6 August 2020
[131-20]

Supporting document 1

Risk and technical assessment report – Application A1186

Soy leghemoglobin in meat analogue products

Executive summary

A genetically modified (GM) strain of the yeast *Pichia pastoris* has been developed for the production of soy leghemoglobin. Leghemoglobin is a globin protein, containing an iron-bound haem B prosthetic group and is typically expressed in the root nodules of leguminous plants.

A preparation of *P. pastoris* containing the soy leghemoglobin will be added to meat analogue products at levels of not more than 0.8% (w/w) to replicate the nutritional source of iron, flavour and aroma of myoglobin, a protein found in the muscle tissue of animals. Soy leghemoglobin does have additional functions that align with the definition of a food additive (i.e. flavouring and aroma). FSANZ considered permissons for soy leghemoglobin in three separate Code standards/schedules (as a GM food, as a food additive, and as a nutritive substance) but we decided that consideration as a food additive was not justified. Regulation as a nutritive substance allowed FSANZ to include permissions for it as a source of iron in Schedule S17—3, and review data and information on the bioavailability of iron in the ingredient.

The production of soy leghemoglobin is achieved through the expression of a leghemoglobin gene from soybean (*Glycine max*) as well as overexpression of several genes from the *Pichia* host. The overexpression of host genes support the production of the leghemoglobin protein. The soy leghemoglobin preparation, also known as LegH Prep, produced by the production strain MXY0541, is a soluble component of a fermentation culture that has undergone mechanical lysis, heat inactivation and partial purification. The preparation will contain novel soy leghemoglobin and also proteins and genetic material from the *Pichia* host.

In conducting the risk assessment of the soy leghemoglobin and the LegH Prep, a number of criteria have been addressed, including the safety of the *P. pastoris* host strain, novel proteins, toxicity of the LegH Prep and a nutritional and dietary intake assessment. The safety assessment of the source organism and novel proteins concluded there were no public health and safety concerns. The source organism is a well characterised yeast with a recognised safe history of use for the production of food enzymes. It is neither pathogenic nor toxigenic.

The novel soy leghemoglobin was shown to be equivalent to that expressed in soybean and

was shown to be expressed as a holoprotein[1]. Analyses of the potential allergenicity or toxicity of the soy leghemoglobin and the *Pichia* proteins did not identify any significant similarities to known allergens or toxins. The proteins were shown to be susceptible to pepsin digestion and were denatured at standard cooking temperatures and in acidic conditions that mimic the stomach environment. The shelf life and specifications of the LegH Prep are also appropriate for addition to meat analogue products.

The applicant submitted *in vitro* genotoxicity studies in bacterial and mammalian cells and an oral toxicity study in rats. These studies are intended to confirm the outcome of the compositional and bioinformatic analysis conducted as a part of the safety assessment. No hazard was identified in the submitted studies. LegH Prep was not genotoxic *in vitro* and did not cause adverse effects in short-term toxicity studies in rats. The no observed adverse effect level (NOAEL) of freeze-dried LegH Prep in a 28-day dietary toxicity study in rats was 1536 mg/kg bw/day, the highest dose tested. This dose corresponds to 1421 mg/kg bw/day total organic solids (TOS).

Mean and P90 estimated dietary intakes of LegH Prep at the maximum proposed use level were 20 – 60 mg/kg bw/day TOS and 45 – 124 mg/kg bw/day TOS, respectively. Mean and P90 estimated dietary intakes of LegH Prep at the likely use level were 11 – 32 mg/kg bw/day TOS and 24 – 68 mg/kg bw/day TOS, respectively. The estimated intakes of LegH Prep for both scenarios are considered to be conservative and over-estimate exposure as it is unlikely that consumers will eat meat analogue products containing soy leghemoglobin in the same amounts or with the same frequency they currently consume minced meat and poultry products, and vegetarian meat alternatives (particularly over a long period of time).

