogue products containing soy leghemoglobin, will provide consumers with more choices for plant-based food products in the Australia/New Zealand marketplace. Consumers (both meat-eaters and vegetarians alike) who seek out plant-based alternatives will have the option to purchase meat analogue products that are designed to mimic the nutrition (*i.e.*, provide a source of iron), flavour, and aroma of the animal-derived counterpart. Soy leghemoglobin is a key ingredient that is responsible for some of these attributes. Consumers will benefit from having access to a nutritious and flavourful alternative to foods derived from animals, with a much-reduced environmental impact.

The proposed amendments will not place any additional economic costs to the consumers. Consumers are free to choose between purchasing meat analogue products containing soy leghemoglobin, or comparator products of other plant-based meat analogue products on the market.

b) Impact on the Industry

The approval of Impossible Foods' soy leghemoglobin, and introduction of meat analogue products containing this ingredient, is not expected to negatively impact the industry. Meat analogue products are already available for purchase in Australia/New Zealand, and they are targeted towards individuals who seek out such products for various reasons of personal preference (*e.g.*, health, ethical, religious, environmental). The introduction of meat analogue products containing soy leghemoglobin will promote culinary innovations within Australia/New Zealand and may benefit the local retailers (grocery stores, restaurants) who carries such products.

c) Impact on the Government

The approval of Impossible Foods' soy leghemoglobin in Australia/New Zealand, and the subsequent marketing of meat analogue products containing this ingredient, is not expected to have any negative impact on government agencies (*e.g.*, increased regulatory costs). A shift toward the increased consumption of plant-based protein sources in the diet may be beneficial for the environment and for public health.

3.2 Impact on International Trade

Meat analogue products containing soy leghemoglobin derived from *P. pastoris* are already being marketed in the United States (U.S.), Singapore, Hong Kong, and Macao, and Impossible Foods is currently in the process of obtaining market authorisation in other jurisdictions. Approval of Impossible Foods' soy leghemoglobin for use in meat analogue products in Australia/New Zealand will promote international trade and reduce technical barriers to trade, while continuing to protect public health and safety.

4. Information to Support the Application

This application is prepared in accordance with the relevant sections within the *Food Standards Australia New Zealand Application Handbook* (from 01 March 2016), including the following:

- Chapter 3.1 General requirements for applications
- Chapter 3.5 Guidelines for applications for new foods
    - Guideline 3.5.1 Foods produced using gene technology
    - Guideline 3.5.2 Novel foods
- Chapter 3.3 Guidelines for applications for substances added to food
    - Guideline 3.3.3 Substances used for a nutritive purpose

Information is provided in this application to enable the objectives specified in Section 18 of the Food Standards Australia New Zealand (FSANZ) Act, which includes:

- 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 the soy leghemoglobin and *P. pastoris* published since Impossible Foods first notified the U.S. Food and Drug Administration (FDA) of their conclusion that their soy leghemoglobin is Generally Recognized as Safe (GRAS), a comprehensive search of the published scientific literature was conducted for the period spanning from 2016 through May 2019 using the electronic search tool, ProQuest Dialog™. 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, and ToxFile®.

As detailed in Section C, no published studies were identified that suggested allergic, toxic or adverse health effects related to consumption of soy leghemoglobin or *P. pastoris* proteins.

5. Assessment Procedure

Impossible Foods considers the Major Procedure (Subdivision F of the FSANZ Act) to be the most appropriate assessment procedure for this application.

6. Confidential Commercial Information (CCI)

Confidential commercial information, in relation to food, is defined in Subsection 4(1) of the FSANZ Act as meaning:

- A trade secret relating to food; or

- Any other information relating to food that has a commercial value that would be, or could reasonably be expected to be, destroyed or diminished if the information were disclosed.

Impossible Foods requests the information contained within the following appendices be considered confidential commercial information:

- Appendix III Details of the Manufacturing Process
- Appendix IV Internal Methods of Analysis

The information contained within these appendices is not publicly available and release of these data would be at a commercial disadvantage to Impossible Foods. The company has invested considerable capital to develop the production organism and to optimise the production process for their soy leghemoglobin. A non-confidential description of the production organism and the manufacturing process employed is provided in Section B.4. Impossible Foods has also invested in the development and validation of the methods of analyses for the soy leghemoglobin. The types of techniques that are used to detect and quantify soy leghemoglobin within the LegH Prep are named in Table B.6.1-1, and a non-confidential description of the method of analysis for detecting soy leghemoglobin in finished food products (*e.g.*, meat analogues) is provided in Section B.7.

7. Other Confidential Information

Impossible Foods requests that the information contained in Appendices III (Details of Production Strain and Manufacturing) and IV (Internal Methods of Analysis) remain confidential. These Appendices contain Confidential Commercial Information, as justified in Point 6 above. No other confidential information is included in this application.

8. Exclusive Capturable Commercial Benefit (ECCB)

As mentioned, Impossible Foods will not sell their soy leghemoglobin ingredient to other food manufacturers, or directly to consumers for use in foods. The soy leghemoglobin will be used only as an ingredient in meat analogue products that are manufactured by Impossible Foods, and only the finished meat analogue products containing the soy leghemoglobin will be sold in Australia/New Zealand.

Thus, it is anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the FSANZ Act, which states:

> "*An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food regulatory measure under Section 22 if:*
>
> *(a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that would be prepared in relation to the application; and*
>
> *(b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application*".

9. International and Other National Standards

There are no Codex Alimentarius Commission standards that are relevant to Impossible Foods' soy leghemoglobin for use in meat analogue products. In the U.S., Impossible Foods' soy leghemoglobin has GRAS status for uses in ground beef analogue products, at levels of not more than 0.8% soy leghemoglobin. The GRAS status of soy leghemoglobin was notified to the offices of the U.S. FDA and filed by the Agency without questions under GRAS Notice number (GRN) 737 (U.S. FDA, 2018a). Impossible Foods intends on expanding the uses of soy leghemoglobin in the U.S. to include other meat analogue products (*i.e.*, pork analogues); under the proposed uses, soy leghemoglobin would be added to beef analogue products at up to 0.45%, and to pork analogue products at up to 0.25%.

In August 2018, the Agri-Food & Veterinary Authority (AVA) of Singapore[1] concluded that they have no objections to the use of Impossible Foods' soy leghemoglobin as a food additive/ingredient, and permission to begin commercial sales of products containing the ingredient was granted at that time. On 27 February 2019, soy leghemoglobin was included into the *List of Other Food Additives/Ingredients that are Permitted Under the Singapore Food Regulations* (SFA, 2019), which specifies that: "*Soy leghemoglobin derived from genetically modified Pichia pastoris may be used in plant-based meat analogues at levels up to 0.45% w/w*".

[1] The Agri-Food & Veterinary Authority of Singapore has been recently restructured, with the food-related services now being under the purview of the Singapore Food Agency (SFA) (effective 1 April 2019).

Based on the U.S. FDA's "no questions" response letter to the GRAS Notice for soy leghemoglobin, and the acceptance of the Singapore AVA for its use as a food additive/ingredient, soy leghemoglobin is considered to meet the food regulatory requirements in Hong Kong and it has been in commerce there. By extension, products containing soy leghemoglobin have also been distributed into Macao.

10. Statutory Declaration

A signed Statutory Declaration is provided in Appendix I.

11. Checklist

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

A. EXCLUSIVE USE OF NOVEL FOODS

This section is completed in accordance with Section A of Guideline 3.5.2 – Novel foods – of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), which states the following information are to be provided:

- A statement as to whether the application is seeking exclusive permission for the novel food. If exclusive permission is sought, the application must include details of the following:
    - The specific class of food; and
    - The brand of the food, including the name the food will be marketed under (if known).

Impossible Foods is not seeking exclusive permission to market their soy leghemoglobin for 15 months, should it be approved by FSANZ as a novel food.

B. TECHNICAL INFORMATION

Technical information on the soy leghemoglobin produced by Impossible Foods is described in this section. Specifically, this section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel foods) and Guideline 3.5.1 (Foods produced using gene technology) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019). The corresponding sections of this application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	Required Information Described in the Guideline	Section of the Application where this is Addressed
Guideline 3.5.2 – Novel foods	B. Technical information on the novel food	
	B.1 Information on the type of novel food	Section B.1
	B.2 Information on the purpose of adding a novel food ingredient to food	Section B.2
	B.3 Information on the physical and chemical properties of the novel food or novel food ingredient	Section B.3
	B.4 Information on the impurity profile for a typical preparation	Section B.5
	B.5 Manufacturing process for a novel food ingredient	Section B.4
	B.6 Specification for identity and purity for a novel food ingredient	Section B.6
	B.7 Analytical method for detection of a novel food ingredient	Section B.7
Guideline 3.5.1 – Foods produced using gene technology	A. Technical information on the food produced using gene technology	
	A.1 Nature and identity of the genetically modified food	Sections B.4, C.5
	A.2 History of use of the host and donor organisms	Sections B.4, C.1, C.5
	A.3 The nature of the genetic modification	Section B.4
Guideline 3.3.3 – Substances used for a nutritive purpose	A. Information on the use of the nutritive substance	
	A.1 Information on the purpose of the use of a nutritive substance in food	Section B.2
	A.2 General data requirements for supporting evidence	Section B.2
	B Technical information on the use of the nutritive substance	
	B.1 Information to enable identification of the nutritive substance	Section B.3, B.4
	B.2 Information on the chemical and physical properties of the nutritive substance	Section B.3
	B.3 Information on the impurity profile	Section B.5
	B.4 Manufacturing process	Section B.4
	B.5 Specification for identity and purity	Section B.6
	B.6 Analytical method for detection	Section B.7
	B.7 Information on the proposed food label	Sections B.3, F.3

B.1 Information on the Type of Novel Food

Impossible Foods obtains soy leghemoglobin by the submerged fermentation of a *P. pastoris* strain that has been genetically modified to express this protein. Following fermentation, the cells are lysed, then physical separation techniques are applied to remove insoluble materials and concentrate the soy leghemoglobin. The resulting liquid concentrate (LegH Prep) is a mixture containing the soy leghemoglobin, residual *P. pastoris* (yeast) proteins, and added stabilisers (*e.g.*, sodium ascorbate and sodium chloride).

Of the major novel food categories listed in Section 3.5.2 – Novel Foods – of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), the soy leghemoglobin produced by Impossible Foods is most appropriately classified as:

- Food ingredients derived from a new source

B.2 Information on the Purpose of Adding a Novel Food Ingredient to Food

Impossible Foods intends to use soy leghemoglobin as a component within their meat analogue products. These products are largely intended for consumption by the general population (age 2 years and older). The primary purpose of adding soy leghemoglobin to meat analogue products is to mimic the flavour and aroma of the animal-derived counterpart. In addition, soy leghemoglobin has nutritive value as a source of iron, being analogous to the role of myoglobin as an iron source in red meat. Once cooked and digested, both soy leghemoglobin and animal-based myoglobin release identical heme B molecules into the digestive system (see Section C.2). Studies using cell models of iron bioavailability have shown that the bioavailability of iron in soy leghemoglobin is equivalent to that of bovine myoglobin when in a food-like substrate (Proulx and Reddy, 2006). Unlike conventional red meat analogue products (*i.e.*, veggie or soy burgers) that typically provide only non-haem iron, the addition of soy leghemoglobin to meat analogue products specifically, will provide levels of haem iron that are comparable to those present in an equivalent serving of red meat. A further discussion of the nutritional impact of soy leghemoglobin is provided in Section E.

Thus, the addition of soy leghemoglobin to meat analogue products will enhance both the flavour and the nutritional profile of those products. These products offer the juicy, meaty taste that meat-eaters crave, and they also appeal to vegetarians and vegans since it is made without animal products.

B.3 Information on the Physical and Chemical Properties

B.3.1 Common or Usual Name

Soy leghemoglobin[2] (UniProtKB/Swiss-Prot #: P02236, GeneInfo Identifier [GI] 126241) is the principal component of LegH Prep. An appropriate common or usual name to identify this ingredient in food products is “soy leghemoglobin”. The label of food products sold by Impossible Foods containing soy leghemoglobin will disclose an allergen warning, (*i.e.*, “Contains Soy”), and Impossible Foods will provide training materials and information to restaurants who purchase the product indicating it is a soy-protein based product.

[2] In this application, the terms “soy leghemoglobin” and “soy leghemoglobin protein” are used interchangeably to refer to the characterizing component in the LegH Prep.

B.3.2 Identity of Soy Leghemoglobin

Soy leghemoglobin is a small 16 kDa holoprotein (protein + heme cofactor) expressed within the nitrogen-fixing root nodule of the soybean plant (*Glycine max*), and it exists in symbiotic relationship with heme producing bacteria within the root nodules (O'Brian *et al*., 1987). The soybean nodule contains 4 major species of leghemoglobin (Lba, Lbc_1, Lbc_2, and Lbc_3) (Fuchsman and Appleby, 1979), along with several minor components that are post-translationally modified forms of the major species (Whittaker *et al.*, 1981). The 4 major soy leghemoglobin species are encoded by closely related genes, and the protein sequence differ by only a few amino acids (Brisson and Verma, 1982).

Soy leghemoglobin c2, which is encoded by the *LGB2* gene[3] in the soybean plant, is expressed during fermentation with a genetically modified *P. pastoris* production strain developed by Impossible Foods. The resulting LegH Prep that is produced is specified to contain up to 9% soy leghemoglobin on a wet weight basis. At least 65% (w/w) of the total proteins in the LegH Prep is accounted for by soy leghemoglobin; the remainder represents residual yeast proteins from the *P. pastoris* production strain.

Of note, the *LGB2* gene encoding for leghemoglobin c2 in the soybean plant (*Glycine max*) was synthesised and codon-optimised for expression in *P. pastoris*. The amino acid sequence of soy leghemoglobin c2 expressed by the genetically modified *P. pastoris* is identical to that of the naturally occurring soy leghemoglobin c2, as indicated below.

Amino Acid Sequence for Leghemoglobin c2 in *Glycine max* (Soybean)
NCBI Reference Sequence: NP_001235248.2 (145 aa)
1 MGAFTEKQEA LVSSSFEAFK ANIPQYSVVF YTSILEKAPA AKDLFSFLSN GVDPSNPKLT
61 GHAEKLFGLV RDSAGQLKAN GTVVADAALG SIHAQKAITD PQFVVVKEAL LKTIKEAVGD
121 KWSDELSSAW EVAYDELAAA IKKAF

B.4 Manufacturing Process

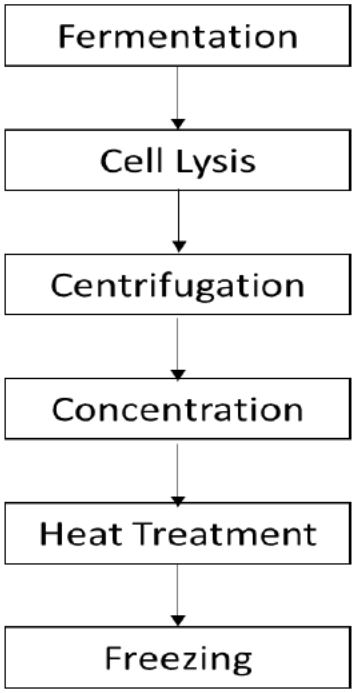
B.4.1 Overview

LegH Prep is manufactured by Impossible Foods in compliance with current Good Manufacturing Practices (cGMP). A schematic overview of the manufacturing process is presented in Figure B.4.1-1 below. All materials used in the production of LegH Prep are standard ingredients used in the food/enzyme industry and are suitable for their intended use. These materials are food-grade or high-quality chemical- and pharmaceutical-grade obtained from approved suppliers. The process used to isolate soy leghemoglobin from a well-characterised fermentation medium complies with the Enzyme Technical Association's guidelines for microbially derived recombinant proteins (Enzyme Technical Association, 2005; Taylor and Baumert, 2013).

A full description of the manufacturing process is provided in Appendix III **[CONFIDENTIAL]**, which includes proprietary information on the raw materials and processing aids, and details of the development and characterisation of the production strain. A non-confidential description of the *P. pastoris* production strain and its construction is presented in Sections B.4.2 and B.4.3 below. This is followed by a non-confidential summary of the fermentation and recovery steps in Section B.4.4. A description of the quality control measures is provided in Section B.4.5.

[3] May be alternatively denoted as the *LBC2* gene.

Figure B.4.1-1 Schematic Overview of the Manufacturing Process for LegH Prep



B.4.2 Description of the *Pichia pastoris* Production Strain

B.4.2.1 Taxonomy

P. pastoris is a methylotrophic yeast that has a long history of use as an expression host for the production of recombinant enzymes and other proteins of interest to industrial, food, and pharmaceutical practice (Cereghino and Cregg, 2000). Based on work by Yamada *et al.* (1995) and Dlauchy *et al.* (2003) comparing partial sequences of 18S and 26S rRNAs to other methanol assimilating yeasts, the species identity was renamed *Komagatella phaffia* (Kurtzman, 2009); however, many scientific resources continue to use the term *Pichia pastoris*, and for the sake of clarity the name *P. pastoris* will be retained throughout this application.

The taxonomic identity of the *P. pastoris* production organism is presented in Table B.4.2.1-1.

Table B.4.2.1-1 Taxonomic Identity of the Production Organism

Name	*Komagatella phaffii* (synonym *Pichia pastoris*)
Kingdom	Fungi
Phylum	Ascomycota
Class	Hemiascomycetes
Order	Saccharomycetales
Family	Endomycetaceae
Genus	*Komagatella* (synonym *Pichia*)
Species	*Phaffii*

B.4.2.2 Donor Organisms

Initially, the *P. pastoris* MXY0291 production strain was used by Impossible Foods to produce their soy leghemoglobin. Details of the MXY0291 production strain have been summarised in the U.S. GRAS Notice submitted for soy leghemoglobin, which was issued a "no questions" response from the U.S. FDA (GRN 737 – U.S. FDA, 2018a). The only heterologous donor gene introduced to the production strain is the *LGB2* gene encoding leghemoglobin from the soybean plant (*Glycine max*), which was synthesised and codon-optimised for expression in *P. pastoris*. Other changes introduced into the production strain include an upregulation of the native heme biosynthesis pathway and some transcriptional machinery of the *Pichia* genome. Additionally, Mxr1, a transcriptional activator of the native *P. pastoris* alcohol oxidase promoter (*pAOX1*), is overexpressed. Impossible Foods has since further optimised the MXY0291 strain for the production of soy leghemoglobin; the details of these optimisations are provided in Appendix III **[CONFIDENTIAL]**. These additional modifications are minor and do not affect the safety profile of the LegH Prep.

The only recombinant DNA in the MXY0291 or the optimised MXY0541 production strain that is not already native to *P. pastoris* is the *LGB2* gene encoding for soy leghemoglobin. However, as mentioned, the *LGB2* gene is generated by Impossible Foods through DNA synthesis, which ensures that only the leghemoglobin protein from the soybean plant is expressed.

B.4.2.3 Recipient (Host) Organism

The *P. pastoris* production strain is constructed from the recipient strain Bg11 (internally referenced as MXY0051) using a series of genetic transformations. The recipient strain is purchased from BioGrammatics Inc. (Carlsbad, CA) and represents a safe strain lineage with a long history of use in production of food enzymes (see Section B.4.2.4). As indicated in Figure B.4.2.3-1, the *P. pastoris* recipient strain Bg11 and its precursor Bg10 are derived from the well-characterised strain Y-11430, which is deposited in the collection at the Northern Regional Research Laboratories (NRRL). The lineage of *P. pastoris* strain NRRL Y-11430 is discussed in brief below. A detailed description of the strain lineage of NRRL Y-11430 is presented in a GRAS Notice that has been submitted to the U.S. FDA (GRN 204 – U.S. FDA, 2006).

The first *P. pastoris* strains were isolated from an oak tree and a chestnut tree and were deposited in the collection at the NRRL[4] (see Figure B.4.2.3-1, and www.biogrammatics.com). Yeast strains screened by Phillips Petroleum for growth on methanol included 2 *P. pastoris* strains, designated NRRL Y-1603 (American Type Culture Collection [ATCC] accession 28485) (ATCC, 2016a) and NRRL YB-4290 (NCAUR, 2019). Phillips Petroleum identified a *P. pastoris* strain with improved growth characteristics. The strain was designated 21-1 and deposited at NRRL, as NRRL Y-11430 (Wegner, 1986). This strain is now available from ATCC as 76273 (ATCC, 2016b). No records are available confirming that NRRL Y-1603 or NRRL YB-4290 is the progenitor of NRRL Y-11430, but it seems likely that 1 of them is the progenitor strain (Madden, 2014). NRRL Y-11430 was the progenitor strain for GS115, a histidine auxotrophic mutant (his4-) (Cregg *et al*., 1985), a common *P. pastoris* strain provided in commercial kits by Invitrogen Corporation, and widely used as the parental strain of many biotechnology products. Additionally, the GS115-derived strain SMD1168 is used for the GRAS approved production of BD16449 Phospholipase C (GRN 204 – U.S. FDA, 2006). Like GS115, the BioGrammatics, Inc. strain, Bg10 is also a derivative of NRRL Y-11430, and genomic sequencing data performed by BioGrammatics, Inc. confirm the similarity of NRRL Y-11430, Bg10 and GS115 (Figure B.4.2.3-1). Additional taxonomic history of

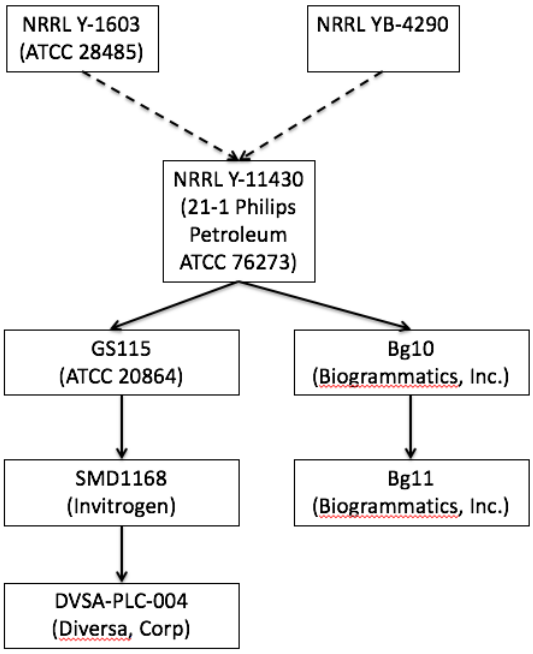
[4] The NRRL collection is now known as the Agriculture Research Service Culture Collection and is at the Microbial Genomics and Bioprocessing Research Unit (MGB) of the National Center for Agricultural Utilization Research (NCAUR) in Peoria, IL.

these strains is available in a 2009 manuscript by C. Kurtzman (Kurtzman, 2009) and on the BioGrammatics, Inc. webpage (biogrammatics.com).

BioGrammatics, Inc. further developed the NRRL-Y-11430 strain to remove the native *P. pastoris* plasmids. Polymerase chain reaction (PCR) primers unique to the plasmids were used to screen multiple single colony isolates for the presence of the plasmids. One isolate without plasmids was selected to become the wild-type BioGrammatics, Inc. strain, Bg10. Genomic sequence from Bg10 indicates the plasmids are no longer present, and, benchmarks the similarity of Bg10 with NRRL-Y11430, as well as with GS115. Like NRRL Y-11430 and GS115, Bg10 does not contain antibiotic-resistance genes.