The margins of exposure (MOEs) between the NOAEL of 1421 mg/kg bw/day TOS in the rat oral toxicity study and estimated dietary exposures at the maximum proposed use level ranged between 20 – 70 for mean exposures and between 10 – 30 at the 90th percentile. At likely use levels, MOEs for mean and P90 estimated dietary intakes ranged between 40 – 130 and 20 – 60, respectively. These MOEs are not considered to be of concern given that: a sufficient body of knowledge exists on the safety of the source organism, the soy leghemoglobin and *Pichia* proteins will be digested like other other dietary proteins and do not share any significant similarities to known allergens or toxins; and the conservative nature of the dietary exposure assessment which is likely to overestimate intakes over a long period of time.

The nutrition assessment concluded that haem iron from soy leghemoglobin is likely to have similar bioavailability to haem iron from mammalian haem proteins (e.g. myoglobin present in muscle tissue). The absence of meat proteins in the proposed meat analogue products may decrease the bioavailability of haem iron from soy leghemoglobin. However, because iron absorption is regulated tightly by the body, and meat analogue products have higher total iron content due to higher content of non-haem iron relative to comparison foods, any decrease in haem iron bioavailability should not result in a nutritional disadvantage to consumers in Australia and New Zealand.

The estimated intakes of iron (with the additional iron contribution from soy leghemoglobin) for all population age/sex groups assessed for both the Australian and New Zealand populations are below the ULs for iron. The estimated iron intakes in FSANZ's assessment, for both the *maximum proposed use level* and *likely use level* scenarios, are considered to be conservative and an overestimation of actual iron intakes. It is unlikely that consumption of meat analogue products containing soy leghemoglobin would pose a risk of iron exceedances to the Australian and New Zealand populations, including at levels up to 0.8% soy leghemoglobin.

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[1] A holoprotein is a protein bound to a prosthetic group (e.g. haem)

As of March 2020, the applicant advised they had sold approximately 100,000,000 quarter-pound (113 g) servings of meat analogue products containing soy leghemoglobin. Its post-marketing surveillance has identified one complaint per 600,000 servings based on the current formulation (released on the market in the US in early 2019), but none of these complaints has been confirmed as an adverse event due to consumption of these products.

The assessment of soy leghemoglobin and the LegH Prep concluded there are no public health and safety concerns associated with its use in meat analogue products at the proposed levels.

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Abbreviations

AOAC	Association of Official Agricultural Chemists
AOX1	alcohol oxidase 1 gene
AOX2	alcohol oxidase 2 gene
bw	body weight
cGMP	current good manufacturing practice
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Da	dalton
DAS2	dihydroxyacetone synthase 2 gene
DNA	deoxyribonucleic acid

EFSA	European Food Safety Authority
EHEC	enterohemorrhagic *Escherichia coli*
EPA	Environmental Protection Agency (USA)
FDA	Food and Drug Administration (USA)
FSANZ	Food Standards Australia New Zealand
g	gram
GLP	good laboratory practice
GM	genetically modified
GRAS	generally recognised as safe (US FDA)
GRN	GRAS notification number (US FDA)
kg	kilogram
L	litre
LOD	limit of detection
mg	milligram
m	minute
mL	millilitre
mmol	millimolar
MOE	margin of exposure
NHMRC	National Health and Medical Research Council
NOAEL	no observed adverse effect level
NRV	nutrient reference value
NRRL	Northern Regional Research Laboratory (US Department of Agriculture)
N-terminal	amino-terminal of a protein
OECD	Organisation for Economic Co-operation and Development
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ppm	parts per million
QPS	qualified presumption of safety
SDS	sodium dodecyl sulfate
Spp.	species
TOS	total organic solids
U	units
µg	microgram
µL	microliter
UL	upper level of intake
UPLC	Ultra performance liquid chromatography
USA	United States of America
UTR	untranslated region
UV	ultraviolet
w/w	weight for weight

1 Introduction

FSANZ received an application from Impossible Foods Inc. to permit the use of soy leghemoglobin in meat analogue products. Soy leghemoglobin is a component of a cell lysate preparation from a genetically modified (GM) yeast, *Pichia pastoris*. The yeast has been modified to express the leghemoglobin gene from soybean *(Glycine max)* and other host proteins that support the expression of leghemoglobin. Leghemoglobin is a globin protein, containing an iron-bound haem B prosthetic group and is typically expressed in the root nodules of leguminous plants. The soy leghemoglobin preparation (LegH Prep) also contains proteins and genomic DNA from the *Pichia* production strain.