P. pastoris is a methylotrophic yeast that is capable of using methanol as sole carbon source. Alcohol oxidase 1 (Aox1) is the primary enzyme responsible for methanol metabolism, and strains lacking this enzyme have a reduced rate of methanol utilisation and are therefore preferred in industrial fermentations due to decreased heat generation and rate of oxygen consumption. BioGrammatics, Inc. deleted the gene encoding for Aox1 from Bg10 using homologous recombination to generate a strain that grows more slowly on methanol-containing induction media. The antibiotic resistance gene and background vector sequences used during homologous recombination were subsequently removed to generate a clean, antibiotic resistance gene-free Bg11 strain (Figure B.4.2.3-1), which was purchased by Impossible Foods for the construction of their production strain.

Figure B.4.2.3-1 Strain Lineage of Recipient Strain Bg11 (MXY0051)



B.4.2.4 History of Use

As mentioned, the soy leghemoglobin produced by Impossible Foods is expressed intracellularly within the *P. pastoris* production strain, and isolation of the protein requires cell lysis, which is then followed by physical separation methods. Some residual amounts of the *P. pastoris* production strain, including residual protein (representing up to 35% of the total protein) and DNA (approximately 300 mg/L), remains in the final LegH Prep.

A comprehensive discussion on the safety of the production strain developed by Impossible Foods to produce their soy leghemoglobin is presented in Section C of this application. In brief, the *P. pastoris* production strain is derived from a strain lineage (NRRL YB-11430) that is well-characterised and has a long history of safe use in the manufacture of various food enzymes and pharmaceutical agents (Cereghino and Cregg, 2000; see Section C.5.1). *Pichia* is non-pathogenic and non-toxigenic (see Section C.5.2), and *P. pastoris* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority when used for enzyme production (EFSA, 2017). Moreover, all of the recombinant DNA that are introduced into the production strain are native to *P. pastoris*, with the exception of the gene encoding for leghemoglobin in the soybean plant (*Glycine max*), which is obtained through DNA synthesis.

Given the long history of safe use for this strain lineage, no safety concerns are anticipated from the use of the *P. pastoris* production strain in the manufacture of Impossible Foods' soy leghemoglobin ingredient.

B.4.2.5 Antibiotic Resistance

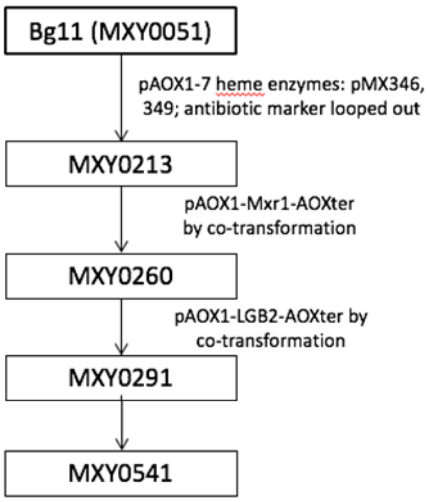
The production strain does not contain any antibiotic resistance genes (see Appendix III **[CONFIDENTIAL]**). Moreover, the final stages of processing for the LegH Prep includes a heat treatment step to ensure that no viable *P. pastoris* production strain remains.

B.4.3 Nature of the Genetic Modifications

B.4.3.1 Overview

To enable the expression of soy leghemoglobin, a series of transformations involving different expression constructs was applied to the Bg11 recipient strain (internally referenced as MXY0051) to generate the *P. pastoris* production strain. A schematic of the different transformation steps, along with the intermediate strains generated, is presented in Figure B.4.3.1-1. A non-confidential description of each step used to obtain the MXY0291 production strain, as detailed in GRN 737, is provided in Sections B.4.3.2 to B.4.3.4. Impossible Foods has since introduced several minor modifications to further optimised the MXY0291 strain for the production of soy leghemoglobin. A non-confidential description of the modifications made and the construction of the optimised MXY0541 production strain are described in Section B.4.3.5.

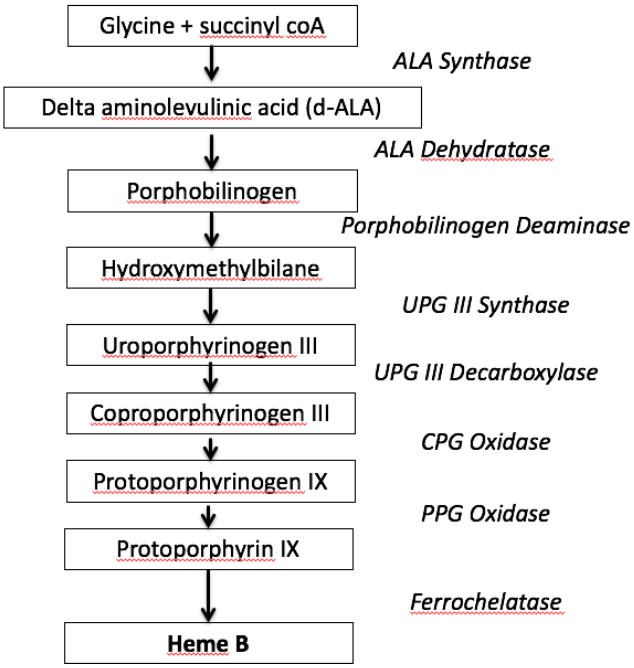
Figure B.4.3.1-1 Construction of the *Pichia pastoris* Production Strain MXY0541



B.4.3.2 Construction of MXY0213: Strain Overexpressing the Heme Biosynthesis Pathway

To increase the intracellular concentration of heme to generate sufficient heme bound soy leghemoglobin protein, the heme biosynthetic pathway of Bg11 was upregulated. Heme biosynthesis is the result of an 8-step pathway, each catalysed by a distinct, highly conserved enzyme (see Figure B.4.3.2-1). Genes encoding all 8 enzymes of the *Pichia* heme biosynthesis pathway were amplified from the *Pichia* genome and cloned into 2 plasmids: pMX349 and pMX346. The 2 plasmids were linearized using restriction enzyme (Pme1) digestion and sequentially transformed into the recipient strain Bg11 leading to integration of the entire cassette expressing the sets of heme enzymes in the genome. Following each round of transformation, the integrated antibiotic resistance genes flanked by lox restriction sites were removed from the strain using cre-lox recombination. This resulted in MXY0213, a stable strain that contained extra copies of the native *Pichia* heme biosynthesis enzymes under extra copies of the native *pAOX1* promoter.

Figure B.4.3.2-1 The Highly Conserved Pathway for Heme Biosynthesis



Note: Enzymes catalysing each step are shown on the right in *italics*.

B.4.3.3 Construction of MXY0260: Strain Overexpressing Mxr1 and the Heme Biosynthesis Pathway

Mxr1 is a transcriptional activator of the *pAOX1*. This promoter has been demonstrated to produce high levels of recombinant proteins by *P. pastoris* strains when cultivated on glycerol as a carbon source and methanol as an inducer of *pAOX1* (Cereghino and Cregg, 2000). The presence of Mxr1 leads to improved production of the recombinant soy leghemoglobin protein. A linear cassette of DNA containing the modified *P. pastoris* MXR1 gene under the control of *pAOX1* promoter and AOX1 terminator (AOX1TT) was introduced into MXY0213 by co-transformation using an empty plasmid vector containing 2 antibiotic resistance genes.

Due to the cloning strategy, the overexpressed Mxr1 protein contains 6 extra amino acids on its N-terminus compared to the native *P. pastoris* Mxr1. Mass spectrometry analysis demonstrates that neither the modified Mxr1 protein nor the native Mxr1 protein is detectable in the final LegH Prep (see Appendix III **[CONFIDENTIAL]**). The pAOX1-MXR1-AOX1ter DNA cassette and an empty plasmid vector containing an antibiotic resistance gene were co-transformed into MXY0213. This enabled selection of transformants containing the empty vector, which were then screened by colony PCR for integration of the cassette at the pAOX1 locus. Transformants containing the desired MXR1 integration were subsequently cured of the empty vector and confirmation of plasmid loss was demonstrated by screening for antibiotic sensitivity and PCR (see Appendix III **[CONFIDENTIAL]**). The resulting strain was MXY0260, the parent to the production strain MXY0291.

B.4.3.4 Construction of MXY0291: Production Strain Overexpressing Soy Leghemoglobin

The *LGB2* gene encoding for leghemoglobin in the soybean plant (*Glycine max*) was synthesised and codon-optimised for expression in *P. pastoris*. A linear cassette of pAOX1-LGB2-AOX1ter was PCR amplified and introduced into MXY0260 by co-transformation as described above. Quantitative polymerase chain reaction (qPCR) and protein expression assays identified the production strain, MXY0291, which contains 16 copies of the recombinant *LGB2* gene. As described above, antibiotic sensitivity and PCR were used to demonstrate effective curing of the plasmid following co-transformation.

B.4.3.5 Construction of MXY0541: Optimised Production Strain Overexpressing Soy Leghemoglobin

In an effort to further increase production of leghemoglobin, additional copies of *LGB2* were introduced. Optimisations to the heme biosynthesis pathway were also made. Details of these modifications are described in Appendix III **[CONFIDENTIAL]**.

B.4.3.6 Molecular Characterisation of the Genetic Modifications

The genome of the MXY0291 production strain initially developed by Impossible Foods has been well-characterised and completely sequenced, and it is confirmed to contain the following sequences in addition to the background *P. pastoris* DNA:

- Sixteen copies of pAOX1-LGB2-AOX1ter
- One copy of pAOX1-MXR1-AOX1ter
- One copy of a portion of pMX349 (no antibiotic resistance genes, no origin of replication)
- Two to three copies of a portion of pMX346 (no antibiotic resistance genes, no origin of replication)

The additional genetic changes that have been introduced into MXY0291 to generate the current optimised production strain (MXY0541) have been verified by standard PCR and quantitative PCR based methods (see Appendix III **[CONFIDENTIAL]**). Moreover, the genome of MXY0541 has been completely sequenced and been confirmed to contain the following additional changes:

- Several copies of a CPG oxidase cassette
- Additional copies of the *LGB2* gene

B.4.3.7 Stability of the Genetic Changes

All changes introduced into both the initial (MXY0291) and current (MXY0541) production strains are stably integrated in the genome and confirmed to be present after >150 to 200 generations of growth on non-selective growth media. No plasmid sequences are present in the production strain.

B.4.4 Fermentation and Recovery Processes for the LegH Prep

Soy leghemoglobin is expressed during submerged fed-batch fermentation using the *P. pastoris* production strain. Frozen cell banks of the *P. pastoris* production organism, maintained at -80°C in 20% v/v glycerol, are used as the source inoculum for production of the LegH Prep. The master cell bank is stored at multiple locations. Working cell banks are prepared from the master cell bank and are tested for microbial purity, specific growth rate, and soy leghemoglobin yield prior to production fermentation. The fermentation broth is periodically analysed microscopically to ensure culture purity. Process parameters including pH, temperature, agitation, dissolved oxygen, methanol concentration and glycerol concentration are routinely monitored throughout fermentation. Fermentations that incur microbial contamination and/or other process deviations that affect safety and/or quality are sterilised by steam in place and discarded.

Once fermentation is complete, the *P. pastoris* cells in the fermentation broth are lysed by suitable processes such as bead mill mechanical shearing or high-pressure homogenisation. Insoluble material within the lysate is removed by suitable processes such as centrifugation. Ultrafiltration or other suitable processes are used to concentrate soy leghemoglobin. Heat treatment is then used to remove the remote potential for growth of any possible *P. pastoris* cells remaining. The resulting concentrated sample is formulated with suitable stabilisers (*e.g.*, sodium ascorbate and sodium chloride), and stored as a frozen liquid.

B.4.5 Quality Controls

LegH Prep is manufactured in accordance with cGMP. The manufacturing process employs in-process controls to ensure the purity of the final product and minimise the amount of potential impurities to the level that is technically feasible.

Impossible Foods tests the fermentation broth to ensure the absence of microbiological contaminants specified in Section B.6 (*i.e.*, *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* [*E. coli*] O157:H7). In addition, the final product from every LegH Prep production run is tested for these microbiological parameters. Failure to comply with the specifications outlined in Section B.6, would result in the batch being evaluated and/or discarded (in the case of the presence of a pathogen), the execution of additional sanitisation standard operating procedures in compliance with Impossible Foods' internal food-safety standards, and a root cause analysis.

B.5 Information on the Impurity Profile

Impossible Foods has established acceptable limits for heavy metal and microbiological contaminants in the specifications for their LegH Prep (see Section B.6). Additionally, in accordance with the recommendations for safety evaluation by the International Food Biotechnology Committee (IFBC, 1990a), Impossible Foods has demonstrated that viable colonies of the *P. pastoris* production strain are not detected in the LegH Prep, supporting the effectiveness of the process controls for removal and/or inactivation of the production organism.

B.6 Specifications

B.6.1 Product Specifications

Food-grade specifications for the LegH Prep are presented in Table B.6.1-1 below. The LegH Prep is a liquid specified to contain up to 9% soy leghemoglobin as measured by ultra-performance liquid chromatography (UPLC), with a protein purity of at least 65% as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining, and gel densitometry. Upper limits have also been established for heavy metals, and microbiological parameters to ensure the purity of the final product.

Details of the methods of analysis that were internally developed and validated by Impossible Foods for the evaluation of the specification parameters for the LegH Prep (*i.e.*, content and purity of the soy leghemoglobin protein) are provided in Appendix IV **[CONFIDENTIAL]**. All other parameters are assessed by well-recognised and validated methods of analysis.

Table B.6.1-1 Product Specifications for LegH Prep

Source		
Genetically modified strain of *Pichia pastoris*		
Parameter	**Specification**	**Method**
Soy leghemoglobin protein (w/w)[a]	≤9%	UPLC (internal method)
Soy leghemoglobin protein purity (w/w)[b]	≥65%	SDS-PAGE (internal method)
Fat (w/w)	≤2%	AOAC 989.05
Carbohydrates (w/w)	≤6%	AOAC 986.25
Ash (w/w)	≤4%	AOAC 945.46
Solids (w/w)	≤26%	Calculated as 100 minus moisture
Moisture (w/w)	≤90%	AOAC 948.12
Heavy Metals		
Lead (ppm)	<0.4	EPA 3050/6020 USP 730
Arsenic (ppm)	<0.05	EPA 3050/6020 USP 730
Mercury (ppm)	<0.05	EPA 3050/6020 USP 730
Cadmium (ppm)	<0.2	EPA 3050/6020 USP 730
Microbiological Parameters		
Escherichia coli EHEC (inclusive of O157:H7)	Absent by test	AOAC RI 020801
Salmonella spp.	Absent by test	AOAC OMA 2011.03
Listeria monocytogenes	Absent by test	AOAC OMA 2010.02

ppm = parts per million; SDS-PAGE = sodium dodecyl sulphate–polyacrylamide gel electrophoresis; UPLC = ultra-performance liquid chromatography.
[a] Soy leghemoglobin protein may exceed 9% if additional water (moisture) is removed during the concentration step of the manufacturing process. Additional concentration (*i.e.*, less water) does not change the composition of the dry solids.
[b] The balance of the proteins in the preparation is residual *Pichia* proteins.

B.6.2 Batch Analysis

Batch analyses for 5 independent commercial batches of LegH Prep are provided in Table B.6.2-1. The results of these batch analyses demonstrate that the final LegH Prep complies with the established product specifications. Certificates of Analyses for these batches are provided in Appendix V.

Table B.6.2-1 Batch Analyses for LegH Prep

Parameter	Specification	Manufacturing Batch No.[a]				
		PP-PGM216-015-101	PP-PGM216-088-101	PP-PGM2-18-162	PP-PGM2-18-176	PP-PGM2-18-190
Soy leghemoglobin protein (w/w)[b]	≤9%	6.74%	6.39%	4.8%	4.5%	4.6%
Soy leghemoglobin protein purity (w/w)[c]	≥65%	82%	71%	77%	68%	76%
Fat (w/w)	≤2%	0.05%	<0.01%	1.5%	1.5%	1.7%
Carbohydrates (w/w)	≤6%	1.72%	0.99%	3.6%	4.1%	3.8%
Ash (w/w)	≤4%	1.87%	0.67%	0.9%	0.9%	0.9%
Solids (w/w)[d]	≤26%	14.85%	12.55%	19.8%	20.3%	20.0%
Moisture (w/w)	≤90%	85.15%	87.45%	80.2%	79.7%	80.0%
Heavy Metals						
Lead (ppm)	<0.4	<0.01	<0.01	<0.01	<0.01	<0.01
Arsenic (ppm)	<0.05	0.01	<0.01	0.03	0.03	0.04
Mercury (ppm)	<0.05	<0.005	<0.005	<0.005	<0.005	<0.005
Cadmium (ppm)	<0.2	<0.001	<0.001	<0.001	<0.001	<0.001
Microbiological Parameters						
Escherichia coli EHEC (inclusive of O157:H7)	Absent by test	Complies	Complies	Complies	Complies	Complies
Salmonella spp.	Absent by test	Complies	Complies	Complies	Complies	Complies
Listeria monocytogenes	Absent by test	Complies	Complies	Complies	Complies	Complies

ppm = parts per million.

[a] Batch numbers PP-PGM216-015-101 and PP-PGM216-088-101 represent LegH Prep that is produced from the initial MXY0291 strain. Batch numbers PP-PGM2-18-162, PP-PGM2-18-176, and PP-PGM2-18-190 represent LegH Prep produced from the current MXY0541 strain. Analytical data from additional batches of the LegH Prep produced by either strain are provided in Appendix III. There are no differences in the overall composition of the LegH Prep produced by MXY0291 from those produced by MXY0541.

[b] Soy leghemoglobin protein may exceed 9% if additional water (moisture) is removed during the concentration step of the manufacturing process. Additional concentration (*i.e.*, less water) does not change the composition of the dry solids.

[c] The balance of the proteins in the preparation is residual *Pichia* proteins.

[d] The solids content of LegH Prep is composed of total protein, fat, carbohydrate, and ash. The total protein content includes both soy leghemoglobin and *Pichia* proteins. The maximum total protein is ≤14% w/w by calculation (*i.e.*, maximum soy leghemoglobin content [9% w/w] divided by the minimum protein purity (65% w/w)). The sum of the total protein (≤14% w/w), fat (≤2% w/w), carbohydrate (≤6% w/w), and ash (≤4% w/w) results in a solids specification of ≤26% w/w. The balance of LegH Prep is water.

B.6.3 Stability

B.6.3.1 Stability During Bulk Storage

LegH Prep may be stored at -20°C as a frozen liquid for at least 24 months with no observable change in soy leghemoglobin stability or performance in meat analogue products (see Table B.6.3.1-1).

Table B.6.3.1-1 Stability of the LegH Prep Under Recommended Storage Conditions at -20°C

Lot	Months	Soy Leghemoglobin Titre by UPLC (%)	Soy Leghemoglobin Protein Purity (w/w)	pH
PP-PGM2-16-102-101	0	6.29	84.9%	7.38
	3.2	6.09	78.9%	7.35
	5.7	6.11	73.4%	7.03
	9.0	6.12	84.1%	7.29
	26.6	5.58	84.0%	7.31

Table B.6.3.1-1 Stability of the LegH Prep Under Recommended Storage Conditions at -20°C

Lot	Months	Soy Leghemoglobin Titre by UPLC (%)	Soy Leghemoglobin Protein Purity (w/w)	pH
PP-PGM2-16-144-101	0	6.73	77.3%	7.01
	2.8	6.02	74.3%	7.96
	5.5	6.29	71.3%	8.61
	25.0	6.08	78.7%	8.34
PP-PGM2-16-200-101	0	6.95	86.3%	6.77
	3.1	6.50	85.7%	6.89
	6.0	6.72	91.0%	6.76
	23.3	6.40	82.9%	6.98
PP-PGM2-17-079-111	0	7.89	77.0%	7.35
	6.8	7.73	85.7%	6.50
	10.3	7.28	79.1%	6.63
	15.3	7.20	80.8%	6.30
PP-PGM2-17-086-111	0	8.76	92.0%	7.19
	6.5	8.92	90.3%	6.51
	10.1	8.25	86.6%	6.60
	15.1	8.44	84.2%	6.32
PP-PGM2-17-121-111 (6DV)	0	8.09	76.0%	7.08
	13.9	7.44	83.1%	6.38
PP-PGM2-17-128-111	0	7.60	76.0%	7.24
	5.9	7.98	85.2%	6.36
	8.9	7.88	78.7%	6.41
	13.7	7.50	81.8%	6.28
50D-LH-021	0.0	6.82	83.7%	6.71
	9.6	6.23	91.0%	6.16
50D-LH-022	0.0	5.49	84.9%	6.64
	9.4	5.21	91.1%	6.00
50D-LH-035	0.0	7.52	83.7%	6.60
	7.1	6.91	81.1%	6.24
50D-LH-037	0.0	7.46	83.9%	7.07
	6.7	7.24	79.2%	6.24

UPLC = ultra-performance liquid chromatography.

B.6.3.2 Stability in Meat Analogue Products

Impossible Foods has demonstrated that soy leghemoglobin is stable within their meat analogue products when stored for up to 9 days at 4°C, or up to 6 months at -20°C. The stability of soy leghemoglobin, as determined by its recovery from the meat analogue product, following storage at 4°C and -20°C is presented in Table B.6.3.2-1 and Table B.6.3.2-2, respectively.

Table B.6.3.2-1 Stability of Soy Leghemoglobin in a Meat Analogue Product at 4°C Storage

Sample ID	Days	% Target Recovery	% Relative Standard Deviation
50D-LH-035_Burger_1	0	104	2.8
50D-LH-035_Burger_2		105	
50D-LH-035_Burger_3		103	

Table B.6.3.2-1 Stability of Soy Leghemoglobin in a Meat Analogue Product at 4°C Storage

Sample ID	Days	% Target Recovery	% Relative Standard Deviation
50D-LH-035_Burger_4		100	
50D-LH-035_Burger_5		98	
50D-LH-037_Burger_1		98	1.8
50D-LH-037_Burger_2		102	
50D-LH-037_Burger_3		102	
50D-LH-037_Burger_4		101	
50D-LH-037_Burger_5		98	
50D-LH-035_5d_Burger_1	5	88	3.4
50D-LH-035_5d_Burger_2		95	
50D-LH-035_5d_Burger_3		87	
50D-LH-035_5d_Burger_4		89	
50D-LH-035_5d_Burger_5		92	
50D-LH-037_5d_Burger_1		101	4.4
50D-LH-037_5d_Burger_2		101	
50D-LH-037_5d_Burger_3		106	
50D-LH-037_5d_Burger_4		100	
50D-LH-037_5d_Burger_5		111	
50D-LH-035_9d_Burger_1	9	85	85.2
50D-LH-035_9d_Burger_2		85	
50D-LH-035_9d_Burger_3		85	
50D-LH-035_9d_Burger_4		85	
50D-LH-035_9d_Burger_5		87	
50D-LH-037_9d_Burger_1		91	91.3
50D-LH-037_9d_Burger_2		92	
50D-LH-037_9d_Burger_3		92	
50D-LH-037_9d_Burger_4		91	
50D-LH-037_9d_Burger_5		91	

Table B.6.3.2-2 Stability of Soy Leghemoglobin in a Meat Analogue Product at -20°C Storage

Sample ID	Day	% Target Recovery	% Relative Standard Deviation (RSD)
50D-LH-035-B-6M-1	6 months	87.6	1
50D-LH-035-B-6M-2		85.4	
50D-LH-035-B-6M-3		85.2	
50D-LH-035-B-6M-4		85.2	
50D-LH-035-B-6M-5		87.1	
50D-LH-037-B-6M-1		85.4	
50D-LH-037-B-6M-2		85.2	
50D-LH-037-B-6M-3		85.1	
50D-LH-037-B-6M-4		87.5	
50D-LH-037-B-6M-5		86.2	

B.7 Analytical Detection Method

Soy leghemoglobin is measured in samples *via* a UPLC-based assay developed and validated by Impossible Foods. The UPLC method detects and quantitates the amount of soy leghemoglobin in a liquid sample (LegH Prep) and on extracts of the Impossible Foods meat analogue (burger) product. Details of the confidential analytical method is provided in Appendix IV **[CONFIDENTIAL]**.

C. SAFETY ASSESSMENT

Information and data to support the safety of Impossible Foods' soy leghemoglobin under its proposed conditions of use is described in this section. Specifically, this section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel foods), Guideline 3.5.1 (Foods produced using gene technology), and Guideline 3.3.3 (Substances used for a nutritive purpose) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019). The corresponding sections of this application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	Required Information Described in the Guideline	Section(s) of the Application where this is Addressed
Guideline 3.5.2 – Novel foods	C.6 Food ingredients derived from a new source	
	C.6.1 Information on the safety of the source organism	Sections C.1, C.5
	C.6.2 Information on the composition of the novel food ingredient derived from a new source	Sections B.6, C.1
	C.6.3 Information on the toxicity of the novel food ingredient derived from the new source	Sections C.1 to C.6
	C.6.4 Safety assessment reports prepared by international agencies or other national government agencies	Section C.7
Guideline 3.5.1 – Foods produced using gene technology	B. Characterisation and safety assessment of new substances	
	B.1 Characterisation and safety assessment of new substances	
	A full description of the biochemical function and phenotypic effects of all new substances (*e.g.*, a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions	Sections B.4.3, C.1, C.2
	Information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.	Sections C.1, C.2
	Information on whether any new protein has undergone any unexpected post-translational modification in the new host	Not applicable.
	Where any ORFs have been identified (in subparagraph A.3(c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs.	Sections C.1, C.6
	B.2 New proteins	
	Information on the potential toxicity of any new proteins	Sections C.1 to C.6
	Information on the potential allergenicity of any new proteins	Sections C.1 to C.6
	B.3 Other (non-protein) new substances	Sections C.1, C.4, C.5
	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants	Not applicable.
	B.5 Compositional analyses of the food produced using gene technology	Sections B.6, C.1
Guideline 3.3.3 – Substances used for a nutritive purpose	C Information related to the safety of the nutritive substance	
	C.1 Information on the toxicokinetics and metabolism of the nutritive substance and, if necessary, its degradation products and major metabolites	Sections C.3, C.6.3
	C.2 Information from studies in animals or humans that is relevant to the toxicity of the nutritive substance and, if necessary, its degradation products and major metabolites	Sections C.1 to C.6
	C.3 Safety assessment reports prepared by international agencies or other national government agencies, if available	Section C.7

C.1 Approach of the Safety Assessment

The safety assessment of Impossible Foods' LegH Prep, as described herein, has been conducted in accordance with the general principles outlined in the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), and the *Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms* from the CODEX Alimentarius (CAC/GL 46-2003 - Codex Alimentarius, 2003).

The 2-tier testing strategy for the safety evaluation of protein products of biotechnology developed by International Life Sciences Institute (ILSI) was applied as a tool for part of the safety assessment process (Delaney *et al*., 2008; Hammond *et al*., 2013). The Tier I step involves hazard characterisation of the protein and includes consideration of biological function, mode of action or intended use application of the protein, assessment of the history of safe use, a comparison of the amino acid sequence of the protein to other known proteins (*i.e*., allergens and toxins). In instances where information from the Tier I assessment are sufficiently robust to characterise the hazard of the protein, safety of the protein may be supported without the need for further evaluation through traditional toxicological testing paradigms. Where gaps or safety concerns are identified at the Tier I stage, Tier II testing may involve toxicological studies or other hypothesis-based testing strategies to answer specific safety related questions.

An outline of the 2-tier safety assessment approach as it applies to the LegH Prep is presented in Figure C.1-1. As part of the Tier I assessment, Impossible Foods noted that leghemoglobin is derived from soybeans (*Glycine max*), a donor organism that has a safe history of consumption and is not known to produce protein toxins. The structure function of soy leghemoglobin is well-characterised and based on its structural homology to other heme proteins consumed in the diet (*e.g*., myoglobin), it can therefore be concluded that the protein would not display undesirable biological activities that are of concern for use as a food ingredient. Bioinformatic evaluations did not identify soy leghemoglobin as homologous to any protein within available databases of known and putative allergens and toxins. *In vitro* studies also have demonstrated that soy leghemoglobin is susceptible to pepsin-based digestion (Jin *et al*., 2018). Limitations or data-gaps identified in the Tier I safety assessment included the fact that soy leghemoglobin does not have a direct history of consumption as the protein is expressed exclusively in the root nodule of the plant. Although this data-gap was not considered substantive as it applied to the safety assessment, a series of preclinical toxicity studies were conducted as part of the Tier II assessment process to provide corroborating information on the safety conclusions derived from Tier I. These included repeated-dose toxicology testing in Sprague-Dawley rats and a battery of *in vitro* genotoxicity studies involving LegH Prep, which contain soy leghemoglobin as the characterizing component (Fraser *et al*., 2018). The results of these studies confirm that the LegH Prep is innocuous as no evidence of toxicity or risk of genotoxicity was observed. The no-observed-adverse-effect level (NOAEL) from a 28-day feeding study in rats was derived as 1,536 mg LegH Prep dry solids/kg body weight/day, the highest dose tested in the study, which corresponded to 750 mg soy leghemoglobin/kg body weight/day (Fraser *et al*., 2018). Protein toxins, as a structural class, can be defined as acute toxins (Pariza and Foster, 1983; Delaney *et al*., 2008; Hammond *et al*., 2013), and therefore the results of a short-term toxicity study, in conjunction with findings from the Tier I assessment, are sufficient to support that soy leghemoglobin (and the LegH Prep in which it is delivered) do not represent a safety hazard for food use.

The safety assessment also included a comprehensive evaluation of the safety of the production organism, and potential fermentation products derived thereof that may be transferred to the LegH Prep. As the LegH Prep is a protein preparation, the safety assessment approach for food enzymes produced from traditional practices and from techniques of modern biotechnology was considered a relevant tool for the safety evaluation process. The safety assessment of food enzymes involves a

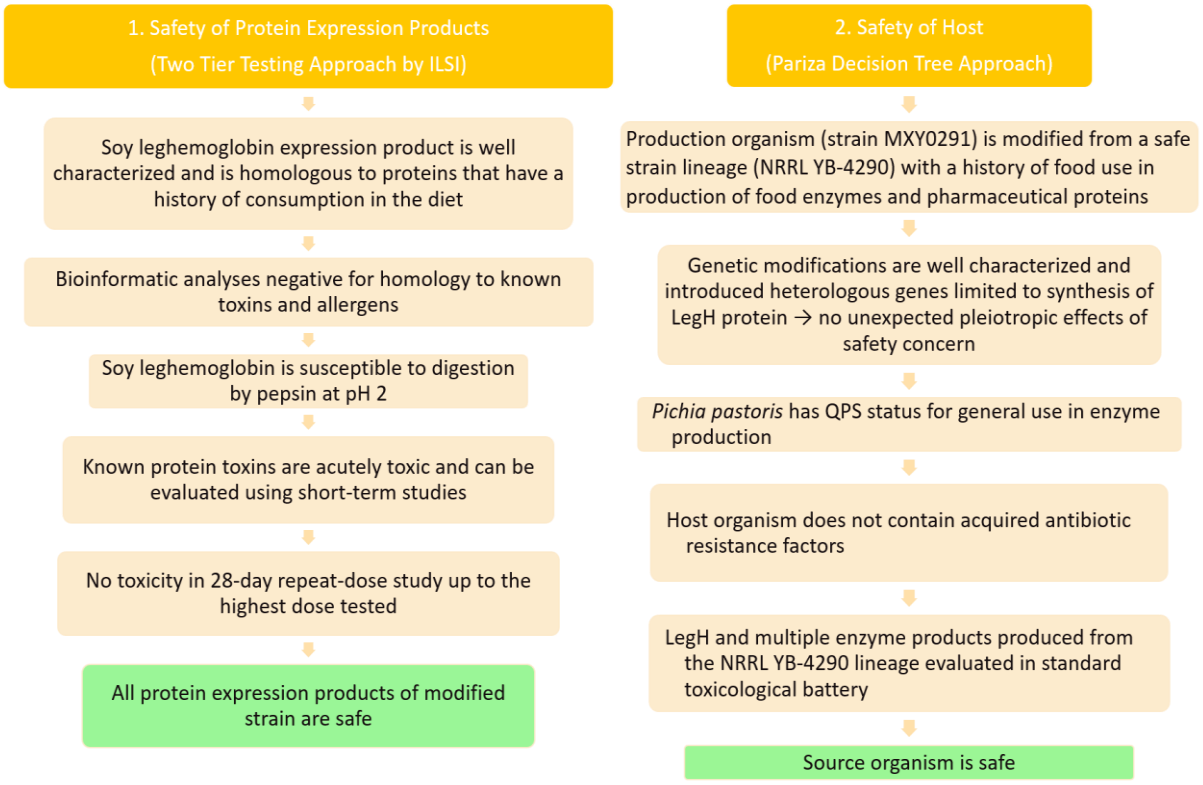
decision tree approach that places emphasis on independent hazard characterisation of the enzyme and the production strain lineage. The safety of non-enzyme constituents originating from the fermentation process (*i.e.*, the production organism) can be supported through an understanding of the safety of the strain lineage. Where a production organism has an established history of safe use in the production of food enzymes, and where these enzyme preparations have been the subject of previous safety evaluations, safety conclusions on the strain lineage can be extended to enzymes produced from modified progenitors of the lineage provided that specific questions on the nature of the genetic modifications are addressed (*e.g.*, the genetic modifications do not introduce unknown pleiotropic effects, absence of potentially transferable antibiotic resistance genes). A similar approach to the safety assessment of genetically modified products has been accepted by the European Food Safety Authority (EFSA), who has stated that:

> "*In the case Genetically Modified Microorganisms (GMM) for which the species of the recipient strain qualifies for the QPS status, and for which the genetically modified state does not give rise to safety concerns, the QPS approach can be extended to genetically modified production strains*" (EFSA, 2018, 2019).

Therefore, toxicological studies would not be required if the GMM production strain is derived from a strain lineage with QPS status, and if the genetic modification raises no safety concerns.

Impossible Foods notes that the *P. pastoris* production strain is derived from a species with QPS status (EFSA, 2017) and from a strain lineage (NRRL YB-11430) that is well-characterised and represents a progenitor of several derivatives that have a long-history of use in the manufacture of food enzymes and pharmaceutical agents (Cereghino and Cregg, 2000). In fact, all *P. pastoris* strains are derivatives of NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, Ill.) (Cregg, 2018). Numerous recombinant protein preparations from this lineage have been the subject of toxicological evaluations confirming that the strain lineage does not produce natural protein toxins or other secondary metabolites that are of toxicological concern when cultivated under conventional fermentation conditions used for protein production. For example, phospholipase C produced from a recombinant strain of *P. pastoris* has been the subject of formal safety evaluations under the GRAS procedure (GRN 204), which was without evidence of toxicity in toxicological evaluations (Ciofalo *et al*., 2006). Other enzyme preparations from the NRRL YB-11430 also have been the subject of repeat-dose toxicological evaluations without evidence of toxicity (Zhu *et al*., 2012; Driss *et al*., 2014). Impossible Foods is not aware of any published evidence that this strain lineage contains the biosynthetic capacity to produce undesirable substances provided the genetic transformations are well-characterised. Impossible Foods notes that their production strain does not contain antibiotic resistance genes, and the only foreign DNA inserted into the organism is a gene encoding for the synthesis of soy leghemoglobin; therefore, none of the genetic transformations would be expected to produce pleiotropic effects of safety concern. Impossible Foods' soy leghemoglobin is expressed intracellularly within *P. pastoris* and isolation of the protein preparation involves cell lysis and physical separation methods; these production processes are virtually identical to the production processes that are applied for food enzymes. Accordingly, the available data and information characterizing the safety of NRRL YB-11430, in particular repeated-dose toxicology studies that have been conducted with enzyme preparations obtained from this strain lineage, support the conclusion that the strain lineage is a safe and suitable expression system for production of protein preparations used in food. Therefore, this history of safe use for the strain lineage can be extended to the production strain developed by Impossible Foods for the production of soy leghemoglobin.

Figure C.1-1 Safety Assessment Approach for LegH Prep



Based on the hazard characterisation of the recombinantly expressed protein (soy leghemoglobin), the *Pichia* proteins, and the production strain (Figure C.1-1), the LegH Prep can be concluded safe and suitable for use in ground meat analogue products marketed in Australia/New Zealand. Detailed description of the data and information supporting the aforementioned safety assessment are presented in Sections C.2 to C.6 below.

C.2 History of Safe Consumption and Functions of Heme Proteins

Haemoglobin proteins are found in most organisms, including bacteria, protozoa, fungi, plants and animals (Hardison, 1998). Heme proteins are classified as globin/non-globin and symbiotic/non-symbiotic. Haemoglobin, myoglobin, and leghemoglobin are examples of globin proteins. Cytochrome oxidases, hemocyanins, and methemalbumin are examples of non-globin heme proteins (Everse, 2004; Jokipii-Lukkari *et al*., 2009). Plant haemoglobins are classified according to function as symbiotic or non-symbiotic (Gupta *et al*., 2011). Symbiotic haemoglobins are found predominantly in leguminous plant species. The most studied symbiotic haemoglobins are the leghemoglobins of nitrogen-fixing legumes where they facilitate oxygen diffusion within root tissues. Non-symbiotic haemoglobins have been identified in a wide range of legume and nonlegume plants. The highest expression levels for nonsymbiotic plant haemoglobin are observed in metabolically active or stressed tissue (Anderson *et al*., 1996).

Soy has been shown to express 3 haemoglobin proteins: symbiotic, nonsymbiotic, and truncated (Lee *et al*., 2004). The proteins share a common evolutionary origin (Vinogradov *et al*., 2007) and, based on structural studies and homology modelling, share a common 3-dimensional structure involving an alpha helical globin-fold wrapped around a heme B molecule (Ellis *et al*., 1997) (Appendix VI). The members of this protein family are all involved in selective transport, storage or buffering of oxygen levels in cells and tissues (Vinogradov and Moens, 2008). The shared and well-characterised physiology

of these proteins strongly supports the inference that the shared 3-dimensional structure of these globin proteins evolved to bind oxygen.

Symbiotic haemoglobins, found predominantly in legume species, function in the nitrogen fixation process in concert with the bacterium *Rhizobium* where they facilitate oxygen diffusion within host tissues. Symbiotic plant haemoglobins, which evolved from non-symbiotic haemoglobins (Wajcman and Kiger, 2002; Gupta *et al*., 2011), are commonly referred to as leghemoglobins. Leghemoglobins' structure and their oxygen binding mechanism are similar to those of animal muscle myoglobin proteins (Hargrove *et al*., 1997). The primary sequence of soy leghemoglobin is not homologous to the primary sequences of mammalian myoglobins. However, the primary sequence of soy leghemoglobin does not contain significant homology to any known allergens or toxins, and therefore does not present a known safety concern (see Section C.6).

Non-symbiotic plant haemoglobins from soy, barley, rice, corn, and mung beans are widely consumed in the diet. Anderson *et al*. (1996) demonstrated that the nonsymbiotic haemoglobin in soy was expressed in various plant tissues including stems, shoots, cotyledon, leaves, and root hair. These soy tissues are commonly consumed in the diet in the form of soybean sprouts. Sprouted barley, which is widely used in the beverage industry (malted barley) and in the baking industry (malted barley flour), has been shown to express haemoglobin 1 day after imbibition (Duff *et al*., 1998). Non-symbiotic haemoglobins are expressed in the rice embryo as well as in the coleoptiles and seminal root of sprouted rice, which is consumed as part of the diet as well (Lira-Ruan *et al*., 2011). Non-symbiotic haemoglobin is expressed in corn seedlings and may provide a good source of bioavailable heme in mature corn seeds (Bodnar, 2011). Impossible Foods has detected non-symbiotic haemoglobin in mung bean sprouts by mass spectrometry. The 3-dimensional structure of soy leghemoglobin is highly similar to the non-symbiotic haemoglobins of corn, rice, and barley (Appendix VI). Although there are no crystal structures for non-symbiotic haemoglobins from soy or mung beans, based on the highly similar structures of non-symbiotic haemoglobins from corn, rice and barley to each other and to soy leghemoglobin, Impossible Foods expects that they (soy and mung) are likewise structurally similar.

Thus, haemoglobin proteins of plant and animal sources are widely consumed in the human diet and represent a highly bioavailable source of dietary iron for human nutrition. Proulx and Reddy demonstrated that soy leghemoglobin and bovine haemoglobin showed similar iron bioavailability within a food matrix, both of which were higher than free iron (Proulx and Reddy, 2006). Furthermore, plant-derived haemoglobins are already prevalent in our food system through malted grain products and sprouted seeds, grains, rice and beans (pulses) (Anderson *et al*., 1996; Duff *et al*., 1998; Lira-Ruan *et al*., 2011).

The heme B moiety plays a central role in oxygen binding, and the structure of the globin protein serves to isolate the heme from other molecules by creating a small binding pocket inaccessible to most other molecules (Ellis *et al*., I 997). Thus, heme B-containing globin proteins remain largely inert so long as the 3-dimensional structure is maintained. When globin proteins are heated, as in cooking, or exposed to a low pH environment, as in the human stomach, the protein unfolds, and the heme B molecule is released (Appendix VI). Impossible Foods has shown that heme B, released when myoglobin is heated to cooking temperature, plays a major role in generating the flavours and aromas characteristic of cooked meat (see Appendix VI).

The abundant consumption of heme B is widespread in humans and other animals, as heme proteins are abundant in animal tissues consumed as meat and are also present in the leaves and other routinely consumed parts of plants. Thus, there is overwhelming evidence that heme B-containing proteins, which are functionally equivalent to Impossible Foods' soy leghemoglobin, have been safely consumed throughout human history.

There is no evidence that any of the globin subfamily that contains the plant haemoglobins have any biochemical activities other than the binding of oxygen (O2) or the structurally similar carbon dioxide (CO2), nitrous oxide (NO), and carbon monoxide (CO). The 3-dimensional structure of leghemoglobin contains no additional active sites to distinguish it from widely consumed heme proteins, nor is there any biochemical or physiological evidence that this protein has any enzymatic activity or other function outside of controlled binding to oxygen.

Thus, there is no evidence to suggest that soy leghemoglobin in food will behave any differently from the myriad of other functionally equivalent and widely consumed globin proteins in the human diet. However, due to a lack of widespread human consumption, Impossible Foods has used rigorous scientific procedures to evaluate soy leghemoglobin for potential toxicity or allergenicity, with results confirming that soy leghemoglobin (and the LegH Prep in which it is delivered) is non-toxic and poses negligible risk of allergenicity.

C.3 Absorption, Distribution, Metabolism and Excretion (ADME)

As discussed by Delaney *et al*. (2008), proteins that are unstable to the gastrointestinal system will not retain their functional attributes and therefore are of lower inherent risk to exhibit safety concerns following ingestion.

Detailed discussion of the *in vitro* digestibility of the LegH Prep is presented in Section C.6.3. In brief, soy leghemoglobin and *Pichia* proteins present within LegH Prep were shown to be readily digested by pepsin at pH 2. There are no compositional elements in LegH Prep that warrant further characterisation of the absorption, distribution, metabolism and excretion (ADME) profile of the ingredient.

C.4 Toxicological Data

Impossible Foods' LegH Prep has been the subject of comprehensive toxicological assessment, including 28-day oral toxicity studies conducted in rats, an *in vitro* bacterial reverse mutation assay, and an *in vitro* mammalian chromosome aberration test in human lymphocytes. These studies have been reviewed by various qualified scientific experts and by the U.S. FDA as part of the voluntary GRAS Notification procedure (GRN 737 – U.S. FDA, 2018a). These data were also published in a peer-reviewed publication (Fraser *et al*., 2018), and the full study reports of these toxicological studies are provided in Appendix VII.

Although the LegH Prep employed in these studies were produced using the initial MXY0291 production strain, their results are considered to be applicable to the LegH Prep produced by the current optimised MXY0541 strain on the basis that: (i) the composition of the LegH Prep produced by either strains are comparable and meets the same defined specifications; (ii) MXY0541 is a derivative of the MXY0291 strain; (iii) the DNA sequence encoding for the soy leghemoglobin and accordingly, the expressed protein, are identical in both strains (see Appendix III **[CONFIDENTIAL]**); and (iv) the additional modifications made to MXY0541 involve the incorporation of genes that are native to *P. pastoris*; they are only intended to optimise the production of soy leghemoglobin, and no unintended protein products are expected in the final LegH Prep (see Appendix III **[CONFIDENTIAL]**).

C.4.1 Repeated-Dose Toxicity Studies

C.4.1.1 14-Day non-GLP Dietary Toxicity and Palatability Study in Rats (Study 43167)

A non-Good Laboratory Practice (GLP) 14-day dietary toxicity and palatability study was conducted in rats to assess the feasibility of oral administration of LegH Prep (which contains soy leghemoglobin, *Pichia* proteins and other components) and to establish the dose range for a subsequent GLP 28-day dietary toxicology study (Fraser *et al.*, 2018). The study was conducted by Product Safety Labs (Dayton, NJ). The LegH Prep test article was freeze-dried, as freeze drying allowed for increased test article concentration in the feed and facilitated homogeneous dietary mixing. Doses of 0, 125, 250, or 500 mg/kg body weight/day of soy leghemoglobin were administered in the diet to groups of 6 male and 6 female CRL Sprague-Dawley CD® IGS rats for 14 days. Experimental observations included clinical observations, food consumption, body weight, haematology, and liver, spleen and bone marrow weight and histopathology. No treatment-related adverse findings were reported. Therefore, it was concluded that 500 mg/kg body weight/day of soy leghemoglobin would be well-tolerated by rats in a feeding study of longer duration.

C.4.1.2 28-Day GLP Dietary Toxicity Study in Rats (Study 43166)

A GLP 28-day dietary toxicology study was conducted in rats to determine the NOAEL for LegH Prep (containing soy leghemoglobin, *Pichia* proteins, and other components) (Fraser *et al.*, 2018). The study was conducted by Product Safety Labs (Dayton, NJ) and the full study report is provided in Appendix VII. The study was designed based on the U.S. FDA *Redbook Guidelines IV.C.4.a* (U.S. FDA, 2003) and the Organisation for Economic Co-operation and Development (OECD) Test Guideline No. 407 (OECD, 2008), and conducted in accordance with the U.S. FDA GLP (21 CFR Part 58) and OECD Principles of GLP (OECD, 1998).