The purpose of adding soy leghemoglobin to meat analogue products is to replicate the nutrition (source of iron), flavour and aroma of myoglobin, an oxygen transporting haem protein found in the muscle tissue of animals (Ordway and Garry 2004). Though soy leghemoglobin does have additional functions that align with the definition of a food additive (i.e. flavouring and aroma), FSANZ considered three permissions in the Code for the same ingredient was excessive. Regulation as a nutritive substance allowed FSANZ to include permissions for it as a source of Iron in S17—3, and review data and information on the bioavailability of iron in the ingredient.

To prepare the LegH Prep for use in meat analogue products, *Pichia* cells expressing soy leghemoglobin undergo a fermentation process followed by mechanical lysis, with further processing to minimise the level of impurities and to ensure absence of viable *Pichia* cells. The partially purified preparation is then blended with stabilisers. This preparation contains ≤ 14% total protein, of which ≥ 65% is leghemoglobin, with the remainder of the protein consisting of native *Pichia* proteins.

The LegH Prep will not be sold in Australia or New Zealand as an individual product, rather it will be an ingredient in meat analogue products manufactured by the applicant at levels of not more than 0.8% soy leghemoglobin (w/w). The applicant currently manufactures both LegH Prep and their meat analogue products outside of Australia and New Zealand , therefore these products will be imported as frozen, packaged products. In the future, the applicant may establish a co-manufacturing agreement in Australia and New Zealand to produce Impossible meat analogue products. Where possible, locally sourced ingredients would be used with the exception of LegH Prep, which would continue to be manufactured in an Impossible Foods production facility located outside of Australia and New Zealand. Its use would be permitted in Impossible meat analogue products in the co-manufacturers' foods for sale such as ready-meals and burgers etc.

As of March 2020, the applicant advised they had sold approximately 100,000,000 quarter-pound 113g servings. Meat analogue products containing LegH Prep are permitted for sale in retail and catering outlets in the US, Canada, Singapore, Hong Kong and Macao.

2　Risk assessment of soy leghemoglobin and the LegH Prep

2.1 Technical assessment

2.1.1 Identity of the source organism and product

The soy leghemoglobin-containing LegH Prep is produced from a GM *P. pastoris* strain MXY0541. This strain contains several copies of the leghemoglobin gene from soybean (*G. max*) as well as extra copies of endogenous genes to overexpress a transcription factor Mxr1 and the eight enzymes involved in the synthesis of the prosthetic group haem B. A full characterisation of the production strain MXY0541 is provided in Section 2.7.3. Some of the data provided to FSANZ for the risk assessment analyses was obtained from a predecessor of MXY0541, designated MXY0291. The major differences between these two strains is the copy number of the leghemoglobin gene (MXY0291 contains fewer copies) and MXY0541 contains extra DNA sequences associated with one of the haem-synthesis enzyme genes. Further information about MXY0291 can be found in the US FDA notification GRN 737 (US FDA 2018).

2.1.2 Technological purpose and justification

Soy leghemoglobin, a component of the LegH Prep, is to be added to meat analogue products produced by Impossible Foods. The meat analogue products are largely intended for consumption by the general population (aged 2 years and older). There are multiple purposes for adding soy leghemoglobin to meat analogue products, including to provide the nutrition (i.e. source of iron), flavour, and aroma of myoglobin.

2.1.3 LegH Prep specifications

The specifications for the LegH Prep are outlined in Table 1, which shows the combined minimum and maximum results for strains MXY0291, MXY0541 and the individual average results for each strain. The preparation contains up to 9% soy leghemoglobin, which has a minimum protein purity of 65%. Acceptable limits for heavy metal and microbiological contaminants have been established. Additionally, no viable *P. pastoris* cells are present, which is in accordance with the recommendations for safety evaluation by the International Food Biotechnology Committee (IFBC, 1990). The inability to detect viable *Pichia* provides evidence of effective process controls for the removal and/or inactivation of the production organism. Details of the methods of analysis that were internally developed and validated by Impossible Foods for the evaluation of the specification parameters (i.e. content and purity of the soy leghemoglobin protein) were provided as confidential information. All other parameters are assessed by recognised and validated methods of analysis.

Table 1: **Specifications and batch analyses for the LegH Prep product**

Parameter	Specification (%)	Min (%)[1]	Max (%)[1]	Avg (%) MXY0291[2]	Avg (%) MXY0541[3]
Total protein (w/w)[4]	≤14	Average of 12.73%			
Soy leghemoglobin (w/w)	≤ 9	4.50	7.20	6.62	5.85
Soy Leghemoglobin Purity (%)	≥ 65	68.00	86.00	80.20	76.00
Fat (w/w)	≤ 2	0.03	1.70	0.05	0.80
Carbohydrates (w/w)	≤ 6	0.99	4.10	1.82	2.83
Ash (w/w)	≤ 4	0.09	3.60	2.11	1.82

Solids (w/w)[5, 6]	≤ 26	12.55	20.30	15.61	19.08
Moisture (w/w)	≤ 90	79.7	87.45	84.37	80.92
	Range				
Lead (ppm)	< 0.4	<0.01			
Arsenic (ppm)	< 0.05	<0.01 - 0.04			
Mercury (ppm)	< 0.05	<0.005			
Cadmium (ppm)	< 0.2	<0.001 - 0.003			
***Escherichia coli* EHEC (inc O157:H7)**	Absent by test	Absent			
***Salmonella* spp.**	Absent by test	Absent			
Listeria monocytogenes	Absent by test	Absent			

1. the results are combined data from MXY0291 & MXY0541 batch analyses
2. the number of batches analysed = 5
3. the number of batches analysed = 6
4. total protein is averaged from 3 batches
5. solids = Sum of total protein, fat, carbohydrate and ash
6. total organic solids (TOS) content = 22% (100% – (% Ash + % Water + % Diluent))

2.1.4 Product Stability

Stability of the LegH Prep prepared from nine batches of MXY0541, stored at the recommended -20°C were measured via an ultra-performance liquid chromatography assay developed and validated by Impossible Foods. Three parameters were measured: percent soy leghemoglobin, percent purity of soy leghemoglobin of total protein and pH. All data was compared to observed values on the day of production. Measurements were taken at variable times for up to 24 months. The results showed no significant changes in protein stability. The results are comparable to those obtained with the LegH Prep from MXY0291.

Stability of the soy leghemoglobin was also determined in the meat analogue product stored for up to 9 days at 4°C and 6 months at -20°C. There was minimal loss of the soy leghemoglobin at day 5 and up to 10-15% loss at day 9. Similarly, after storage at -20°C for 6 months, there was up to 15% loss of the soy leghemoglobin.

FSANZ considers the stability (shelf-life) of products containing soy leghemoglobin would be determined by the same factors that determine shelf life in minced (ground[2]) meat. Frozen product can be affected by factors that are not microbiological such as: meat colour and appearance; rancidity caused by chemical oxidation of fats at low temperature, and changes in texture caused by extended enzymatic activity; product drying during storage, e.g. freezer burn; or flavour and odour changes caused from protein denaturation or non-enzymatic browning reactions. Similarly, spoilage that can occur in the chilled product can be physical, chemical or microbiological. The two principal spoilage mechanisms that affect the chilled shelf-life of meat are microbial growth and oxidation of myoglobin causing browning of lipids causing rancidity.