The LegH Prep test article (containing soy leghemoglobin, *Pichia* proteins, and other components) was freeze-dried lot PP-PGM2-16-088-101, which was given a new sub-lot of PP-PGM2-16-088-301. As in the 14-day dietary study, freeze drying allowed for increased test article concentration in the feed and facilitated homogeneous dietary mixing. Groups of 10 male and 10 female CRL Sprague-Dawley CD® IGS rats were administered 0 (control), 512, 1,024, and 1,536 mg/kg/day of freeze-dried LegH Prep (LegH Prep solids) in the diet for 28 days. These dietary doses were selected to correspond to 250, 500, and 750 mg/kg/day of soy leghemoglobin, in the low-, medium-, and high-dose groups, respectively (Table C.4.1.2-1). The maximum dose of 750 mg/kg/day soy leghemoglobin was selected to achieve a concentration greater than 100-fold above the anticipated 90th percentile estimated daily intake (EDI). The control group received unformulated feed. To maintain target dietary doses throughout the study, concentrations in the test diets were calculated based on the most recent group body weight and food consumption data.

Table C.4.1.2-1 Dosing Information in a 28-Day Rat Feeding Study (Study 43166)

Group	Number of Animals per Group (M/F)	Target Exposure of Test Substance LegH Prep Dry Solids (mg/kg bw/day)	Target Exposure of Soy Leghemoglobin (mg/kg bw/day)
Control	10/10	0	0
Low-dose	10/10	512	250
Mid-dose	10/10	1,024	500
High-dose	10/10	1,536	750

bw = body weight; F = female; M = male.

Experimental observations included ophthalmologic evaluations, clinical observations, body weights, food consumption, clinical pathology including blood chemistry, haematology, coagulation, and urinalysis, gross necropsy, organ weights, and histopathology.

There were no mortalities, clinical observations, ophthalmology, body weight, body weight gain, food consumption, or food efficiency changes attributable to the administration of the test article. Additionally, there were no test article-related changes in haematology, serum chemistry or urinalysis parameters for males or female rats. Changes in coagulation parameters were limited to a non-dose-dependent increase in activated partial thromboplastin time in mid- and high-dose males. Since this finding was small in magnitude, with no corresponding pathological or clinical findings, it was not considered to be toxicologically relevant.

There were no test article-related differences in organ weights between test groups. Further no test article-related effects were reported during necropsy, macroscopic, or histopathological examinations in the male and female animals, with a single exception of an increased incidence in the metestrus stage of the oestrous cycle in the low- and high-dose groups. As discussed in detail below, the control and treated animals used in this study all had distributions of oestrous cycle stages that deviated significantly from published reports, suggesting the possibility of a sampling artefact unrelated to the administration of the test article. A follow-up study described below in Section C.4.1.3 demonstrated that the observed distributions were very likely due to sampling and assessing oestrous cycle distribution on a single day, rather using a longitudinal study that would assess the totality of the oestrous cycle and is not indicative of an adverse health effect.

Due to the oestrous cycle distributions reported in the control group as well as the test animals in the 28-day oral toxicity study, Impossible Foods elected to carry out a more extensive and rigorous longitudinal study focusing on the effects of the LegH Prep on the oestrous cycle of a larger group of female rats. The results of this study, described below, provide strong evidence that the oestrous cycle distribution of a group of rats on a given day commonly deviates greatly from their distribution over time, and provides a highly unreliable picture of oestrous cycle function.

C.4.1.3 28-Day Investigative Study in Rats with 14-Day Oestrous Cycle Pre-Screen (Study 44856)

To directly address the oestrous cycle distributions observed in the 28-day oral toxicity study, a non-GLP investigative 28-day dietary study was conducted in rats with a focus on oestrous cyclicity (Product Safety Labs, Dayton, NJ) (Fraser *et al*., 2018). The full study report is provided in Appendix VII. The study design included a 14-day oestrous cycle pre-screen to ensure that only animals with regular cyclicity advanced to the test article-dosing phase. The oestrous cycle was monitored daily for the last 14 days of the 28-day dosing period, which is consistent with OECD Test Guideline No. 421 for oestrous cycle evaluation (OECD, 2016a). At study termination, the reproductive organs were evaluated macroscopically and microscopically.

Groups of 15 female CRL Sprague-Dawley CD® IGS rats were administered freeze-dried LegH Prep[5] (containing soy leghemoglobin, *Pichia* proteins, and other components) in the diet at doses of 0 (control), 512, 1,024, and 1,536 mg/kg body weight/day. These doses were selected to correspond to 0, 250, 500, and 750 mg/kg body weight/day of soy leghemoglobin in the control, low-dose, mid-dose, and high-dose groups, respectively. The control group received unformulated feed. To maintain target dietary dose levels throughout the study, concentrations in the test diets were calculated based on the most recent group body weight and food consumption data.

[5] The same batch of LegH Prep (PP-PGM2-16-088-301) used in the 28-day oral toxicity study (Study 43166) was used in the 28-day investigative study with 14-day estrous cycle pre-screen (Study 44856).

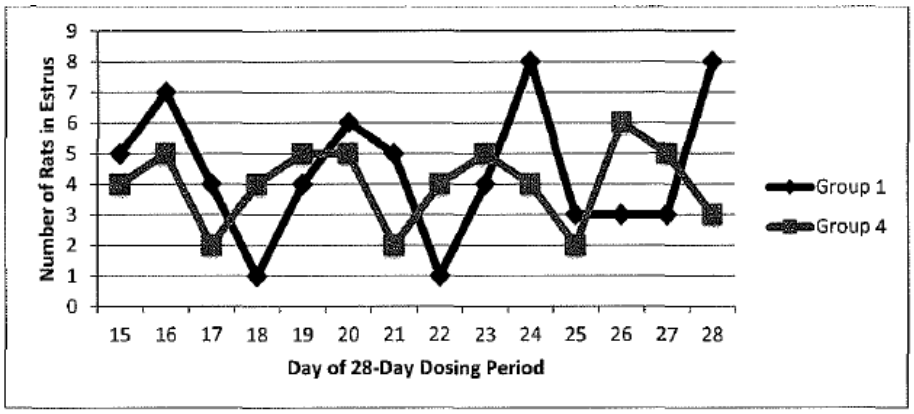
Prior to the 28-day dosing phase, oestrous was determined daily for 14 days, by vaginal lavage, to ensure that each animal had an average oestrous cycle length that was consistent with the published literature. Oestrous was also determined daily for the last 14 days of the 28-day dosing period to detect any changes in average oestrous cycle length as a result of LegH Prep consumption. By monitoring the oestrous cycle over time in each rat, the study avoided the sampling artefact of the previous study. The oestrous cycle was not monitored for the first 14-days of the dosing period to avoid over-manipulating the animals. Additional experimental observations included clinical observations, body weights, food consumption, gross necropsy, reproductive organ weights (uterus and ovaries with oviducts), and histopathology on reproductive organs (vagina, cervix, uterus, ovaries, and oviducts).

During the 14-day pre-dosing period, there was no significant change in average oestrous cycle length between groups, and all animals showed regular oestrous cyclicity. Therefore, all animals were advanced to the 28-day dosing phase. During the dosing phase, there were no clinical observations attributable to the administration of LegH Prep. There were no changes in body weight, rate of body weight gain, food consumption, and food efficiency attributable to LegH Prep administration. The mean number of oestrous cycles for female rats in the low- to high-dose groups were comparable to the control group throughout the study. There were no macroscopic and microscopic observations or organ weight changes attributed to the LegH Prep administration. Therefore, under the conditions of this study and based on the toxicological endpoints evaluated, administration of LegH Prep at doses up to 1,536 mg/kg body weight/day total dry solids (corresponding to 750 mg/kg body weight/day of soy leghemoglobin) did not cause any effect in oestrous cyclicity or reproductive organ pathology of female Sprague Dawley rats.

The results from Study 44856 fully address the potential concerns raised by Study 43166 and demonstrate that LegH Prep does not affect the female rat oestrous cycle. Each point is discussed below in greater detail.

Despite intrinsically normal oestrous cycles, the distribution of oestrous cycle stages on any given day can often be extremely deviant from the within-rat distribution over time (Figure C.4.1.3-1). Indeed, had the animals been analysed by necropsy on Day 18 of the dosing period, one would have drawn a completely different conclusion regarding the test article effect on oestrous cycle than had the necropsy been performed on Day 21. Thus, to avoid sampling artefacts, proper evaluation of the effect of a test substance on the oestrous cycle requires an extended longitudinal observation as performed in this study, in which no test article-dependent effect on the female oestrous cycle length or progression was reported.

Figure C.4.1.3-1 Number of Rats in the Oestrus Phase of the Oestrous Cycle on Each Day (Study 44856)



There was no difference in mean number of oestrous cycles between groups in either the pre-dosing or dosing phase of the study. All animals showed oestrous cyclicity that was consistent with the published literature (Westwood, 2008). The daily oestrous cycle monitoring that was performed in this study follows OECD 421 guidelines and demonstrates that all groups were cycling normally as expected based on published literature (Westwood, 2008; OECD, 2016a).

Study 43166 showed a decrease in uterine weights that corresponded to a decreased incidence of fluid filled uteri in low- and high-dose group females. In Study 44856, there was no significant difference in organ weights for the uterus or ovaries with oviducts between groups. Moreover, the presence of fluid filled uteri did not differ across groups (Table C.4.1.3-1). Published literature demonstrates that the presence of fluid filled uteri and uterine weight correlates with oestrous cycle stage (Westwood, 2008). Our results from Study 44856 reveal a similarly consistent correlation. Thus, the simplest explanation for the decrease in uterine weights observed for the low-dose and high-dose groups in Study 43166 is that the animals within those groups had a different distribution of oestrous cycle stages that typically correspond to lower uterine weight in healthy rats, compared with the control and mid-dose groups.

Table C.4.1.3-1 Summary of Necropsy Observations in the Uterus (Study 44856)

	Control 0 mg/kg/day Soy Leghemoglobin (n=15)	Low-dose 250 mg/kg/day Soy Leghemoglobin (n=15)	Mid-dose 500 mg/kg/day Soy Leghemoglobin (n=15)	High-dose 750 mg/kg/day Soy Leghemoglobin (n=15)
Number of uteri submitted for examination	13	14	15	15
Fluid filled	2	1	1	2

In Study 44856, Impossible Foods commissioned Karen Regan, DVM, DACVP, DABT (Regan Path/Tox Services, Inc., Ashland, OH) for histological evaluation of the female reproductive organs. Dr. Regan has extensive experience in the evaluation of rat reproductive systems, and currently serves as the U.S. FDA advisory committee member for reproductive toxicology. Prior to finalizing the pathology report, Impossible Foods shared the draft report for Study 43166 with Dr. Regan to ensure that she would look for the potential effects noted in that study.

In Study 44856, Dr. Regan performed a blind oestrous cycle determination, as well as a histological assessment on the vagina, uterus, ovaries, oviducts, and cervix of the control and high-dose group animals. Dr. Regan concluded that there were no test article-related microscopic observations in the reproductive tissues examined. All animals were considered to be cycling normally, with the exception of a single control animal that appeared to have a prolonged oestrous based on the morphology of the ovaries and uterus. This control animal finding was considered to be spontaneous and incidental because of the lack of similar findings in animals at the higher dose levels. Within the control and high-dose groups, all animals had evidence of old and recent corpora lutea and follicles at various stages of development in the ovaries, and had reproductive tissue morphology consistent with the stage of the cycle they were in. One low-dose group animal had prolonged oestrous based on morphology of the ovaries, including large atretic follicles, multiple corpus lutea at a similar state of atresia, and presence of squamous metaplasia of the uterus. These findings were considered spontaneous and incidental due to the lack of similar findings at higher dose levels. One control animal had large atretic follicles observed in both ovaries, and 1 high-dose group animal had luteinised follicles (follicles with evidence of luteinisation in the wall but have not ovulated) in both ovaries. Both of these observations are reported as background findings in rats of the strain and age used in this study (Dixon *et al.*, 2014) and were considered incidental because of their singular occurrences.

In summary, in Study 44856, Dr. Regan and Product Safety Labs concluded that there was no test substance-related effect on reproductive macroscopic or microscopic observations, reproductive organ weights, or oestrous cyclicity.

C.4.1.4 Pathology Peer Review on 28-Day GLP Dietary Toxicity Study in Rats (Study 43166)

Because no test article-related effects on the female oestrous cycle were seen in Study 44856, Impossible Foods commissioned a pathology peer review on the reproductive organs from Study 43166. The review pathologist (Dr. Regan) received and evaluated histological slides for the cervix, ovaries, oviducts, uterus and vagina, along with the corresponding macroscopic and microscopic finding noted by the study pathologist. Both the study pathologist and review pathologist met and performed an in-person slide review in June 2017 and reached a consensus evaluation that is reflected in the pathology report for Study 43166. Both pathologists were in agreement on the oestrous cycle staging; however, the presence of old and recent corpora lutea suggests that the animals were cycling normally. Moreover, Study 44856 clearly illustrates that that there is no test article-dependent effect on oestrous cyclicity. In summary, although the study pathologist for Study 43166 initially reported a possible change in the oestrous cycle, following peer review, a consensus was reached that there were no test article-dependent effects on the female oestrous cycle and reproductive organs.

C.4.1.5 No-Observed-Adverse-Effect Level

In the 28-day GLP dietary toxicity study in rats (Study 43166), there were no test article-related adverse effects observed in the male or female animals at the maximum dose tested. Therefore, the NOAEL for administration of LegH Prep solids in the diet of male and female Sprague Dawley rats was the maximum dose tested, 1,536 mg/kg body weight/day, which corresponds to 750 mg/kg body weight/day of soy leghemoglobin.

C.4.2 Mutagenicity/Genotoxicity

The genotoxic potential of LegH Prep (Batch No. PP-PGM2-16-015-101) (containing soy leghemoglobin, *Pichia* proteins, and other components) was evaluated in a bacterial reverse mutation assay and an *in vitro* mammalian chromosome aberration test in human lymphocytes. The studies were conducted in accordance with OECD Testing Guideline Nos. 471 and No. 473, respectively, as well as the OECD Principles of GLP (as revised in 1997) (OECD, 1997, 1998, 2016b). These studies used the standard liquid formulation of LegH Prep since, unlike the animal feeding studies, freeze drying was not required for test article administration.

C.4.2.1 Bacterial Reverse Mutation Assay

A bacterial reverse mutation test was conducted to evaluate the mutagenic potential of LegH Prep (Fraser *et al*., 2018). The full study report is provided in Appendix VII. LegH Prep was tested up to a maximum concentration of 74,000 µg/plate, which corresponded to a maximum soy leghemoglobin concentration of 5,000 µg/plate, in *Salmonella typhimurium* (*S. typhimurium*) TA1535, TA1537, TA98, TA100, and *E. coli* WP2 uvrA. The main test was conducted using the plate incorporation method, both in the presence and absence of metabolic activation (S9), and the confirmation test was conducted using the pre-incubation method. No signs of precipitation or contamination were reported in any of the strains. Further, for both the main test and the confirmation test, no signs of toxicity were reported in any test strains in the presence or absence of S9. Based on the results of this study, LegH Prep is non-mutagenic at concentrations up to 74,000 µg/plate.

C.4.2.2 In Vitro Mammalian Chromosome Aberration Test in Human Lymphocytes

An *in vitro* chromosome aberration assay was conducted to investigate the potential of soy leghemoglobin and LegH Prep to induce structural chromosome aberrations in human lymphocytes (Fraser *et al*., 2018). The full study report is provided in Appendix VII. The treatment interval was 4 hours without and with metabolic activation (Experiment I) and 24 hours without metabolic activation (Experiment II). Tests were performed in duplicate cultures, and 150 metaphases per culture were scored for structural chromosomal aberrations. The metaphases were prepared 24 hours after start of treatment with the test item.

In Experiment I, test concentrations of soy leghemoglobin were 500, 1,000, 2,500, or 5,000 µg/mL. In Experiment II, test concentrations of soy leghemoglobin were 100, 200, 500, or 1,000 µg/mL. In Experiment II, precipitation occurred at concentrations 500 µg/mL and higher during the fixation of the cells. In contrast to Experiment I, in Experiment II (long-term treatment), the test item was not removed by repeated washing steps, as the treatment period is stopped by the fixation step directly. When the cells were spread on the object slides, the precipitation appeared as a greenish lacquer coat, visible by eye and with the aid of an inverted microscope. The evaluation of aberration rates was not affected.

In each experiment, percent relative mitotic index was measured for each soy leghemoglobin concentration. A relative mitotic index greater than 45% is required to accurately measure chromosome aberrations. In Experiment I, without metabolic activation, the decrease below 70% relative mitotic index was seen at concentrations of 1,000 µg/mL (69%), 2,500 µg/mL (56%), and 5,000 µg/mL (54%). In Experiment I, with metabolic activation, no decrease below 70% relative mitotic index was observed. As noted by OECD Guideline No. 473 (OECD, 2016b), mitotic index is an indirect measurement of toxicity that can be influenced by a number of factors such as time and cell cycle disruption, and additional data such as cell cycle delay is often helpful in assessing toxicity. In the current experiments, cell cycle delay was assessed in the cell proliferation using the Bromodeoxyuridine (BrdU) technique. No biologically significant decrease in proliferation was noted in Experiment I, and the levels of the mitotic index remained above the 45% required to accurately assess chromosomal aberrations. Further, as noted in the study report, the cytotoxicity is likely even lower than shown in Experiment I without metabolic activation, as there appeared to be a detoxification in the Experiment I with metabolic activation.

In Experiment II, without metabolic activation, cytotoxic effects regarding the mitotic index were reported at concentrations of 500 µg/mL (69%), 1,000 µg/mL (53%), 2,000 µg/mL (26%), 3,000 µg/mL (13%), 4,000 µg/mL (38%), and 5,000 µg/mL (42%).

In Experiments I and II, no biologically relevant increase in the frequencies of polyploid cells was reported at concentrations up to 5,000 µg/mL. In Experiment I, no biologically relevant decreases of the proliferation index were reported at concentrations up to 5,000 µg/mL. In Experiment II, the values of the proliferation index of the negative controls were 1.56. The proliferation index of the 500 and 1,000 µg/mL groups were 1.23 and 1.12. Decreases in the proliferation index of 79% at 500 µg/mL and 72% at 1,000 µg/mL were observed. These decreases were not a consequence of chromosome aberrations.

In Experiments I and II, no biologically or statistically significant increase of the aberration rates was reported after treatment with LegH Prep containing soy leghemoglobin compared to the solvent control cultures. The x2 Test for trend was performed to test whether there was a concentration-related increase in chromosomal aberrations. No statistically significant increase was reported in all experimental conditions. Ethyl methanesulfonate (EMS) (400 or 900 µg/mL) and cyclophosphamide (CPA) (7.5 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the efficiency of the test system to indicate potential clastogenic effects.

Under the conditions of these studies, it was concluded that LegH Prep did not induce structural chromosomal aberrations in human lymphocyte cells. Therefore, LegH Prep is considered to be non-clastogenic in this chromosome aberration test.

C.5 Safety of the Production Organism

C.5.1 Origins and History of Use

As discussed, soy leghemoglobin is produced in the well-characterised expression host *P. pastoris* (Cereghino and Cregg, 2000). *Pichia* is non-pathogenic and non-toxigenic. *Pichia* belongs to the same family of yeast (Saccharomycetaceae) as several yeast genera widely used in food: Saccharomyces, Torula, Yarrowia, Dekkera, and Brettanomyces. Brettanomyces, a yeast traditionally used in brewing Belgian beers, belongs to the same sub-family of yeast as *Pichia* (the *Pichiaceae*). Yeast extract (from *Saccharomyces cerevisiae* [S. cerevisiae] and Torula) is frequently directly consumed in substantial quantities in human diets. Impossible Foods' genetically modified *Pichia* production strain complies with the OECD criteria for Good Industrial Large Scale Practice (GILSP) microorganisms (OECD, 1992). It also meets the criteria for a safe production microorganism as described by Pariza and Foster, Pariza and Johnson, and several expert groups (Pariza and Foster, 1983; Berkowitz and Maryanski, 1989; IFBC, 1990b; SCF, 1992; OECD, 1993; FAO/WHO, 1996; Jonas *et al*., 1996; Pariza and Johnson, 2001). Furthermore, the production strain is derived from a strain lineage considered to meet EFSA's QPS status when used for enzyme production (EFSA, 2017).

Pichia has been used to express recombinant proteins for use in human food (*e.g*., phospholipase C [GRN 204 – U.S. FDA, 2006]) and U.S. FDA-approved therapeutics. Moreover, the American Association of Feed Control Officials has approved the *E. coli* enzyme phytase derived from the fermentation of recombinant *P. pastoris* for use in animal feed (AAFCO, 2019). EFSA has approved the use of phytase produced from the fermentation of recombinant *Komagataella pastoris* (formerly named as *Pichia pastoris*) for use in animal feed (EFSA, 2016). *P. pastoris* is also the host used for production of nitrate reductase (The Nitrate Elimination Co. Lake Linden, MI), an enzyme used for treatment of potable water. *P. pastoris* itself has been approved by the U.S. FDA as an animal feed protein source allowed in broiler feed up to 10% of the total feed (FDA 21 CFR Part 573, 1993) (U.S. FDA, 1993).

As such, Impossible Foods' *P. pastoris* production strain is derived from a strain lineage with a long history of safe use. All genetic modifications made to generate the production strain are well-characterised by full genome sequencing and conform to the guidelines for generating safe production strains for the recombinant production of food ingredients (Olempska-Beer *et al*., 2006). LegH Prep does not contain any viable production organism or antibiotic resistance genes. Impossible Foods has been able to isolate detectable amounts of *Pichia* DNA from the final LegH Prep solution, and it has determined that about 300 mg of *Pichia* DNA will be present in about 1 L of LegH Prep. Impossible Foods has used mass spectrometry to identify the *P. pastoris* proteins that are present in LegH Prep at ≥1% of the total protein fraction. As described below in Section C.6, the sequence of each protein was analysed to ensure that the *Pichia* proteins present in LegH Prep do not contain significant homology to known allergens. All of the identified co-purifying proteins have highly conserved orthologues in yeast species used in food, such as *S. cerevisiae*.

C.5.2 Information on the Pathogenicity and Toxicity of the Production Microorganism

According to the definitive source of yeast taxonomy, as well as a thorough literature search, there are no indications that *P. pastoris* has been associated with animal or human illness.

P. pastoris does not produce active toxins (Pariza and Johnson, 2001). *P. pastoris* has been placed in the Biosafety Level 1 (BSL-1) class by the ATCC organisation, indicating *Pichia* is a well-characterised agent not known to cause disease in healthy human adults, and to be of minimal hazard to laboratory personnel and the environment (CDC, 1999). Toxicity studies done in support of the above-referenced *P. pastoris*-approved animal feed also demonstrated that *P. pastoris* is neither pathogenic nor toxigenic (FDA 21 CFR Part 573 – U.S. FDA, 1993). Moreover, systemic toxicity and genotoxicity testing performed on LegH Prep demonstrate that the residual *Pichia* proteins and cellular components present in LegH Prep are non-toxic.