Generally, meat is considered to be past its shelf-life when it is no longer acceptable to the consumer as a result of key attribute deterioration such as colour, flavour, texture, aroma or nutrient content. Another determinant of shelf-life would also be when a food-safety issue arises, where the food product may make consumers unwell (CSIRO 2006). FSANZ conclude that the mechanisms inducing spoilage of meat would similarly affect meat analogue products containing soy leghemoglobin and therefore have no concerns around the stability information provided by Impossible Foods.

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[2] The applicant refers to 'ground' meat products, however in Australia and New Zealand the term 'minced' meat is used and from herein will be referred to as such.

2.1.5 Conclusion

Analyses of the data demonstrated that the LegH Prep containing the soy leghemoglobin is stable in chilled and frozen forms and within the final meat analogue product. FSANZ concludes that, in the form and prescribed amounts, soy leghemoglobin is technologically justified.

2.2 History of use

Soy leghemoglobin

Leghemoglobin is a haem-containing protein, with the highest levels of expression found in the roots of leguminous plants, more specifically in root nodules infected with nitrogen-fixing bacteria known as rhizobia (Kubo 1939; Appleby and Bergersen 1980; Appleby 1984). The edible portion of a legume is the seed or seed pod not the roots, thus leghemoglobin at the levels found in the root does not have a history of use in food.

While roots are not commonly used as food, plants do express a range of haemoglobins within the phytoglobin family (Hill et al. 2016) that share a high degree of structural similarity. These phytoglobins are expressed in leaf, stem and rapidly growing tissue in all higher plants (Garrocho-Villegas et al. 2007; Smagghe et al. 2009; Hill 2012). Expression of these phytoglobins is dependent on growth stage and reported levels are low (1-100 nM) compared to that found for leghemoglobin in root tissue (1-3 mM) (Appleby 1984, Arechaga-Ocampo et al. 2001; Lira-Ruan et al. 2001; Ross et al. 2001). The level of soy leghemoglobin present in the meat analogue products falls with the range found in plants and is equivalent to the reported levels of myoglobin found in beef (Cho et al. 2014; Texas A&M Institute 2019).

The role of leghemoglobin is to regulate oxygen levels in the root nodules, providing enough oxygen for cells undergoing cellular respiration while maintaining a moderately hypoxic environment to ensure nitrogen-fixation can occur (Appleby and Bergersen 1980; Ott et al. 2005). This oxygen transporting role is similar to the animal myoglobin, which is a haem protein that regulates oxygen levels in muscle tissue and is therefore present in meat products. Beyond function, leghemoglobin shares a common evolutionary origin as well as similar secondary and tertiary structure to myoglobin (Vainshtein et al. 1975; Arredondo-Peter et al. 1998). There is no evidence that myoglobin or other commonly ingested haem proteins are either allergenic or toxic to humans and myoglobin is considered to have a long history of safe use in food.

The soy leghemoglobin within the LegH Prep is produced by a GM strain of *P. pastoris* containing the leghemoglobin gene *LBC2* from soybean (*G. max*), encoding the leghemoglobin C2 protein (P02236[3]). The introduced gene encodes a protein of 145 amino acids with a theoretical monoisotopic mass of 15515.08 Da (ExPASy Compute MW tool[4]). When bound to haem, the holoprotein will have an expected mass of 16,131 Da, noting that the haem component has a mass of 616 Da (Li et al. 1993). The characterisation and assessment of this novel protein is presented in Sections 2.3.1, 2.3.2 and 2.3.3.