The use of *P. pastoris* in the production of a phospholipase C enzyme preparation for use as a de-gumming agent for vegetable oils was concluded to be GRAS for use by Diversa Corporation in 2006. This conclusion was notified to the U.S. FDA in June 2006 (GRN 204 – U.S. FDA, 2006), and the Agency responded with a "no questions" letter. Pivotal data that supported the safety of using *P. pastoris* in the production of a phospholipase C enzyme preparation included a 90-day gavage toxicity study conducted in Sprague Dawley rats (20 animals/sex/group) administered the enzyme preparation at doses of 0, 500, 1,000, or 2,000 mg/kg body weight/day. The investigators concluded, and the U.S. FDA agreed, that the NOAEL for this study was 2,000 mg/kg body weight/day, the highest dose study. Although the phospholipase C enzyme preparation was a secreted product from *P. pastoris* compared to the lysing/extraction of LegH Prep, the data support that the host microorganism, *P. pastoris* does not impart metabolites that would be a safety concern.

The safety and probiotic properties of live *P. pastoris* has been evaluated in several animal models that support that this strain is not toxic or pathogenic. Studies conducted in chicks support that *P. pastoris* is safe for use in feed as a probiotic and can increase feed efficiency compared to untreated control feeds (Gil de los Santos *et al*., 2012, 2018). In another publication, França *et al*. (2015) administered live *P. pastoris* X-33 strain to male BALB/c mice to evaluate persistence and susceptibility of the microbe to digestive processes, as well as safety and antibacterial properties against *S. typhimurium* inoculations. In the persistence investigation, live *P. pastoris* (~7 log CFU/animal) were administered by gavage and faeces were collected for 72 hours. In the safety and antibacterial investigation, the microbe was administered to groups of 10 or 15 male mice by either gavage or in the diet for 20 days and compared to control groups receiving *Saccharomyces boulardii* (*S. boulardii*). After 20 days of dosing, 5 animals per group were euthanised to evaluate safety and subjected to histopathologic evaluations (intestine, liver, spleens), while the remainder of the animals were challenged with a 5 log CFU/mouse dose of *S. typhimurium*. Survival was measured 10-days post-challenge dose.

Following acute oral dosing, 76.8% of *P. pastoris* was reported to survive the gastrointestinal tract and live counts were measured in the fresh faeces at 24 hours (6.84 log CFU/g), 48 hours (2.87 log CFU/g), but no growth was observed in faeces collected at 72 hours post-dosing. These data support that although *P. pastoris* may survive digestive processes, it does not appear to persist or grow in the gastrointestinal tract. In the repeated-dose study, no clinical signs of toxicity were observed, and histopathologic evaluations did not reveal any signs of toxicity. Survival after *S. typhimurium* challenge was statistically significantly improved in the 2 groups receiving *P. pastoris* (gavage and diet) and no *S. typhimurium* was detected in faeces or translocated into the liver or spleen compared to untreated animals and decreased levels of live bacteria were detected in the intestines compared to controls.

Overall, the available evidence supports that *P. pastoris* is non-toxigenic and non-pathogenic. It should also be noted that no viable *P. pastoris* production strain remains in the final LegH Prep ingredient.

C.6 Allergenicity

C.6.1 Approach for the Assessment of Potential Allergenicity

The potential allergenicity of soy leghemoglobin as well as the *Pichia* proteins present in LegH Prep were assessed in the same manner as used for the novel proteins expressed in genetically engineered foods. The Codex Alimentarius Commission developed an assessment scheme for the analysis of the potential allergenicity of proteins derived from biotechnology (Codex Alimentarius, 2009). This assessment is a multi-factorial approach which includes assessing the source of the protein for allergenicity, the sequence homology of the protein to known allergens, resistance to pepsin degradation and, if there is a high suspicion of allergenicity, specific serum screening. This analysis provides a likelihood of allergenic response by considering the totality of the evidence. Several prominent organisations support this approach: the 1996 Allergy and Immunology Institute of the International Life Sciences Institute and International Food Biotechnology Council (ILSI-IFBC) decision tree, the 1996 Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) consultation on biotechnology and food safety, the 2000 FAO/WHO consultation on food derived from biotechnology, the 2001 FAO/WHO consultation on allergenicity assessment of genetically-modified foods, the 2002 Codex *ad hoc* task force on safety assessment of biotechnology, and the 2003 Codex Alimentarius Commission guidelines to assess the allergenicity of genetically modified crops (FAO/WHO, 1996, 2000, 2001; Metcalfe *et al.,* 1996; Codex Alimentarius, 2002, 2009).

Initially, the *P. pastoris* MXY0291 production strain was used by Impossible Foods to produce their soy leghemoglobin. As detailed in the GRAS Notice submitted to the U.S. FDA (GRN 737 – U.S. FDA, 2018a), Impossible Foods had enlisted Dr. Richard E. Goodman, research professor at the Food Allergy Research and Resource Program (FARRP) of the University of Nebraska, to assess the potential allergenicity and toxicity of soy leghemoglobin and the *P. pastoris* proteins present in LegH Prep at ≥1% of the total protein fraction, consistent with the Codex recommendations. Approximately 17 *P. pastoris* proteins were found to be present in LegH Prep at ≥1% of the total protein fraction. These proteins are consistent from batch to batch and were identified by Impossible Foods using mass spectrometry. The multifactorial approach employed by Dr. Goodman, which included a comprehensive literature search, sequence homology, and pepsin digestion assessments, is widely used in the food industry to assess the allergenic potential of new proteins (Fuchs *et al.*, 1993; Noteborn *et al.,* 1995; Harrison *et al.,* 1996; Reed *et al.,* 1996; Hashimoto *et al.,* 1999; Momma *et al.,* 1999; Hileman *et al*., 2006; Goodman *et al*., 2007; Moran *et al*., 2014). A summary of Dr. Goodman's evaluation of the LegH Prep is provided in Appendix VIII. The comprehensive literature search conducted by Dr. Goodman (see Appendix IX-a, Appendix IX-b), and as updated by Impossible Foods for this submission, did not identify any published literature to suggest that consumption of soy leghemoglobin or residual *P. pastoris* proteins would be associated with allergic, toxic or adverse health effects. As part of the evaluation for LegH Prep produced using the initial MXY0291 production strain, Dr. Goodman also performed bioinformatic searches for sequence homology, and a full pepsin digestion study, the results of which are provided in Appendix IX and X. These data were also published by Goodman and colleagues in a peer-reviewed publication in 2018 (Jin *et al*., 2018).

Impossible Foods has since introduced a few additional minor modifications to the *P. pastoris* MXY0291 production strain to further optimise for the expression of soy leghemoglobin, which resulted in the current MXY0541 strain. The residual proteins in LegH Prep produced by MXY0541 are largely the same as those previously identified in the LegH Prep produced by the initial MXY0291 strain, or otherwise represent proteins that are native to the *P. pastoris* production organism (see Appendix III **[CONFIDENTIAL]**). As such, the LegH Prep produced by the optimised MXY0541 strain is similarly not anticipated to pose any significant risk of allergy or toxicity to consumers. This is supported by

additional bioinformatic analysis conducted on the LegH Prep produced using the optimised MXY0541 strain.

Overall, based on a weight-of-evidence approach, it was concluded that consumption of soy leghemoglobin, as well as the residual *P. pastoris* proteins present in Impossible Foods' LegH Prep, raise no health or safety concern as they do not pose any significant risk of allergy or toxicity to consumers. An overview of the data and information supporting the assessment for allergenicity is presented in Sections C.6.2 to C.6.5 that follow.

C.6.2 Bioinformatic Searches for Sequence Homology

Dr. Goodman's assessment determined if the amino acid sequence of soy leghemoglobin or the *P. pastoris* proteins in LegH Prep contained sufficient similarity with any known allergen or toxin to suggest possible cross-reactivity. Soy leghemoglobin and *P. pastoris* protein sequences were compared to the Allergen Online Database (version 16, January 2016; www.allergenonline.org – FARRP, 2016). Additionally, the sequences of soy leghemoglobin and P. *pastoris* proteins were searched against the entries in the National Center for Biotechnology Information (NCBI)-Entrez database, first without any keyword selection, and again with keywords "allergen", "toxin" or "toxic". Soy leghemoglobin protein did not produce significant (>35%) homology to known allergens or toxins (see Appendix IX-a and Jin *et al.*, 2018).

All of the residual *P. pastoris* proteins remaining in the LegH Prep have homologs that are ubiquitous in nature. A search of the NCBI database for sequences related to each of these residual proteins, using BLASTP without keyword limits, identified good alignments with related proteins from many moulds and yeasts. These alignments included *S. cerevisiae* and *Saccharomyces bayanus*, which are commonly used in making wine, bread, and beer, and *S. boulardii*, which is widely used as a probiotic (Moyad, 2008; Liu *et al*., 2016; Muñoz-Bernal *et al*., 2016). The long history of consumption of these close homologs of the residual *P. pastoris* proteins, with no reports of allergenicity or toxicity, offers strong general evidence for their safety in food (Appendix IX-b).

Bioinformatics searches with the 17 most abundant residual *P. pastoris* proteins found in the LegH Prep produced by the original MXY0291 strain, identified a few related protein sequences with sufficient similarity to exceed the Codex suggestion for potential cross reactivity (>35%) (Table C.6.2-1). However, the sequence-related putative allergens identified in this search were not potent, common allergens, nor were any of them known to be allergenic when ingested. Moreover, comparison of the same *P. pastoris* proteins with all proteins in the NCBI Protein database identified far more significant matches to proteins found in commonly consumed fungi, including baker's yeast (*Saccharomyces* species). Similar results were obtained in the updated search conducted on the 11 most abundant residual *P. pastoris* proteins that were identified in the LegH Prep produced by the current optimised MXY0541 strain (Table C.6.2-2).

While a number of organisms with known toxicity (*e.g.*, *Bacillus* sp., *Enterococcus faecalis*, *Streptomyces* sp., *Clostridium* sp.) contained proteins with sequences similar to those of the *P. pastoris* proteins of interest, these proteins were ubiquitous and highly conserved across diverse species and are not themselves known or suspected to be toxic. A comparison of the sequence-related proteins from toxin-producing species with proteins from diverse non-toxic species revealed far more closely related proteins from sources that are known to be safe and non-toxic (Appendix IX-b).

Table C.6.2-1 Summary of Sequence Alignments for Soy Leghemoglobin and the 17 Most Abundant Residual *Pichia pastoris* Proteins Found in LegH Prep Produced by MXY0291

Protein Name	GeneInfo Identifier	Accession	No. of Amino Acids	AllergenOnline Matched Allergen[a]	AllergenOnline Best ID (%)	*Saccharomyces* sp. Best ID (%)
Soy leghemoglobin	126241	P02236.2	145	n/a	n/a	n/a
Alpha aminoadipate reductase	238030060	CAY67983.1	1400	n/a	n/a	60%
Cobalamin-independent methionine synthase	238030843	CAY68766.1	768	Sal k 3	77.50%	77%
Aconitase	254564667	XP_002489444.1	780	n/a	n/a	81%
Transketolase	238030057	CAY67980.I	679	n/a	n/a	70%
Glycerol kinase	238034027	CAY72049.I	621	n/a	n/a	53%
Catalase A	254569930	XP _002492075.1	510	Alt a 10	72.50%	66%
G6PD	-238031000	CAY68923.I	504	Lep d 13	35.40%	64%
Hypothetical protein PAS	238031215	CAY69138.I	525	Cla h 10	72.50%	69%
				Alt a 10	72.50%	
				Lep d 13	35.40%	
Mitochondrial aldehyde dehydrogenase	238033249	CAY71271.1	501	Cla h 10	76.20%	62%
Delta-aminolevulinate dehydratase	238033645	CAY71667.I	341	n/a	n/a	76%
Mitochondrial alcohol dehydrogenase isozyme III	238031179	CAY69102.I	350	Cand a 1	85%	74%
Malate dehydrogenase	238034064	CAY72086.1	342	Mala f 4	70%	57%
				Pis s 2	36.20%	
Putative protein, unknown function	238033788	CAY718IO.I	328	n/a	n/a	86%
Triose phosphase isomerase	238032989	CAY71012.1	248	Tri a 31	62.50%	71%
				Def f 25.0101 (isoform)	60.00%	
				Der f 25.0201 (isoform)	60.00%	
				Cra c 8	57.50%	
Hypothetical protein (cyclophilin) PP7435	328350030	CCA36430.1	161	Mala s 6	87.50%	74%
				Asp f 27	85%	
				Cat r 1	81.30%	
				Der f 29	80%	
				Asp f 11	80%	
				Bet v 7	80%	
				(Unassigned by IUIS) PPIase from *Dauces carota*	78%	
Cytosolic superoxide dismutase	238034030	CAY72052.1	154	Ole e 5	60% to 55%	79%
				23 isoforms		

Table C.6.2-1 Summary of Sequence Alignments for Soy Leghemoglobin and the 17 Most Abundant Residual *Pichia pastoris* Proteins Found in LegH Prep Produced by MXY0291

Protein Name	GeneInfo Identifier	Accession	No. of Amino Acids	AllergenOnline Matched Allergen[a]	AllergenOnline Best ID (%)	*Saccharomyces* sp. Best ID (%)
Mitochondria ATPase Inhibitor	238029769	CAY67692.1	84	n/a	n/a	62%

ID = identity; IUIS = International Union of Immunological Societies; n/a = not available or no answer; PPIase = peptidylprolyl isomerase.
[a] Allergen name in IUIS allergen list unless denoted as unassigned by IUIS.

Table C.6.2-2 Summary of Sequence Alignments for the Most Abundant Residual *Pichia pastoris* Proteins Found in LegH Prep Produced by MXY0541

Protein Name[a]	GeneInfo Identifier	Accession	No. of Amino Acids	AllergenOnline Matched Allergenb	AllergenOnline Best ID (%)	*Saccharomyces* sp. Best ID (%)
Proteins that Represented ≥1% of the Total Protein Fraction in the LegH Prep Produced by MXY0291						
Mitochondrial alcohol dehydrogenase isozyme III	238031179	CAY69102.I	350	Cand a 1	85%	74%
				(Unassigned by IUIS) alcohol dehydrogenase from *Curvularia lunata*	73%	
Mitochondrial aldehyde dehydrogenase	238033249	CAY71271.1	501	(Unassigned by IUIS) aldehyde dehydrogenase from *Davidiella tassiana*	76%	64%
				Alt a 10	74%	
				Tyr p 35	71%	
Delta-aminolevulinate dehydratase	238033645	XP_002493846.1	341	n/a	n/a	64%
hypothetical protein	254568616	XP_002491418.1	525	(Unassigned by IUIS) aldehyde dehydrogenase from *Davidiella tassiana*	72%	71%
				Alt a 10	73%	
				Tyr p 35	64%	
				(Unassigned by IUIS) allergen from *Lepidoglyphus destructor*	35%	
Proteins that are Abundant Only in the LegH Prep Produced by MXY0541						
NAD(+)-dependent formate dehydrogenase	254572123	XP_002493171.1	365	n/a	n/a	61%
Uroporphyrinogen decarboxylase	254572169	XP_002493194.1	361	n/a	n/a	74%

Table C.6.2-2 Summary of Sequence Alignments for the Most Abundant Residual *Pichia pastoris* Proteins Found in LegH Prep Produced by MXY0541

Protein Name[a]	GeneInfo Identifier	Accession	No. of Amino Acids	AllergenOnline Matched Allergenb	AllergenOnline Best ID (%)	*Saccharomyces* sp. Best ID (%)
Translational elongation factor EF-1 alpha	254567507	XP_002490864.1	459	n/a	n/a	89%
Hexokinase-2	254567173	XP_002490697.1	496	n/a	n/a	58%
acetate--CoA ligase	8198044	XP_002491701.1	668	n/a	n/a	67%
Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3	254568470	XP_002491345.1	333	Tri a 34.0101	88%	81%
Transketolase, similar to Tkl2p	254571911	XP_002493065.1	707	n/a	n/a	45%

ID = identity; IUIS = International Union of Immunological Societies; n/a = not available or no answer.
[a] Of the 11 residual proteins that are present in the LegH Prep from the current MXY0541 strain, there are 7 proteins that were not present at ≥1% of the total protein fraction in the LegH Prep from the MXY0291 strain. Even though overlap existed across 4 of the residual proteins, the bioinformatic searches were repeated for all 11 residual *P. pastoris* proteins, given that the AllergenOnline and NCBI-Entrez databases have been updated since the searches were initially conducted.
[b] Allergen name in IUIS allergen list unless denoted as unassigned by IUIS.

C.6.3 *In vitro* Digestibility and Stability

In vitro studies have been conducted to evaluate the stability of the soy leghemoglobin and *Pichia* proteins within the LegH Prep to pepsin degradation in simulated gastric fluid. Several peer reviewed studies have shown that low *in vitro* pepsin digestibility is an important risk factor for food allergy (Astwood *et al.*, 1996; del Val *et al.*, 1999). Bannon *et al.* (2003) reviewed a broad range of published pepsin digestion studies and found a strong positive predictive value of the digestion protocol when comparing the stability of allergenic and non-allergenic dietary proteins (Bannon *et al.*, 2003). A published multi-laboratory study demonstrated the rigor and reproducibility of using pepsin digestion to evaluate the stability of a number of food allergens and non-allergenic proteins across 9 laboratories (Thomas *et al.*, 2004). The pepsin digest protocol used by Dr. Goodman to investigate the digestibility of the proteins in the LegH Prep is identical to the robust procedure used in Thomas *et al.* (2004). In addition to the recommended ratio of 10 U pepsin enzyme to 1 µg target protein, a more stringent ratio of 1 U enzyme to 1 µg target protein was also employed.

The digestibility study conducted by Dr. Goodman on the LegH Prep obtained using the initial *P. pastoris* MXY0291 production strain is summarised in detail in Appendix X, and the results have also been published (Jin *et al.*, 2018). This study demonstrated that soy leghemoglobin, as well as the residual *P. pastoris* proteins, are readily digested by pepsin at ratios of 10 U pepsin enzyme to 1 µg target protein and 1 U enzyme to 1 µg target protein, as confirmed with SDS-PAGE analysis (Appendix X; Jin *et al.*, 2018). It is the expert opinion of Dr. Goodman that using a lower than standard activity of pepsin in this assay is not scientifically justified due to insufficient published data on the sensitivity of known allergens and non-allergenic proteins under these conditions, and thus the inability to interpret the results.

C.6.4 Assessment of Potential Soy and Legume Cross-Reactivity

C.6.4.1 Soy Cross-Reactivity

It is the expert opinion of Dr. Steven L. Taylor, co-founder and co-director of the FARRP at the University of Nebraska, that Impossible Foods does not need to perform experiments to demonstrate that LegH Prep does not cross-react with soy-allergic individuals (Appendix VIII). Although soybeans are acknowledged as a commonly allergenic food, soy allergy appears to occur almost exclusively in young infants, and while 0.4% of children are allergic to soy, the large majority of them outgrow it by the age of 10 (Savage *et al*., 2010). Dr. Taylor indicated that given the limited size of adult population of soy-allergic individuals, it would be difficult to acquire enough subjects to perform an oral challenge study, and that it would be difficult to even identify a sufficient number of well-characterised soy-allergic subjects to be sources of blood serum for serum immunoglobin E (IgE)-binding studies (Appendix VIII). It should also be highlighted that soy leghemoglobin is not identified among the known soybean allergens, nor is it detectably present in the edible soybean seeds. Leghemoglobin is natively expressed in the root of the soybean plant, whereas the major soy allergens (Gly m 4, Gly m 5, and Gly m 6) are located in the soybean seed. These allergens are completely absent from LegH Prep, which is produced by *P. pastoris* genetically engineered to express only soy leghemoglobin. This physical separation, as well as the lack of sequence homology to known soy allergens, indicates that soy leghemoglobin is highly unlikely to elicit a reaction in a soy-allergic consumer.

Additionally, soy leghemoglobin was evaluated to determine if this protein had the potential to become a novel food allergen. In accordance with the consensus recommendations of the Codex Alimentarius Commission, it is the opinion of Dr. Taylor that sequence homology and pepsin digest analyses are the most predictive methods known to date to assess allergenicity of novel proteins. Therefore, besides these 2 tests, there are no additional tests that Impossible Foods could perform that would strengthen the evidence against potential allergenicity of soy leghemoglobin.

Even so, Impossible Foods will notify consumers by labelling their products containing soy leghemoglobin with the statement, “Contains Soy”. In addition to the allergen statement on the business to business labelling, Impossible Foods will provide training materials and information about the product to restaurants who purchase the product, indicating it is a soy-protein based product. Because Impossible Foods will identify the potential allergen on its label, there is no necessity to prove that soy-allergic individuals will not react to soy leghemoglobin.

C.6.4.2 Legume Cross-Reactivity

Clinical cross-reactivity among various foods from the legume family is rare (Bernhisel-Broadbent and Sampson, 1989). In the largest study reported to date, in 793 persistent peanut-allergic subjects, 9.5% were considered allergic to other legumes by oral challenge including 48 to soy, 19 to pea, 7 to lentil, 4 to chickpea, and 3 to green bean (Neuman-Sunshine *et al*., 2012). Based upon the prevalence and severity of peanut allergy, potential cross-reactions between soy leghemoglobin and peanut allergens is the key area of potential concern. The various peanut allergens are very well identified and characterised. No significant sequence homology exists between soy leghemoglobin and any of the peanut allergens (see Section C.6.2). Moreover, soy leghemoglobin is found in the root of the soybean plant and bears no structural resemblance or sequence homology to these seed storage proteins which are found in the peanut kernel. It is the expert opinion of Dr. Taylor that Impossible Foods does not need to perform experiments to demonstrate that LegH Prep does not cross-react with legume-allergic individuals (Appendix VIII).

C.6.5 Assessment of Potential Cross-Reactivity with Meat Allergic Individuals

Tick-bite induced allergy to mammalian meat (*e.g.*, meat, pork) and organs (*e.g.*, liver, kidney) has been reported in the U.S., Europe, Australia and parts of Asia (Steinke *et al.*, 2015). However, the allergic reaction is due to an IgE antibody response to the oligosaccharide galactose-alpha-1,3-galactose (α-gal), which is located on glycoproteins and glycolipids in non-primate mammalian meat and organs (Commins *et al.*, 2016). The allergic reaction is not caused by myoglobin, and therefore consumers with α-gal specific IgE antibodies will not cross-react with soy leghemoglobin.

Impossible Foods is aware of only a single case of meat allergy linked to bovine myoglobin (Fuentes *et al.*, 2004), although implication of bovine myoglobin in this case has been disputed (Fiocchi *et al.*, 2005). The reactions reported in this patient were specific to bovine myoglobin, and not porcine myoglobin, suggesting that this is not a general allergy to oxygen-binding globin proteins, but rather a specific response to a bovine-derived protein. Given the widespread consumption of meats containing oxygen-binding globins at concentrations comparable to those proposed for use of the soy leghemoglobin, the low incidence of meat allergies in general (and the cause of those few reactions is predominantly due to bovine serum albumin sensitivities), and only a single reported case of myoglobin allergy, this argues that these proteins as a class have low allergenicity.

C.7 Safety Assessment Reports Prepared by International Agencies or Other National Government Agencies

C.7.1 United States

Impossible Foods' soy leghemoglobin has GRAS status for use as a flavouring and nutrient source of iron in ground beef analogue products at a level of not more than 0.8% soy leghemoglobin. The GRAS status of soy leghemoglobin was first notified to the U.S. FDA in 2014 by Impossible Foods. The Notice was subsequently withdrawn without prejudice by Impossible Foods, and the U.S. FDA ceased evaluation of the Notice on 10 November 2015 (GRN 540 – Impossible Foods Inc., 2014; U.S. FDA, 2015). An updated GRAS Notification containing published results on toxicological testing conducted with LegH Prep was submitted by Impossible Foods to the U.S. FDA in 2018 and was filed without questions from the Agency under GRN 737 (Impossible Foods Inc., 2018; U.S. FDA, 2018a).

Impossible Foods intends on expanding the uses of soy leghemoglobin beyond ground beef analogue applications in the U.S. The consumption analysis, as described in Section D, support the use of soy leghemoglobin in other types of meat analogue products (*i.e.*, pork analogues) at the intended levels of use.

C.7.2 Singapore

Impossible Foods has received no objection on the use of soy leghemoglobin from genetically modified *P. pastoris* for use in plant-based meat analogues from the Agri-Food & Veterinary Authority of Singapore in August 2018. Soy leghemoglobin is listed in the *List of Other Food Additives/Ingredients that are Permitted Under the Singapore Food Regulations* (SFA, 2019), which specifies that: "*Soy leghemoglobin derived from genetically modified Pichia pastoris may be used in plant-based meat analogues at levels up to 0.45% w/w*". This level is consistent with the estimated amount used in the proposed food categories for the consumption analysis described in Section D.

C.7.3 Hong Kong and Macao

The soy leghemoglobin is considered to meet the food regulatory requirements in Hong Kong, and by extension in Macao, on the basis that the GRAS Notice for soy leghemoglobin was filed without questions from the U.S. FDA (U.S. FDA, 2018b), and that Singapore AVA has accepted its use as a food additive/ingredient (SFA, 2019).

C.7.4 Marketing History

The safety of LegH Prep for human consumption is further supported by the fact that over 20-million 1/4-pound servings of meat analogue products containing the LegH Prep ingredient have been sold in the U.S. since June 2016. In addition to over 9,000 restaurants in the U.S., at least 300 restaurants in Hong Kong, Macao, and Singapore also serve meat analogue products containing soy leghemoglobin without evidence or reports of any safety issues concerning soy leghemoglobin, indicating that meat analogue products containing LegH Prep are well tolerated.

C.8 Summary

Overall, the safety of LegH Prep for its intended conditions of use in meat analogue products can be supported based on the following:

1. LegH Prep, manufactured by Impossible Foods, is produced in a consistent and reliable manner under cGMP, is well-characterised and meets food grade specifications, and is free of undesirable substances;

2. Meat analogue products produced by Impossible Foods will not contain soy leghemoglobin at levels exceeding 0.8%, which is comparable to the myoglobin content of red meat (0.8 to 1.8%);

3. Soy leghemoglobin is produced using a safe and non-toxicogenic strain lineage of *P. pastoris*, a strain lineage that has been the subject of previous premarket safety evaluations;

4. Genetic modifications applied to the host organism are well-characterised and do not impart unexpected pleiotropic effects to the organism;

5. The identity of soy leghemoglobin is well defined, and the structure of the protein is homologous to heme proteins with a long history of safe consumption;

6. Amino acid sequence of soy leghemoglobin is not homologous to known or putative allergens and toxins; and

7. Safety is corroborated by the results of preclinical toxicological tests demonstrating that LegH Prep is without evidence of toxicity or mutagenicity/genotoxicity.

D. INFORMATION ON DIETARY EXPOSURE TO THE NOVEL FOOD

Information is provided in this section on the proposed food uses and use levels for Impossible Foods' soy leghemoglobin, and accordingly, its anticipated level of exposure. This section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel foods) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019). The corresponding sections of this application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline[a]	Required Information Described in the Guideline	Section of the Application where this is Addressed
Guideline 3.5.2 – Novel foods	D.1 A list of the foods or food groups proposed to or which might contain the novel food ingredient or substance	Sections D.1, D.2
	D.2 The proposed level of the novel food ingredient or substance for each food or food group	Section D.1
	D.3 For foods or food groups not currently listed in the most recent Australian or New Zealand (NNSs), information on the likely level of consumption	Section D.2
	D.4 The percentage of the food group in which the novel food ingredient is proposed to be used or the percentage of the market likely to use the novel food ingredient	Section D.4
	D.5 For foods where consumption has changed in recent years, information on likely current food consumption	Section D.2
	D.6 Data to show whether the food, or the food in which the novel food ingredient is used, is likely to replace another food from the diet, if applicable	Sections D.4, F.2
	D.7 Information relating to the use of the novel food or novel food ingredient in other countries, if applicable	Sections C.7, D.2, D.4, F.2

[a] The information requirements outlined in Guideline 3.3.3 (D. Information on dietary intake of the nutritive substance) are virtually identical to those in Guideline 3.5.2; therefore, these requirements are not listed here.

D.1 Proposed Use and Use Level

LegH Prep, a mixture containing soy leghemoglobin, *Pichia* (yeast) proteins, and stabilisers (*e.g.*, sodium chloride and sodium ascorbate), is intended to be used in plant-based meat analogue products to provide the nutrition (*i.e.*, a source of iron), flavour, and aroma of their traditional animal-derived counterpart. The characterizing component in the LegH Prep that is responsible for imparting these characteristics is soy leghemoglobin. The meat analogue products produced by Impossible Foods will contain soy leghemoglobin at levels up to 0.8%, which is comparable to the myoglobin content of red meat (0.8 to 1.8%) (Texas A&M Institute, 2019). The addition of the LegH Prep to meat analogue products is self-limiting due to unacceptable organoleptic properties that would occur at higher use levels.

Impossible Foods' soy leghemoglobin will be marketed exclusively as a component of a finished product in Australia/New Zealand. These products are largely intended for consumption by the general population (age 2 years and older). The meat analogue products containing soy leghemoglobin will be marketed in retail avenues such as grocery store outlets and restaurants. Soy leghemoglobin itself will not be marketed as an ingredient for general purchase and food use by other manufacturers, nor will it be sold directly to consumers.

D.2 Estimated Intake from Proposed Food Uses

D.2.1 Assessment Using U.S. Food Consumption Data

Estimation of soy leghemoglobin consumption has been previously conducted using the average daily intakes for beef in the U.S., based on the consumption data obtained from the National Health and Nutrition Examination Survey (NHANES) conducted in 2007-2008 (Bowman *et al.*, 2013). Details of the assessment are available in the GRAS notice submitted for soy leghemoglobin (GRN 737). Impossible Foods now intends to also use soy leghemoglobin in simulated meat products marketed as pork alternatives (*i.e.*, pork analogues); therefore, an updated exposure assessment was conducted. Although soy leghemoglobin is proposed for addition to meat analogues at levels of not more than 0.8% in the finished product, in practice, the soy leghemoglobin will be added only at levels that would be needed to recreate the flavour and aroma contributed by heme proteins (myoglobin) of their animal-derived counterparts. For instance, to obtain flavouring profiles that are representative of beef and pork products, soy leghemoglobin is added at use levels of 0.45% in beef analogue products and 0.25% in pork analogue products.

Utilizing food consumption records available in the 2013-2014 and 2015-2016 cycles of the NHANES, the Estimated Daily Intake (EDI) of soy leghemoglobin under the expanded conditions of use was derived for beef analogue as well as pork analogue products. The dietary recall component of the NHANES is referred to as What We Eat In America (WWEIA). This continuous survey is a complex multistage probability sample designed to be representative of the U.S. population. The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the U.S.

The Food and Nutrient Database for Dietary Studies (FNDDS) contains food composition data for food codes in the NHANES (*i.e.*, breaks down food codes by ingredients). This database, in combination with the Food Patterns Ingredients Database (FPID)[6], also developed by the U.S. Department of Agriculture (USDA), was used to identify NHANES food codes representative of meat products or mixed dishes containing meat products, to capture the consumption of meat products by the U.S. population.

[6] Database is part of the Food Patterns Equivalents Database (FPED).

Ingredient components for beef and pork products that best serve as a proxy for the types of plant-based meat alternative products that could potentially contain the soy leghemoglobin ingredient were identified based on ingredient description in the FNDDS (see Table D.2.1-1 below). NHANES food codes identified as containing the ingredient components indicated in Table D.2.1-1 were then selected for the assessment. To derive the EDI for soy leghemoglobin, it was assumed that soy leghemoglobin is added at 0.45% to beef products and 0.25% to pork products. These use levels were applied to the selected NHANES food codes. For food mixtures, the recipe fraction of the beef/pork component was used in the analysis. The food consumption data in the NHANES, which was collected from individuals using 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2), was taken as a 2-day average. Using the NHANES consumption data, the EDI of the soy leghemoglobin protein from its proposed uses, expressed in milligrams per day (mg/day), as well as on a body-weight (bw) basis (mg/kg bw/day) using each individual's own body weight, were derived for the U.S. population 2 years and older at the mean and 90th percentile on a per user basis. The analysis was limited to individuals who provided complete recalls (Day 1 and Day 2).

Table D.2.1-1 Representative Ingredient Components of Beef and Pork Products Identified from the FNDDS for the Selection of NHANES Food Codes for the Derivation of the Estimated Daily Intake of Soy Leghemoglobin

Ingredient Description
Blood sausage
Bologna, beef and pork
Bologna, pork
Bratwurst, pork cooked
Braunschweiger (a liver sausage), pork
Cheesefurter, cheese smokie, pork, beef
Chorizo, pork and beef
Dutch brand loaf, chicken, pork and beef
Frankfurter, beef, unheated
Knackwurst, knockwurst, pork, beef
Lebanon bologna, beef
Mortadella, beef, pork
Honey loaf
Pepperoni, beef and pork, sliced
Ham, smoked or cured, ground patty
Pork and beef sausage, fresh, cooked
Salami, cooked, beef
Salami, cooked, beef and pork
Salami, dry or hard, pork, beef
Sandwich spread, pork, beef
Sandwich spread, pork, beef
Smoked link sausage, pork
Sausage, smoked link sausage, pork and beef
Thuringer, cervelat, summer sausage, beef, pork
Sausage, Vienna, canned, chicken, beef, pork
Sausage, Italian, pork, cooked
Frankfurter, beef, pork, and turkey, fat free
Pork sausage, link/patty, fully cooked, microwaved
Beef sausage, pre-cooked
Beef sausage, pre-cooked

Table D.2.1-1 Representative Ingredient Components of Beef and Pork Products Identified from the FNDDS for the Selection of NHANES Food Codes for the Derivation of the Estimated Daily Intake of Soy Leghemoglobin

Ingredient Description
Beef sausage, fresh, cooked
Frankfurter, meat and poultry, unheated
Pork sausage, link/patty, reduced fat, cooked, pan-fried
Kielbasa, fully cooked, grilled
Kielbasa, fully cooked, unheated
Bologna, meat and poultry
Pork sausage, reduced sodium, cooked
Pork, fresh, ground, cooked
Beef, ground, patties, frozen, cooked, broiled
Beans, baked, canned, with franks
Chili with beans, canned
Veal, ground, cooked, broiled
Fast Foods, biscuit, with egg and sausage
Fast foods, biscuit, with sausage
Fast foods, croissant, with egg, cheese, and sausage
Fast foods, english muffin, with cheese and sausage
Fast foods, english muffin, with egg, cheese, and sausage
Fast foods, burrito, with beans and beef
Fast foods, burrito, with beans, cheese, and beef
Fast foods, nachos, with cheese, beans, ground beef, and tomatoes
Fast foods, taco with beef, cheese and lettuce, hard shell
Fast foods, cheeseburger; single, regular patty; plain
Fast foods, cheeseburger; single, regular patty, with condiments
Fast foods, cheeseburger; single, regular patty, with condiments and vegetables
Fast foods, cheeseburger, double, regular patty and bun, with condiments
Fast foods, cheeseburger; single, large patty; plain
Fast foods, hamburger; single, regular patty; plain
Fast foods, hamburger; single, regular patty; with condiments
Fast foods, submarine sandwich, meatball marinara on white bread
Fast foods, hamburger, large, single patty, with condiments
Pizza, meat and vegetable topping, regular crust, frozen,
Pizza, meat and vegetable topping, rising crust, frozen,
Fast Food, Pizza Chain, 14" pizza, pepperoni topping, regular crust
Fast Food, Pizza Chain, 14" pizza, pepperoni topping, thick crust
Fast Food, Pizza Chain, 14" pizza, meat and vegetable topping, regular crust
Fast foods, griddle cake sandwich, egg, cheese, and sausage
Fast foods, griddle cake sandwich, sausage
Fast foods, hamburger; double, large patty; with condiments, vegetables and mayonnaise
Fast foods, hamburger; single, large patty; with condiments, vegetables and mayonnaise
Fast foods, cheeseburger; double, regular patty; with condiments
Fast foods, cheeseburger; single, large patty; with condiments, vegetables and mayonnaise
Fast foods, cheeseburger; single, large patty; with condiments
Fast foods, cheeseburger; double, large patty; with condiments, vegetables and mayonnaise
Fast foods, cheeseburger; double, regular patty; double decker bun with condiments and special sauce

Table D.2.1-1 Representative Ingredient Components of Beef and Pork Products Identified from the FNDDS for the Selection of NHANES Food Codes for the Derivation of the Estimated Daily Intake of Soy Leghemoglobin

Ingredient Description
Fast foods, bagel, with egg, sausage patty, cheese, and condiments
Fast Food, Pizza Chain, 14" pizza, sausage topping, thick crust
Fast Food, Pizza Chain, 14" pizza, sausage topping, thin crust
Fast Food, Pizza Chain, 14" pizza, sausage topping, regular crust
Fast Food, Pizza Chain, 14" pizza, pepperoni topping, thin crust
Fast foods, taco with beef, cheese and lettuce, soft
Fast foods, breakfast burrito, with egg, cheese, and sausage
School Lunch, pizza, pepperoni topping, thin crust, whole grain, frozen, cooked
School Lunch, pizza, pepperoni topping, thick crust, whole grain, frozen, cooked
School Lunch, pizza, sausage topping, thin crust, whole grain, frozen, cooked
School Lunch, pizza, sausage topping, thick crust, whole grain, frozen, cooked
School Lunch, pizza, pepperoni topping, thick crust, whole grain, frozen, cooked
School Lunch, pizza, sausage topping, thin crust, whole grain, frozen, cooked
Spaghetti with meat sauce, frozen entrée
Beef Macaroni, frozen entrée
Pasta with Sliced Franks in Tomato Sauce, canned entree
Ravioli, meat-filled, with tomato sauce or meat sauce, canned
Chili con carne with beans, canned entrée
Pasta with meatballs in tomato sauce, canned entrée
Chili, no beans, canned entrée
Spaghetti, with meatballs in tomato sauce, canned
Lasagna with meat & sauce, frozen entrée
Hot Pockets, meatballs & mozzarella stuffed sandwich, frozen
Corn dogs, frozen, prepared
Beef, ground, 95% lean meat / 5% fat, raw
Beef, ground, 95% lean meat / 5% fat, patty, cooked, broiled
Beef, ground, 95% lean meat / 5% fat, patty, cooked, pan-broiled
Beef, ground, 90% lean meat / 10% fat, raw
Beef, ground, 90% lean meat / 10% fat, patty, cooked, broiled
Beef, ground, 90% lean meat / 10% fat, patty, cooked, pan-broiled
Beef, ground, 85% lean meat / 15% fat, raw
Beef, ground, 85% lean meat / 15% fat, patty, cooked, broiled
Beef, ground, 85% lean meat / 15% fat, patty, cooked, pan-broiled
Beef, ground, 85% lean meat / 15% fat, crumbles, cooked, pan-browned
Beef, ground, 80% lean meat / 20% fat, raw
Beef, ground, 80% lean meat / 20% fat, patty, cooked, broiled
Beef, ground, 80% lean meat / 20% fat, patty, cooked, pan-broiled
Beef, ground, 80% lean meat / 20% fat, crumbles, cooked, pan-browned
Beef, ground, 75% lean meat / 25% fat, raw
Beef, ground, 75% lean meat / 25% fat, patty, cooked, broiled
Beef, ground, 75% lean meat / 25% fat, patty, cooked, pan-broiled
Beef, ground, 75% lean meat / 25% fat, crumbles, cooked, pan-browned
Beef, ground, 75% lean meat / 25% fat, loaf, cooked, baked
Soup, hot and sour, Chinese restaurant

Table D.2.1-1 Representative Ingredient Components of Beef and Pork Products Identified from the FNDDS for the Selection of NHANES Food Codes for the Derivation of the Estimated Daily Intake of Soy Leghemoglobin

Ingredient Description
Soup, wonton, Chinese restaurant
Taquitos, frozen, beef and cheese, oven-heated
Pizza rolls, frozen, unprepared
Turnover, meat- and cheese-filled, tomato-based sauce, reduced fat, frozen
Turnover, filled with egg, meat and cheese, frozen
Sausage, egg and cheese breakfast biscuit
Restaurant, family style, chili with meat and beans
Restaurant, Italian, lasagna with meat
Restaurant, Latino, empanadas, beef, prepared
Bologna, beef and pork, low fat
Bologna, beef, low fat
Turkey and pork sausage, fresh, bulk, patty or link, cooked
Pork sausage rice links, brown and serve, cooked
Frankfurter, meat and poultry, low fat
Beans, chili, barbecue, ranch style, cooked
Beef, bologna, reduced sodium
Frankfurter, low sodium
Beef, ground, patty, frozen

D.2.2 Estimated Daily Intake

The estimated mean and 90th percentile intake of the representative processed beef and pork products for the U.S. population 2 years and older on a per user basis are summarised in Table D.2.2-1. On a body weight basis, the amount of beef and pork products consumed by the total U.S. population at the mean and 90th percentile was estimated at 0.7 and 1.5 g/kg body weight/day, respectively. These intake values are considered an overestimation of the consumption of meat analogue products that may contain soy leghemoglobin, as soy leghemoglobin will not be added to all categories of meat analogue products, and meat analogue products will not completely replace beef and pork products that are consumed in the diet.

Table D.2.2-1 Estimated Daily Intake (EDI) of Representative Beef and Pork Products in the U.S. on a Per User Basis, Using 2-Day Averages from NHANES (2013-2016 Data)

Population Group	EDI (g/day)		EDI (g/kg bw/day)	
	Mean	90th Percentile	Mean	90th Percentile
U.S. population, ages 2+	46	97	0.7	1.5

The mean and 90th percentile EDI for the soy leghemoglobin protein, among the U.S. population 2 years and older on a per user basis, are shown in Table D.2.2-2. The mean and 90th percentile EDI of the soy leghemoglobin in meat analogue products was estimated to be 187 and 394 mg/day (2.9 and 6.1 mg/kg body weight/day), respectively.

Table D.2.2-2 Estimated Daily Intake (EDI) of Soy Leghemoglobin Protein in the U.S. on a Per User Basis, Using 2-Day Averages from the NHANES (2013-16 Data)

Population Group	EDI of soy leghemoglobin (mg/day)	EDI of soy leghemoglobin (mg/kg bw/day)

	Mean	90th Percentile	Mean	90th Percentile
U.S. population, ages 2+	187	394	2.9	6.1

D.2.3 Summary of Anticipated Dietary Exposures to Soy Leghemoglobin in Meat Analogues

To reiterate, soy leghemoglobin is intended to be used in meat analogues marketed in Australia/New Zealand at levels not exceeding 0.8%, which is comparable to the myoglobin content of beef (0.8 to 1.8%) (Texas A&M Institute, 2019). Soy leghemoglobin will only be available to consumers as a component of finished meat analogue products marketed by Impossible Foods. It will not be marketed as an ingredient for general purchase or food use by other manufacturers, nor will it be sold directly to consumers, which further limits its use.

An exposure assessment of soy leghemoglobin under its proposed conditions of use in meat analogues products (at use levels of up to 0.8%) had been conducted using the food consumption data available in the U.S. NHANES. In the assessment, it was assumed that soy leghemoglobin will be added to various beef and pork products that are representative of the types of plant-based meat alternative products that could potentially contain the ingredient. The mean and 90th percentile EDI of the soy leghemoglobin was estimated to be 187 mg/day (2.9 mg/kg body weight/day) and 394 mg/day (6.1 mg/kg body weight/day), respectively.

Impossible Foods' meat analogue products that are formulated with soy leghemoglobin deliver approximately the same amount of heme protein as those provided by the intake of animal-derived meats. Therefore, if consumers substitute Impossible Foods' meat analogue products for its conventionally animal-derived counterpart, the overall consumption of heme proteins is approximately the same. The vast majority of heme proteins consumed in the diet are myoglobins in meat and poultry products (EFSA, 2015). For the U.S. population, the mean consumption of meat and poultry products is reported at 154 g/day (Bowman *et al.*, 2013). Assuming an average myoglobin concentration for meat and poultry products of 0.5% (Yip and Dallman, 1996), the average myoglobin consumption would be 0.77 g/day. The mean and 90th percentile EDI of soy leghemoglobin among U.S. population 2 years and older, which was derived at 0.19 g/day and 0.39 g/day, respectively (Table D.2.2-2), is well below the estimated daily intake of myoglobin in the U.S. from meat and poultry products. As discussed further in Section E, the addition of soy leghemoglobin to meat analogue products will also provide levels of haem iron that are comparable to those present in an equivalent serving of red meat.

D.3 Composition of Meat Analogue Products Containing Soy Leghemoglobin

Soy leghemoglobin provides a 'meat-like' flavour and aroma to the meat analogue products to which it is added and contributes to its nutritional quality (*i.e.*, provides a source of iron). In addition to the soy leghemoglobin component, the meat analogue products manufactured by Impossible Foods will contain various plant proteins, which may include (but are not limited to) commercially available protein sources permitted for sale in the Australia and New Zealand marketplaces (*e.g.*, soy, pea, mung bean, lentil, corn, potato and wheat). Although standards of identity exist for various food products within the Code (*e.g.*, Standard 2.2.1 for meat and meat products), there is no standard for plant-based meat analogues. However, Schedule 17 of the Code does include provisions regarding the addition of vitamins and minerals to "analogues derived from legumes", including "analogues of meat, where no less than 12% of the energy value of the food is derived from protein, and the food contains 5 g protein per serve of the food", which are reproduced in Table D.3-1 below. For comparison, the levels of vitamins and minerals that are in mean analogue products manufactured by Impossible Foods (*e.g.*, Impossible™ Burger) are also provided, along with the content of these nutrients that have been reported in ground beef (raw)

and in veggie/soy burgers according to the United States Department of Agriculture (USDA) Food Composition Databases.

In general, the levels of vitamins and minerals in the Impossible™ Burger are comparable to those that are present in ground beef or veggie/soy burgers. The only vitamin or mineral in the Impossible™ Burger to exceed the maximum permitted amount set forth in Schedule 17 of the Code is folate. However, folate is not an added component to the Impossible™ Burger (*i.e.*, it is present as a component of other added ingredients[7]), and some veggie/soy burgers have been reported to contain even higher levels of folate.