Pichia pastoris

Pichia pastoris is a cosmopolitan organism, commonly found on rotting wood. Since this organism was first characterised over 40 years ago, it has been chosen as a preferred organism for the production of heterologous proteins. Genomic sequencing analyses have led to the reclassification of *P. pastoris* into two distinct species: *Komagataella phaffii* and *K.*

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[3] www.uniprot.org/uniprot/P02236
[4] web.expasy.org/compute_pi/

pastoris (Yamada et al. 1995; Kurtzman 2009; De Schutter et al. 2009; Sturmberger et al. 2016). The strain used to produce soy leghemoglobin is from a lineage derived from an original isolate NRRL Y-11430 (Figure 1), which has been reclassified as *K. phaffi*.
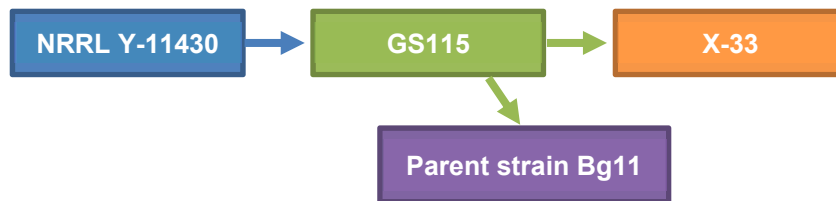

Figure 1: Lineage of the <u>Pichia pastoris</u> host organism Bg11. NRRL Y-11430 is the one of the original isolates from which many of the commercialised <u>P. pastoris</u> strains such as GS115 (Cregg et al. 1985), were derived for the production of food and industrial chemicals.

Both *Komagataella* organisms have been classified as Biosafety Level 1 organisms based on the <u>United States Public Health Service Guidelines</u> (US PHS). They have also been granted qualified presumption of safety (QPS) status by the European Food Safety Authority (EFSA) for the production of enzymes to be added to food (EFSA 2017; 2018). The restriction of QPS status to the production of enzymes is a reflection of the body of knowledge relating predominantly to use of *K. phaffi* in protein expression systems and as a model organism (EFSA 2018).

P. pastoris belongs to the *Saccharomycetaceae* family of budding yeast and is related to *Saccharomyces cerevisiae*, commonly known as baker's and brewer's yeast and *S. cerevisiae* var. *boulardi*, a common organism marketed as a probiotic yeast. *S. cerevisiae* and *S. cerevisiae* var. *boulardii* both have a long history of safe use in food. While there is limited evidence that *P. pastoris* has been consumed by humans, this organism does have a long history of safe use for the production of pharmaceuticals and industrial chemicals, including a number of enzyme processing aids approved by EFSA, US FDA and FSANZ (Spohner et al. 2015). Furthermore, a search of the literature did not identify any potential safety concerns associated with *P. pastoris*, *K. phaffi* or *K. pastoris* and no reports of adverse effects from products produced from *P. pastoris* strains were identified.

While *P. pastoris* may not have a history of use in food, a strain identified as X-33 has been investigated as a feed supplement in chicken and mice. X-33 is a mutation revertant of GS115, which is the parental strain from which Bg11 is derived (Figure 1). In these animal studies, no adverse effects were reported from the ingestion of viable *P. pastoris* X-33 (Gil de los Santos et al. 2012; Franca et al. 2015; Gil de los Santos et al. 2018). These data support the evidence that *P. pastoris* X-33 does not produce toxins or other compounds that might cause adverse effects.

A comparison of the three strains NRRL Y-11430, GS115 and X-33 (Figure 1) at the genome, transcriptome and proteome level have identified the differences between these highly related strains (Brady et al. 2019; Braun-Galleani et al. 2019). The presence of toxin encoding genes was not reported for any of these strains. However NRRL Y-11430 does contain two linear cytoplasmic plasmids, commonly known as killer plasmids (Banerjee and Verma 2000; Sturmberger et al, 2017). These plasmids have been characterised in a range of *Pichia* species and encode proteins used to protect the organism from pathogens and competitors. While NRRL Y-11430 does contain these plasmids, the host strain Bg11 used for the production of soy leghemoglobin does not contain these plasmids.

The most common *Pichia* proteins present in the LegH Prep have been identified and characterised and the assessment is presented in <u>Section 2.3.4</u>.

The microbiological assessement undertaken by FSANZ did not identify any public health and safety concerns associated with the consumption of the LegH preparation containing *P. pastoris*.