Table D.3-1 Content of Vitamins and Minerals in Meat Analogue Products Containing LegH Prep in Comparison to Ground Beef and Veggie Burgers

Vitamin or Mineral	Permitted Uses as per Schedule 17 (S17—4) of the Code[a]		Amount in the Impossible™ Burger (in Raw Form)		Typical Content per 100 g as Reported by the USDA[b]	
	Max. claim per 100 g (Max. % RDI claim)	Max. permitted amount per 100 g	Average and Typical Range per 100 g	Maximum per 100 g	Ground meat, Raw (80% lean meat, 20% fat)	Veggie Burgers or Soy burgers, Unprepared
Thiamin[c]	0.16 mg (15%)	-	25 mg (24 to 27 mg)	27 mg[c]	0.04 mg	2.65 mg
Riboflavin[c]	0.26 mg (15%)	-	0.3 mg (0.3 to 0.38 mg)	0.4 mg[c]	0.15 mg	0.24 mg
Niacin[c]	5.0 mg (50%)	-	4.7 mg (4.1 to 5.3 mg)	6.0 mg[c]	4.2 mg	3.8 mg
Vitamin B6[c]	0.5 mg (30%)	-	0.3 mg (0.25 to 0.39 mg)	0.5 mg[c]	0.3 mg	0.3 mg
Vitamin B12[c]	2.0 µg (100%)	-	2.7 µg (2.4 to 3.0 µg)	3.0 µg[c]	2.1 µg	2.0 µg
Folate	No claim permitted	10 µg	100 µg DFE (89 to 117 µg DFE)	125 µg DFE	7 µg DFE	124 µg DFE
Iron	3.5 mg (30%)	-	3.7 mg (3.4 to 4.0 mg)	4.0 mg	1.9 mg	2.4 mg
Magnesium	No claim permitted	26 mg	59.7 mg 55.8 to 60.5 mg	65 mg	17 mg	56 mg
Zinc[c]	4.4 mg (35%)	-	4.9 mg (4.4 to 5.3 mg)	6.0 mg[c]	4.2 mg	1.7 mg
Calcium	-	-	150 mg (142 to 152 mg)	155 mg	28 mg	136 mg
Potassium	-	-	540 mg (514 to 550 mg)	600 mg	270 mg	333 mg

DFE = dietary folate equivalents; Max = maximum; USDA = United States Department of Agriculture.
[a] For Analogues of meat, where no less than 12% of the energy value of the food is derived from protein, and the food contains 5 g protein per serve of the food. The meat analogue products containing soy leghemoglobin will meet the criteria regarding protein content. In this example, for instance, 1 serving (113 g) of the Impossible™ Burger will provide approximately 240 calories and 19 g of protein, which corresponds to 32% of its energy value being derived from protein.
[b] Based on the in the USDA Food Composition Databases, available at https://ndb.nal.usda.gov/ndb/search/list (USDA, 2019).
[c] These vitamins are added components to the Impossible™ Burger for nutritional purposes, except for thiamin, which is added for flavouring purposes.

[7] The majority (approximately 96%) of the folate content in the Impossible™ Burger is contributed by the soy protein concentrate and yeast extract. The rest is contributed by the various other minor ingredients in the product.

D.4 Market Share Data

The addition of soy leghemoglobin to meat analogue products is intended to provide the nutrition (*i.e.*, a source of iron), flavour, and aroma of its animal-derived counterpart. It is anticipated that meat analogue products containing soy leghemoglobin will be consumed by individuals who seek out such products for various reasons (*e.g.*, health, ethical, religious). Meat analogue products containing soy leghemoglobin is unlikely to have 100% of the market share. The ingredient will only be used in products manufactured by Impossible Foods, and it will not be sold to other food manufacturers. It is unlikely that meat analogue products containing soy leghemoglobin will fully replace other meat analogue products or conventional animal-derived meat products to obtain 100% market share.

E. INFORMATION ON THE NUTRITIONAL AND HEALTH IMPACT

The nutritional and health impact of Impossible Foods' soy leghemoglobin are described in this section. Specifically, this section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel foods) and Guideline 3.5.1 (Foods produced using gene technology) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019). The corresponding sections of this application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	Required Information Described in the Guideline	Section(s) of the Application where this is Addressed
Guideline 3.5.2 – Novel foods	E. Information on the nutritional and health impact of the novel food	
	E.1. Information to demonstrate that the use of the novel food or novel food ingredient will not cause a nutritional imbalance in the diet.	Section E.1
	E.2 Information to demonstrate that the addition of the novel food ingredient will not create a significant negative public health impact	Section E.2
Guideline 3.5.1 – Foods produced using gene technology	C. Information related to the nutritional impact of the food produced using gene technology	
	Data are required on the anticipated dietary intake of the GM food in relation to the overall diet, together with any information which may indicate a change to the bioavailability of the nutrients from the GM food	Sections D, E.1
	Where the GM food contains an intended nutritional change, information, such as clinical trial data, must be provided to determine the nutritional impact of the GM food.	Sections D.3, E.1
Guideline 3.3.3 – Substances used for a nutritive purpose	E Information related to the nutritional impact of a vitamin or mineral	
	E.1 Information to demonstrate a need to permit the addition of a vitamin or mineral to food	Section E.1
	E.2 Information to demonstrate the permitted addition of the vitamin or mineral has the potential to address the deficit or deliver a health benefit to the population or a population subgroup	Section E.1
	F Information related to the nutritional impact of a nutritive substance other than vitamins and minerals	
	F.1 Information related to the nutritional purpose of the use of the substance in each food	Sections B.2, E.1

E.1 Information to Demonstrate that the Use of Soy Leghemoglobin will not Cause a Nutritional Imbalance in the Diet

E.1.1 Bioavailability and Intake of Iron

As detailed in Section C.2, soy leghemoglobin is a symbiotic heme protein that is naturally present in the root nodule of the soybean plant. Although this part of the plant (and soy leghemoglobin itself) is not widely consumed in the human diet, it is structurally similar to other heme B-containing proteins that have been safely consumed (*e.g.*, animal myoglobins and non-symbiotic plant haemoglobins; see Section C.2 and Appendix VI). The high bioavailability of the haem iron component of soy leghemoglobin makes it suitable for enhancing the dietary profile of many processed foods (Carpenter and Mahoney, 1992). Impossible Foods has conducted experiments to demonstrate that soy leghemoglobin will denature at high temperatures (as applied during cooking), and at low pH (as in the human stomach) to release the iron-containing heme B molecule (see Appendix VI). Additionally, Proulx and Reddy (2006) reported that purified soy leghemoglobin and bovine haemoglobin exhibited similar iron bioavailability within a food matrix, both of which were higher than free iron. Therefore, once cooked and digested, the soy leghemoglobin produced by Impossible Foods will serve as a dietary source of iron, analogous to the role of animal-based myoglobin as an iron source in meat.

Soy leghemoglobin is proposed for use at levels of not more than 0.8% in meat analogue products produced by Impossible Foods, which is comparable to the myoglobin content of meat (0.8 to 1.8%) (Texas A&M Institute, 2019). These meat analogue products contain both heme and non-haem iron; the haem iron is contributed by the soy leghemoglobin, while the non-haem iron is provided by the other ingredients (primarily soy protein concentrate) used to formulate the meat analogue products. As an example, the estimated amount of haem and non-haem iron that would be provided in a single serving (113 g) of a representative meat analogue product containing soy leghemoglobin (*i.e.*, the Impossible™ Burger) can be compared to the amount provided by an equivalent amount of ground beef. This comparison is presented in Table E.1.1-1.

Although the Impossible™ Burger contains higher amounts of total iron as an equivalent serving of ground meat (80% lean meat, 20% fat), the amount of haem iron present is nearly the same at approximately 2 mg per serving (Table E.1.1-1). The amount of non-haem iron in the Impossible™ Burger (2.4 mg per serving) is also comparable to the amount of iron (2.7 g) that would be provided by a 113 g serving of a conventional veggie/soy burger (USDA, 2019). As such, the amount of iron contributed by soy leghemoglobin, and when it is formulated into meat analogue products, are within the ranges of iron that has been reported in the food products that it is meant to replace. It is also notable that the amount of total iron provided per serving of the Impossible™ Burger (4.2 mg) is well below the upper levels of intake derived for iron by the National Health and Medical Research Council (NHMRC) during their evaluation of the Nutrient Reference Values for Australia and New Zealand, which ranges from 20 mg/day (children 1 to 3 years of age) to 45 mg/day (adolescents age 14 to 18 years, and adults 19+ years [including during pregnancy and lactation]) (NHMRC, 2006).

Table E.1.1-1 Amount of Haem and Non-Haem iron Present in a Serving of the Impossible™ Burger in Comparison to an Equivalent Serving of Ground Beef

Parameter	Average Amount per 113 g Serving of the Impossible™ Burger (Raw)[a]	Typical Content per 113 g Serving of Ground Beef, Raw (80% lean meat, 20% fat)
Total Iron	4.2 mg	2.2 mg[b]
Haem iron	1.8 mg	1.5 to 2.0 mg[c]
Non-haem iron	2.4 mg	0.2 to 0.7 mg[c]

[a] Assuming soy leghemoglobin is added to the Impossible™ Burger at a use level of 0.45%.
[b] Based on the in the USDA Food Composition Databases (USDA, 2019), available at https://ndb.nal.usda.gov/ndb/search/list.
[c] According to unpublished data obtained by Impossible Foods, haem iron comprises approximately 90% of the total iron content in meat. Data in the published literature suggests that approximately 70% of the total iron content in meat is haem iron (EFSA, 2015). These values were used to calculate the amount of haem iron provided by the total iron content that have been reported in ground meat. The amount of non-haem iron in ground meat is calculated by subtracting the amount of haem iron from the total iron.

E.1.2 Impact on the Bioavailability of Other Nutrients

As explained in Sections D.3, the overall nutritional composition of the meat analogue products containing soy leghemoglobin will generally be comparable to those of its traditional animal-derived counterpart and to other plant-based analogue products. As such, the addition of soy leghemoglobin to meat analogue products will not have any negative impact on nutrient availability or result in nutrient imbalance.

Similar to other heme-containing proteins that are widely consumed in the diet, the soy leghemoglobin protein itself is not anticipated to adversely impact the bioavailability of other nutrients. Moreover, the meat analogue products containing soy leghemoglobin are formulated using plant-based ingredients that are already consumed in the diet. For example, the primary ingredients in the Impossible™ Burger include water, soy protein concentrate, coconut oil, sunflower oil, and natural flavours[8]. Given that it is formulated using plant-based ingredients (*e.g.*, soy protein concentrate), the meat analogue products containing the soy leghemoglobin do contain some phytate (approximately 415 mg phytate per 113 g serving of the Impossible™ Burger). Plant-based diets are known to be high in phytates, which could potentially affect the absorption of nutrients (*e.g.*, minerals such as iron and zinc) from the diet (NHMRC, 2006). However, the amount of phytate in the Impossible™ Burger is well within the ranges of total dietary phytate intake in adults, which have been reported to range from 300 to 800 mg/day in those with a mixed diet, 700 to 1,400 mg/day in those with a mixed diet containing a high proportion of unrefined cereal grain products and legumes, and as high as 1,600 to 2,500 mg/day for adults on vegetarian diets (EFSA, 2014). Overall, meat analogue products containing soy leghemoglobin are not anticipated to affect the bioavailability of other nutrients, beyond what would be observed from the use of those same ingredients (*e.g.*, soy protein concentrate) in other food products.

[8] Other ingredients that are present in small amounts (2% or less) include: Potato Protein, Methylcellulose, Yeast Extract, Cultured Dextrose, Food Starch Modified, Soy Leghemoglobin, Salt, Soy Protein Isolate, Mixed Tocopherols (Vitamin E), Zinc Gluconate, Thiamine Hydrochloride (Vitamin B1), Sodium Ascorbate (Vitamin C), Niacin, Pyridoxine Hydrochloride (Vitamin B6), Riboflavin (Vitamin B2), Vitamin B12. Taken from: https://faq.impossiblefoods.com/hc/en-us/articles/360018937494-What-are-the-ingredients-.

E.2 Information to Demonstrate that the Addition of the Novel Food Ingredient Will Not Create a Significant Negative Public Health Impact

As described in Section C, the safety of soy leghemoglobin, the LegH Prep, and the *P. pastoris* production strain has been rigorously assessed through scientific procedures, in accordance with the general principles outlined in the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019), and the *Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms* from the CODEX Alimentarius (CAC/GL 46-2003 – Codex Alimentarius, 2003).

Furthermore, as described in Section E.1 above, the addition of soy leghemoglobin to meat analogue products are not expected to have any negative impact on the bioavailability or intakes of nutrients from the diet. Instead, the approval of soy leghemoglobin in Australia/New Zealand will give consumers access to more choices of nutritious and flavourful plant-based protein products.

F. INFORMATION RELATED TO POTENTIAL IMPACT ON CONSUMER UNDERSTANDING AND BEHAVIOUR

The potential impact on consumer understanding and behaviour in relation to products containing soy leghemoglobin is discussed in this section. Specifically, this section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel foods) of the *Food Standards Australia New Zealand Application Handbook* (FSANZ, 2019). The corresponding sections of this application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	Required Information Described in the Guideline	Section(s) of the Application where this is Addressed
Guideline 3.5.2 – Novel foods	F.1 Information to demonstrate the level of consumer awareness and understanding of the novel food or novel food ingredient	Section F.1
	F.2 Information on the actual or potential behaviour of consumers in response to the novel food or novel food ingredient	Section F.2
	F.3 Information to demonstrate that the food(s) containing the novel food ingredient will not adversely affect any population groups	Section F.3

F.1 Information to Demonstrate Consumer Awareness and Understanding of the Novel Food Ingredient

Consumers select plant-based protein products for various reasons of personal preference (*e.g.*, health, ethical, religious, environmental). As noted in a report titled, *The Evolution of Plant Protein – Assessing consumer response*, jointly prepared by the Ministry of Primary Industries (MPI) of New Zealand and Plant & Food Research[9], consumers have become more aware of the long term impacts of food production, and the shift in consumer preferences has led to a new wave of innovative meat and dairy substitutes aimed to reduce the reliance on animal-based production systems for protein (MPI, 2018a). In conjunction with this report, the MPI also released 3 market insight reports, including one titled, *The Impossible Burger – Consumer Insights*, which summarises the results of a case study conducted by the MPI on the Impossible™ Burger (MPI, 2018b). As part of their research, the MPI surveyed residents in California and analysed social media and online data. One finding from this case study is that people who have tried the Impossible™ Burger like it most for its environmental credentials ("they're good for the environment").

The meat analogue products manufactured by Impossible Foods is promoted as being capable of delivering all of the "flavour, aroma, and meatiness of meat from animals", despite being composed of plant-derived components. The products are largely marketed towards meat-eating consumers ("flexitarians"), who are looking for more ethical and environmentally friendly alternative meat products without compromising on attributes such as the taste and texture. Impossible Foods makes it widely known that soy leghemoglobin is the key ingredient in their meat analogue products that is responsible for imparting the nutrition (*i.e.*, source of iron), flavour, and aroma of its traditional animal-derived counterpart. This includes educational information about the soy leghemoglobin ingredient that is available on Impossible Foods' webpage[10].

F.2 Information on Actual/Potential Behaviour of Consumers in Response to the Novel Food Ingredient

To date, over 20-million 1/4-pound servings of meat analogue products containing soy leghemoglobin have been sold in the U.S. since June 2016. In addition to over 9,000 restaurants in the U.S., at least 300 restaurants in Hong Kong, Macao, and Singapore also serve soy leghemoglobin containing meat analogue products (such as burgers, meatballs, sausages, or as fillings in buns and dumplings, *etc.*).

The MPI reported in their case study on the Impossible™ Burger that consumers are most likely to be in their 30s or 40s, with access to higher disposable income, living in a large city with ready access to the product (MPI, 2018b). Less than 2% of respondents surveyed have tried the Impossible™ Burger, and of those who have tried it, 30% reported that they will eat them regularly (MPI, 2018b).

[9] Plant & Food Research is a New Zealand-based Crown Research Institute providing research and development that adds value to fruit, vegetable, arable and seafood food products.

[10] Available at: https://impossiblefoods.com/heme/.

F.3 Information to Demonstrate that the Novel Food Ingredient Will Not Adversely Affect Any Population Groups

As outlined in Section C, the safety of soy leghemoglobin, the LegH Prep, and the *P. pastoris* production strain is well established, and there is no evidence to suggest that the ingredient would pose any allergenicity concerns. Accordingly, the meat analogue products containing soy leghemoglobin is considered to be suitable for consumption by the general population.

Although soybeans are acknowledged as a commonly allergenic food, it is highly unlikely that soy leghemoglobin will elicit a reaction in a soy-allergic consumer, as described in depth in Section C.6. Nevertheless, the label of food products sold by Impossible Foods containing soy leghemoglobin will disclose an allergen warning (*i.e.*, "Contains Soy"), and Impossible Foods will provide training materials and information to restaurants who purchase the product indicating it is a soy-protein based product.

The meat analogue products containing soy leghemoglobin are not a suitable alternative for individuals with haemochromatosis, since the content of haem iron in these products is close to that in meat (see Section D.1). Moreover, the meat analogue products manufactured by Impossible Foods contain additional non-haem iron contributed by the other ingredients present (*i.e.*, soy protein concentrate).

REFERENCES

AAFCO (2019). Chapter 6-Feed terms and ingredient definitions-30. Enzymes [Phytase, *Pichia pastoris*]. In: *2019 Official Publication*. Champaign (IL): American Association of Feed Control Officials Inc., p. 379.

Anderson CR, Jensen EO, LLewellyn DJ, Dennis ES, Peacock WJ (1996). A new hemoglobin gene from soybean: a role for hemoglobin in all plants. Proc Natl Acad Sci U S A 93(12):5682-5687. DOI:10.1073/pnas.93.12.5682.

Astwood JD, Leach JN, Fuchs RL (1996). Stability of food allergens to digestion in vitro. Nat Biotechnol 14(10):1269-1273. DOI:10.1038/nbt1096-1269.

ATCC (2016a). *Komagataella pastoris* (Guilliermond) (ATCC® 28485™). Manassas (VA): American Type Culture Collection (ATCC). Available at: https://www.atcc.org/Products/All/28485.aspx [© 2016].

ATCC (2016b). *Komagataella phaffii* Kurtzman (ATCC® 76273™). Manassas (VA): American Type Culture Collection (ATCC). Available at: https://www.atcc.org/products/all/76273.aspx [© 2016].

Bannon G, Fu T-J, Kimber I, Hinton DM (2003). Protein digestibility and relevance to allergenicity. Environ Health Perspect 111(8):1122-1124. DOI:10.1289/ehp.5812.

Berkowitz D, Maryanski J (1989). Implications of biotechnology on international food standards and codes of practice. In: *Joint FAO/WHO Food Standards Programme / Codex Alimentarius Eighteenth Session*, July 3-12, 1989, Geneva, Switz. (ALINORM 89/39). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO), Codex Alimentarius.

Bernhisel-Broadbent J, Sampson HA (1989). Cross-allergenicity in the legume botanical family in children with food hypersensitivity. J Allergy Clin Immunol 83(2, Part 1):435-440. DOI:10.1016/0091-6749(89)90130-9.

Bodnar AL (2011). *Novel Approaches to Improving Qualities of Maize Endosperm*. (Graduate Theses and Dissertations). (Paper 10271). Ames (IA): Iowa State University Digital Repository@ Iowa State University.

Bowman S, Martin C, Clemens J, Lin B-H, Moshfegh A (2013). *Retail Commodity Intakes: Mean Amounts of Retail Commodities per Individual, 2007/2008*. Washington (DC): Agricultural Research Service, Food Surveys Research Group, Department of Agriculture.

Brisson N, Verma DP (1982). Soybean leghemoglobin gene family: normal, pseudo, and truncated genes. Proc Natl Acad Sci U S A 79(13):4055-4059. DOI:10.1073/pnas.79.13.4055.

Carpenter CE, Mahoney AW (1992). Contributions of heme and nonheme iron to human nutrition. Crit Rev Food Sci Nutr 31(4):333-367. DOI:10.1080/10408399209527576.

CDC (1999). *Biosafety in Microbiological and Biomedical Laboratories*. Atlanta (GA): CDC Office of Health and Safety, pp. 17-53.

Cereghino JL, Cregg JM (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24(1):45-66. DOI:10.1111/j.1574-6976.2000.tb00532.x.

Ciofalo V, Barton N, Kreps J, Coats I, Shanahan D (2006). Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil. Regul Toxicol Pharmacol 45(1):1-8. DOI:10.1016/j.yrtph.2006.02.001.

Codex Alimentarius (2002). *Report of the Third Session of the Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology*, Mar. 4-8, Yokohama, Japan. (Alinorm 03/34). Rome, Italy: Codex Alimentarius). Available at: www.fao.org/fileadmin/user_upload/gmfp/resources/al03_34e.pdf.

Codex Alimentarius (2003). *Guideline for the Conduct of Food Safety Assessment of Foods Produced Using Recombinant-DNA Microorganisms*. (CAC/GL 46-2003; Adopted in 2003, Annexes II and III adopted in 2008). Rome Italy: Codex Alimentarius. Available at: http://www.fao.org/fileadmin/user_upload/gmfp/resources/CXG_046e.pdf.

Codex Alimentarius (2009). *Foods Derived from Modern Biotechnology, 2nd edition*. Geneva, Switz.: World Health Organization (\WHO) / Rome, Italy: Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/docrep/011/a1554e/a1554e00.htm.

Commins SP, Jerath MR, Cox K, Erickson LD, Platts-Mills T (2016). Delayed anaphylaxis to alpha-gal, an oligosaccharide in mammalian meat. Allergol Int 65(1):16-20. DOI:10.1016/j.alit.2015.10.001.

Cregg J (2018). *The Pichia System*. Tuscon (AZ): Pichia Technology from RCT (Research Corporation Technologies), *Pichia pastoris* Protein Expression Platform. Available at: https://pichia.com/science-center/james-m-cregg-ph-d/ [Last accessed: September 20, 2018].

Cregg JM, Barringer KJ, Hessler AY, Madden KR (1985). *Pichia pastoris* as a host system for transformations. Mol Cell Biol 5(12):3376-3385. DOI:10.1128/mcb.5.12.3376.

del Val G, Yee BC, Lozano RM, Buchanan BB, Ermel RW, Lee Y-M, et al. (1999). Thioredoxin treatment increases digestibility and lowers allergenicity of milk. J Allergy Clin Immunol 103(4):690-697. DOI:10.1016/S0091-6749(99)70244-7.

Delaney B, Astwood JD, Cunny H, Conn RE, Herouet-Guicheney C, MacIntosh S, et al. (2008). Evaluation of protein safety in the context of agricultural biotechnology. Food Chem Toxicol 46(Suppl. 2):S71-S97. DOI:10.1016/j.fct.2008.01.045.

Dixon D, Alison R, Bach U, Colman K, Foley GL, Harleman JH, et al. (2014). Nonproliferative and proliferative lesions of the rat and mouse female reproductive system. J Toxicol Pathol 27(3/4, Suppl.):1S-107S. DOI:10.1293/tox.27.1S.

Dlauchy D, Tornai-Lehoczki J, Fülöp L, Péter G (2003). *Pichia* (K*omagataella*) *pseudopastoris* sp. nov., a new yeast species from Hungary. Antonie Van Leeuwenhoek 83(4):327-332. DOI:10.1023/A:1023318829389.

Driss D, Soudani N, Boudawara T, Zeghal N, Chaabouni SE (2014). Toxicological study and oxidative stress evaluation for safety assessment of xylanase preparations in Wistar rats. J Biochem Mol Toxicol 28(11):490-500. DOI:10.1002/jbt.21589.

Duff SM, Guy PA, Xianzhou N, Durnin DC, Hill RD (1998). Haemoglobin expression in germinating barley. Seed Sci Res 8(4):431-436. DOI:10.1017/S0960258500004396.

EFSA (2014). Scientific opinion on Dietary Reference Values for zinc. (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no: EFSA-Q-2011-01233 adopted 19 September 2014 by the European Food Safety Authority). EFSA J 12(10):3844 [76pp]. DOI:10.2903/j.efsa.2014.3844. Available at: https://www.efsa.europa.eu/fr/efsajournal/pub/3844.

EFSA (2015). Scientific Opinion on Dietary Reference Values for iron. (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no EFSA-Q-2011-01214, adopted: 23 September 2015 by European Food Safety Authority). EFSA J 13(10):2454 [115pp]. DOI:10.2903/j.efsa.2015.4254. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4254.

EFSA (2016). Scientific opinion on the safety and efficacy of 3-phytase FLF1000 as feed additive for chickens for fattening and laying hens (EFSA Panel on Additives and Products or Substances used in Animal Feed/FEEDAP) (Question no EFSA-Q-2015-00482, adopted: 19 October 2016 by European Food Safety Authority). EFSA J 14(11):45622 [15 pp.]. DOI:10.2903/j.efsa.2017.5021. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4622.

EFSA (2017). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. (EFSA Panel on Biological Hazards/BIOHAZ) (Question no EFSA-Q-2014-00189, adopted: 30 November 2016, published: 14 March, 2017, corrected: 12 September 2017 by European Food Safety Authority). EFSA J 15(3):4664 [177pp]. DOI:10.2903/j.efsa.2016.4664. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4664.

EFSA (2018). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. (EFSA Panel on Biological Hazards/BIOHAZ) (Question no: EFSA-Q-2016-00828; adopted: 6 December 2017; Published: 24 January 2018 by European Food Safety Authority). EFSA J 16(1):5131 [43pp.]. DOI:10.2903/j.efsa.2018.5131. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5131.

EFSA (2019). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 9: suitability of taxonomic units notified to EFSA until September 2018. (EFSA Panel on Biological Hazards/BIOHAZ) (Question no: EFSA-Q-2016-00830; adopted: 5 December 2018; published: 24 January 2019 by European Food Safety Authority). EFSA J 17(1):5555 [46pp.]. DOI:10.2903/j.efsa.2019.5555. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5555.

Ellis PJ, Appleby CA, Guss JM, Hunter WN, Ollis DL, Freeman HC (1997). Structure of ferric soybean leghemoglobin α nicotinate at 2.3 A resolution. Acta Crystallogr D Biol Crystallogr 53(Part 3):302-310.

Enzyme Technical Association (2005). *Enzyme Technical Association Position on Food Allergen Labeling of Microbial Derived Enzymes Under FALCPA as it Applies to Fermentation Media Raw Materials*. Washington (DC): Enzyme Technical Association. Available at: http://www.enzymeassociation.org/wp-content/uploads/2013/09/Allergen-psn-paper-2.pdf.

Everse J (2004). Heme proteins. In: *Encyclopedia of Biological Chemistry, Volume 2*. Elsevier Inc., pp. 354-361.

FAO/WHO (1996). Biotechnology and Food Safety. Report of a Joint FAO/WHO Consultation, Sep. 30-Oct. 4, 1996, Rome, Italy. (FAO Food and Nutrition Paper No. 61). Geneva, Switz.: World Health Organization (WHO) / Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/ag/agn/food/pdf/biotechnology.pdf.

FAO/WHO (2000). *Safety Aspects of Genetically Modified Foods of Plant Origin*, May 29-June 2, 2000, WHO Headquarters, Geneva, Switz. (Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology Consultations and Workshops. WHO/SDE/FOS/00.6). Geneva, Switz.: World Health Organization (WHO), Food Safety Programme / Food and Agriculture Organization of the United Nations (FAO). Available at: http://www.fao.org/fileadmin/templates/agns/pdf/topics/ec_june2000_en.pdf.

FAO/WHO (2001). *Evaluation of Allergenicity of Genetically Modified Foods*. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology 22-25 January 2001. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: World Health Organization (WHO). Available at: http://www.who.int/foodsafety/publications/gmo-allergenicity/en/.

FARRP (2016). *AllergenOnline Version 16: Home of the FARRP Allergen Protein Database.* Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org/ [Released: January 27, 2016].

Fiocchi A, Restani P, Bouygue GR, Martelli A (2005). Beef allergy in adults and children. Allergy 60(1):126. DOI:10.1111/j.1398-9995.2005.00647.x.

França RC, Conceição FR, Mendonça M, Haubert L, Sabadin G, de Oliveira PD, et al. (2015). *Pichia pastoris* X-33 has probiotic properties with remarkable antibacterial activity against *Salmonella Typhimurium*. Appl Microbiol Biotechnol 99(19):7953-7961. DOI:10.1007/s00253-015-6696-9.

Fraser RZ, Shitut M, Agrawal P, Mendes O, Klapholz S (2018). Safety evaluation of soy leghemoglobin protein preparation derived from *Pichia pastoris*, intended for use as a flavor catalyst in plant-based meat. Int J Toxicol 37(3):241-262 [plus supplementary data]. DOI:10.1177/1091581818766318.

FSANZ (2019). [Sections 3.1.1, 3.3.2, 3.5.2., 3.6.2, 3.6.3]. In: *Food Standards Australia New Zealand Application Handbook. Application Handbook. 1 July 2019*. Canberra, Australia / Wellington, NZ: Foods Standards Australia New Zealand (FSANZ). Available at: http://www.foodstandards.gov.au/code/changes/pages/applicationshandbook.aspx.

Fuchs RL, Ream JE, Hammond BG, Naylor MW, Leimgruber RM, Berberich SA (1993). Safety assessment of the neomycin phosphotransferase II (NPTII) protein. Biotechnology (N Y) 11(13):1543-1547.

Fuchsman WH, Appleby CA (1979). Separation and determination of the relative concentrations of the homogeneous components of soybean leghemoglobin by isoelectric focusing. Biochim Biophys Acta 579(2):314-324. DOI:10.1016/0005-2795(79)90059-X.

Fuentes MM, Palacios R, Garcés M, Caballero ML, Moneo I (2004). Isolation and characterization of a heat-resistant meat allergen: myoglobin. Allergy 59(3):327-331.

Gil de los Santos JR, Storch OB, Fernandes CG, Gil-Turnes C (2012). Evaluation in broilers of the probiotic properties of *Pichia pastoris* and a recombinant *P. pastoris* containing *the Clostridium perfringens* alpha toxin gene. Vet Microbiol 156(3/4):448-451. DOI:10.1016/j.vetmic.2011.11.019.

Gil de los Santos D, Gil de Los Santos JR, Gil-Turnes C, Gaboardi G, Fernandes Silva L, França R, et al. (2018). Probiotic effect of *Pichia pastoris* X-33 produced in parboiled rice effluent and YPD medium on broiler chickens. PLoS One 13(2):e0192904 [11pp]. DOI:10.1371/journal.pone.0192904.

Goodman RE, Taylor SL, Yamamura J, Kobayashi T, Kawakami H, Kruger CL, et al. (2007). Assessment of the potential allergenicity of a milk basic protein fraction. Food Chem Toxicol 45(10):1787-1794. DOI:10.1016/j.fct.2007.03.014.

Gupta KJ, Hebelstrup KH, Mur LA, Igamberdiev AU (2011). Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. FEBS Lett 585(24):3843-3849. DOI:10.1016/j.febslet.2011.10.036.

Hammond B, Kough J, Herouet-Guicheney C, Jez JM (2013). Toxicological evaluation of proteins introduced into food crops [ILSI International Food Biotechnology Committee Task Force on Use of Mammalian Toxicology Studies in Safety Assessment of GM Foods]. Crit Rev Toxicol 43(Suppl. 2):25-42. DOI:10.3109/10408444.2013.842956.

Hardison R (1998). Hemoglobins from bacteria to man: evolution of different patterns of gene expression. J Exp Biol 201(8):1099-1117.

Hargrove MS, Barry JK, Brucker EA, Berry MB, Phillips GN Jr, Olson JS, et al. (1997). Characterization of recombinant soybean leghemoglobin α and apolar distal histidine mutants. J Mol Biol 266(5):1032-1042. DOI:10.1006/jmbi.1996.0833.

Harrison LA, Bailey MR, Naylor MW, Ream JE, Hammond BG, Nida DL, et al. (1996). The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. J Nutr 126(3):728-740. DOI:10.1093/jn/126.3.728.

Hashimoto W, Momma K, Katsube T, Ohkawa Y, Ishige T, Kito M, et al. (1999). Safety assessment of genetically engineered potatoes with designed soybean glycinin: compositional analyses of the potato tubers and digestibility of the newly expressed protein in transgenic potatoes. J Sci Food Agric 79(12):1607-1612. DOI:10.1002/(SICI)1097-0010(199909)79:12<1607::AID-JSFA408>3.0.CO;2-T.

Hileman RE, Bonner HK, Kaempfe TA, Hammond BG, Glenn KC (2006). Safety assessment of Cre recombinase. J Agric Food Chem 54(22):8640-8647. DOI:10.1021/jf061210j.

IFBC (1990a). Biotechnologies and food: assuring the safety of foods produced by genetic modification. Regul Toxicol Pharmacol 12(3, Part 2):vii-S196. DOI:10.1016/S0273-2300(05)80069-8.

IFBC (1990b). Chapter 4: Safety evaluation of foods and food ingredients derived from microorganisms (International Food Biotechnology Council - IFBC). Regul Toxicol Pharmacol 12(3, Part 2):S114-S128. DOI:10.1016/S0273-2300(05)80080-7.

Impossible Foods Inc. (2014). GRAS Notification for Soybean Leghemoglobin Protein Derived from Pichia pastoris. (GRN No. 540). Submitted by Redwood City (CA): Impossible Foods, Inc. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: Link http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=540 [Approved by U.S. FDA, Nov. 10, 2015].

Impossible Foods Inc. (2018). *GRAS Notification for Soy Leghemoglobin Protein Preparation Derived from Pichia Pastoris*. (GRN 737). Submitted by Redwood City (CA): Impossible Foods Inc. to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=737 [Approved by U.S. FDA, Jul 23, 2018].

Jin Y, He X, Andoh-Kumi K, Fraser RZ, Lu M, Goodman RE (2018). Evaluating potential risks of food allergy and toxicity of soy leghemoglobin expressed in *Pichia pastoris*. Mol Nutr Food Res 62(1):1700297 [13pp]. DOI:10.1002/mnfr.201700297.

Jokipii-Lukkari S, Frey AD, Kallio PT, Häggman H (2009). Intrinsic non-symbiotic and truncated haemoglobins and heterologous *Vitreoscilla* haemoglobin expression in plants. J Exp Bot 60(2):409-422. DOI:10.1093/jxb/ern320.

Jonas DA, Antignac E, Antoine J-M, Classen H-G, Huggett A, Knudsen I, et al. (1996). The safety assessment of novel foods. Guidelines prepared by ILSI Europe Novel Food Task Force. Food Chem Toxicol 34(10):931-940. DOI:10.1016/S0278-6915(96)00061-0.

Kurtzman CP (2009). Biotechnological strains of *Komagataella* (*Pichia*) *pastoris* are *Komagataella phaffii* as determined from multigene sequence analysis. J Ind Microbiol Biotechnol 36(11):1435-1438. DOI:10.1007/s10295-009-0638-4.

Lee H, Kim H, An CS (2004). Cloning and expression analysis of 2-on-2 hemoglobin from soybean. J Plant Biol 47(2):92-98. DOI:10.1007/BF03030637.

Lira-Ruan V, Ruiz-Kubli M, Arredondo-Peter R (2011). Expression of non-symbiotic hemoglobin 1 and 2 genes in rice (*Oryza sativa*) embryonic organs. Commun Integr Biol 4(4):457-458. DOI:10.4161/cib.4.4.15468.

Liu JJ, Kong II, Zhang GC, Jayakody LN, Kim H, Xia P-F, et al. (2016). Metabolic engineering of probiotic *Saccharomyces boulardii*. Appl Environ Microbiol 82(8):2280-2287. DOI:10.1128/AEM.00057-16.

Madden KR (2014). *BioGrammatics, Inc. Citation Approval for Strain Lineage: CONFIDENTIAL*. Written Communication to Impossible Foods.

Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL, Fuchs RL (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Crit Rev Food Sci Nutr 36(Suppl.):S165-S186. DOI:10.1080/10408399609527763.

Momma K, Hashimoto W, Ozawa S, Kawai S, Katsube T, Takaiwa F, et al. (1999). Quality and safety evaluation of genetically engineered rice with soybean glycinin: analyses of the grain composition and digestibility of glycinin in transgenic rice. Biosci Biotechnol Biochem 63(2):314-318. DOI:10.1271/bbb.63.314.

Moran DL, Tetteh AO, Goodman RE, Underwood MY (2014). Safety assessment of the calcium-binding protein, apoaequorin, expressed by *Escherichia coli*. Regul Toxicol Pharmacol 69(2):243-249. DOI:10.1016/j.yrtph.2014.04.004.

Moyad MA (2008). Brewer's/baker's yeast (*Saccharomyces cerevisiae*) and preventive medicine: Part II. Urol Nurs 28(1):73-75.

MPI (2018a). *Evolution of Plant Protein – Assessing Consumer Response*. (Plant & Food Research). Wellington, NZ: Ministry for Primary Industries (MPI). Available at: https://www.mpi.govt.nz/news-and-resources/science-and-research/food-research/ [Click the first download on the page].

MPI (2018b). *The Impossible Burger – Consumer Insights*. (Plant & Food Research). Wellington, NZ: Ministry for Primary Industries (MPI). Available at: https://www.mpi.govt.nz/news-and-resources/science-and-research/food-research/ [Click: Impossible burger case study].

Muñoz-Bernal E, Deery MJ, Rodríguez ME, Cantoral JM, Howard J, Feret R, et al. (2016). Analysis of temperature-mediated changes in the wine yeast *Saccharomyces bayanus* var *uvarum*. An oenological study of how the protein content influences wine quality. Proteomics 16(4):576-592. DOI:10.1002/pmic.201500137.

NCAUR (2019). [National Center for Agricultural Utilization Research - Website]. Peoria (IL): National Center for Agricultural Utilization Research (NCAUR), U.S. Department of Agriculture (USDA), Economic Research Service (ERS). https://nrrl.ncaur.usda.gov/.

Neuman-Sunshine DL, Eckman JA, Keet CA, Matsui EC, Peng RD, Lenehan PJ, et al. (2012). The prevalence of clinical cross-reactivity on non-peanut legumes to peanut in patients with persistent peanut allergy. J Allergy Clin lmmunol 129(2, Suppl.):AB23 [abstract 89].

NHMRC (2006). *Nutrient Reference Values for Australia and New Zealand Including Recommended Dietary Intakes*. (Reference number: N35, N36, N37). Canberra, Australia: Australian Government, National Health and Medical Research Council (NHMRC) & Department of Health and Aging. Available at: https://www.nhmrc.gov.au/sites/default/files/images/nutrient-refererence-dietary-intakes.pdf.

Noteborn HPJM, Bienenmann-Ploum ME, van den Berg JHJ, Alink GM, Zolla L, Reynaerts A, et al. (1995). Safety assessment of the *Bacillus thuringiensis* insecticidal crystal protein CRYIA(b) expressed in transgenic tomatoes. In: Engel K-H, Takeoka GR, Teranishi R, editors. *Genetically Modified Foods: Safety Issues*, 208th National Meeting of the ACS, Aug. 21-25, 1994, Washington, DC. (ACS Symposium Series, Vol. 605). Washington (DC): American Chemical Society (ACS), pp. 134-147. DOI:10.1021/bk-1995-0605.ch012.

O'Brian MR, Kirshbom PM, Maier RJ (1987). Bacterial heme synthesis is required for expression of the leghemoglobin holoprotein but not the apoprotein in soybean root nodules. Proc Natl Acad Sci U S A 84(23):8390-8393. DOI:10.1073/pnas.84.23.8390.

OECD (1992). *Safety Considerations for Biotechnology 1992*. Paris, France: Organisation for Economic Co-Operation and Development (OECD). Available at: http://www.oecd.org/dataoecd/8/3/2375496.pdf.

OECD (1993). *Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles*. Paris, France: Organisation for Economic Co-Operation and Development (OECD). Available at: http://www.oecd.org/science/biotrack/41036698.pdf.

OECD (1997). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 471) [Updated & Adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en;jsessionid=1ijgh8o34k0qd.delta.

OECD (1998). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, no. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (2008). Repeated dose 28-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 407) [Adopted: 3 Oct 2008]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en.

OECD (2016a). Reproduction/developmental toxicity screening test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 421) [Updated & Adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-421-reproduction-developmental-toxicity-screening-test_9789264264380-en;jsessionid=k7rpdl8f2jeb.x-oecd-live-02.

OECD (2016b). *In Vitro* mammalian chromosome aberration test. In*: OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 473) [Updated & Adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosomal-aberration-test_9789264264649-en;jsessionid=58ip5ibjcf4kc.x-oecd-live-02.

Olempska-Beer ZS, Merker RI, Ditto MD, DiNovi MJ (2006). Food-processing enzymes from recombinant microorganisms-a review. Regul Toxicol Pharmacol 45(2):144-158. DOI:10.1016/j.yrtph.2006.05.001.

Pariza MW, Foster EM (1983). Determining the safety of enzymes used in food processing. J Food Prot 46(5):453-468 [plus addendum]. DOI:10.4315/0362-028X-46.5.453.

Pariza MW, Johnson EA (2001). Evaluating the safety of microbial enzyme preparation used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173-186. DOI:10.1006/rtph.2001.1466.

Proulx AK, Reddy MB (2006). Iron bioavailability of hemoglobin from soy root nodules using a Caco-2 cell culture model. J Agric Food Chem 54(4):1518-1522. DOI:10.1021/jf052268l.

Reed AJ, Kretzmer KA, Naylor MW, Finn RF, Magin KM, Hammond BG, et al. (1996). Safety assessment of 1-aminocyclopropane-1-carboxylic acid deaminase protein expressed in delayed ripening tomatoes. J Agric Food Chem 44(1):388-394. DOI:10.1021/jf9504071.

Savage JH, Kaeding AJ, Matsui EC, Wood RA (2010). The natural history of soy allergy. J Allergy Clin Immunol 125(3):683-686. DOI:10.1016/j.jaci.2009.12.994.

SCF (1992). Guidelines for the presentation of data on food enzymes (Opinion expressed on 11 April 1991). In: *Food Science and Techniques*. (Reports of the Scientific Committee for Food, 27th Series). Brussels, Belgium: Commission of the European Communities (EEC), Scientific Committee for Food (SCF), pp. 13-22. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_reports_27.pdf.

SFA (2019). *Food Additives Permitted Under the Singapore Food Regulations*. (28 May 2019). Singapore Food Agency (SFA). Available at: https://www.sfa.gov.sg/legislation.

Steinke JW, Platts-Mills TA, Commins SP (2015). The alpha-gal story: lessons learned from connecting the dots. J Allergy Clin Immunol 135(3):589-596. DOI:10.1016/j.jaci.2014.12.1947.

Taylor SL, Baumert JL (2013). *Testing of Microbially Derived Enzymes for Potential Allergens from Fermentation Media Raw Materials*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP).

Texas A&M Institute (2019). *Meat Science: Meat Color*. College Station (TX): Texas A&M Institute. Available at: https://meat.tamu.edu/ansc-307-honors/meat-color/ [Last accessed: June 6, 2019].

Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, et al. (2004). A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regul Toxicol Pharmacol 39(2):87-98. DOI:10.1016/j.yrtph.2003.11.003.

U.S. FDA (1993). Food additives permitted in feed and drinking water of animals: *Pichia pastoris* dried yeast: FDA 21 CFR Part 573; Docket No. 87F-0221. Fed Regist (US) 58(214):59169-59170.

U.S. FDA (2003). C. Guidelines for specific toxicity studies. IV.C.4a. Subchronic toxicity studies with rodents. In: *Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000 [Updated to July 2007]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm078345.htm [July 2007].

U.S. FDA (2006). *Agency Response Letter GRAS Notice No. GRN 000204 [Phospholipase C enzyme preparation from Pichia pastoris expressing a heterologous phospholipase C gene, San Diego (CA): Diversa Corporation]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=204 [Dec. 4, 2006].

U.S. FDA (2018a). *Agency Response Letter GRAS Notice No. GRN 000737 [Soy leghemoglobin Soy leghemoglobin preparation from a strain of Pichia pastoris], Redwood City (CA): Impossible Foods Inc.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=737 [July 23, 2018].

U.S. FDA (2015). Agency Response Letter GRAS Notice No. GRN 000540 [Soybean leghemoglobin from Pichia pastoris, Redwood City (CA): Impossible Foods, Inc.]. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=540 [Nov. 10, 2015].

U.S. FDA (2018b). *Ingredients, Additives, GRAS & Packaging Guidance Documents & Regulatory Information*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/ingredientsadditivesgraspackaging/ [Page Last Updated: 08/09/2018].

USDA (2019). *USDA Food Composition Databases*. (v.3.9.5.2_2019-05-07). Beltsville (MD): U.S. Department of Agriculture (USDA), Nutrient Data Laboratory USDA-ARS. Available at: https://ndb.nal.usda.gov/ndb/search/list.

Vinogradov SN, Hoogewijs D, Bailly X, Mizuguchi K, Dewilde S, Moens L, et al. (2007). A model of globin evolution. Gene 398(1/2):132-142. DOI:10.1016/j.gene.2007.02.041.

Vinogradov SN, Moens L (2008). Diversity of globin function: enzymatic, transport, storage, and sensing. J Biol Chem 283(14):8773-8777 [plus supplementary data]. DOI:10.1074/jbc.R700029200.

Wajcman H, Kiger L (2002). L'hémoglobine, des micro-organismes à l'homme: un motif structural unique, des fonctions multiples [Hemoglobin, from microorganisms to man: a single structural motif, multiple functions]. C R Biol 325(12):1159-1174 [French]. DOI:10.1016/S1631-0691(02)01537-8.

Wegner EH, inventor; Bartlesville, Okla: Phillips Petroleum Company, assignee (1986). *Biochemical Conversions by Yeast Fermentation at High Cell Densities*. US Patent Number 4,617,274 [Oct. 14, 1986]. Available at: https://patents.google.com/patent/US4617274A/en.

Westwood FR (2008). The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol 36(3):375-384. DOI:10.1177/0192623308315665.

Whittaker RG, Lennox S, Appleby CA (1981). Relationship of the minor soybean leghemoglobins d_1, d_2 and d_3 to the major leghemoglobins c_1, c_2 and c_3. Biochem Int 3(2):117-124.

Yamada Y, Matsuda M, Maeda K, Mikata K (1995). The phylogenetic relationships of methanol-assimilating yeasts based on the partial sequences of 18S and 26S ribosomal RNAs: the proposal of *Komagataella* gen. nov. (*Saccharomycetaceae*). Biosci Biotechnol Biochem 59(3):439-444. DOI:10.1271/bbb.59.439.

Yip R, Dallman PR (1996). Iron. In: Ziegler EE, Filer LJ Jr, editors. *Present Knowledge in Nutrition, 7th edition*. Washington (DC): International Life Sciences Institute (ILSI), pp. 277-292.

Zhu L, Tian YJ, Zhu SM (2012). 28-Day repeated dose oral toxicity of human copper-zinc superoxide dismutase from recombinant *Pichia pastori* in rats. Drug Chem Toxicol 35(2):155-161. DOI:10.3109/01480545.2011.589448.