2.3 Characterisation of the novel proteins in the LegH Prep

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects are considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

2.3.1 Biochemical analyses of the soy leghemoglobin in the LegH Prep

The soy leghemoglobin component of the LegH Prep was isolated by UPLC and peptide mapping was performed using mass spectrometry on tryptic-digested peptides. The results confirmed that the amino acid sequence was as expected (Figure 2). The analysis showed absence of the N-terminal methionine (M) and C-terminal alanine (A) and phenylalanine (F). The absence of the N-terminal M is a common post-translational modification. The absence of the C-terminal AF is due to the small size of the peptide (2 amino acids) resulting from the tryptic digest, that would not be highly visible in the spectroscopic analysis. Therefore, coverage of the peptide mapping was 99%. Mass spectrometry of the undigested protein determined that the mass of the full *Pichia* expressed protein was 15384.04 Da.

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  1   -GAFTEKQEA LVSSSFEAFK ANIPQYSVVF YTSILEKAPA AKDLFSFLSN GVDPSNPKLT
 61   GHAEKLFGLV RDSAGQLKAN GTVVADAALG SIHAQKAITD PQFVVVKEAL LKTIKEAVGD
121   KWSDELSSAW EVAYDELAAA IKKAF
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Figure 2: Tryptic peptide map of the leghemoglobin in LegH Prep. Trypsin is an enzyme that digests proteins after lysine (K) residues. These residues are shown in red. The mapping provided sequence coverage of 99%.

As a control for the identification of the protein and mass determination, the three main isoforms of leghemoglobin were isolated from the root nodules of soybean. After purification by column chromatography, the mass spectrum for the three isoforms were identified and the mass of the leghemoglobin C2 protein expressed in soybean was 15384.04 Da.

The observed masses for the *Pichia* and plant-extracted leghemoglobins are the same but differ to the expected mass (15515.08 Da). Taking into consideration the missing N-terminal M in the expressed protein, an adjusted theoretical mass was calculated[2] as 15384.04 Da, which agrees with that observed. This data also indicates that the leghemoglobin expressed in *Pichia* has not undergone post-translational modification, as the observed mass is equivalent to the expected mass.

Mass spectrometry also identified a major peak with a mass of 616.1752 Da, that matches the expected peak for haem B. This major peak was co-associated with other isotopic forms, ranging from 614-619 Da. Further analysis using UV-visual spectroscopy showed that haem dissociated from the soy leghemoglobin when the protein was denatured.

2.3.2 Safety of the expressed leghemoglobin protein – potential allergenicity

Leghemoglobin is normally expressed in the part of the soybean plant (root nodules) that is not typically consumed by humans therefore it is a new protein to the diet. As discussed in

Section 2.2, leghemoglobin has structural similarity to myoglobin, a commonly ingested protein that is not associated with allergic reactions in humans.

To assess potential allergenicity, consideration must be given to whether the new protein is one which certain individuals may already be sensitive as well as whether a protein new to the diet is likely to induce allergic reactions in some individuals (Codex, 2009). There are presently no reliable animal models for the assessment of allergenicity, and no single test that can be applied to a protein to predict whether it is likely to be allergenic to humans. Instead, the assessment follows a step-wise, weight of evidence approach that considers:

a) source of the new protein
b) similarity of the new protein amino acid sequence to known allergens
c) structural properties:
      i. susceptibility to enzymatic degradation
     ii. thermal stability
    iii. acid stability

If the new protein is from a source known to be allergenic, consideration may also be given to specific serum screening using sera obtained from individuals with a clinically validated allergy to the source of the protein as well as oral challenge studies with allergic individuals.

Source of the new protein

The leghemoglobin component of the LegH Prep is sourced from the soybean plant *G. max.* which is known to be allergenic to humans. Certain products derived from soybean require a mandatory allergen warning label[5] in Australia and New Zealand, due to soy being one of the eight common food allergen sources. Some soy products (e.g. refined soybean oil) are exempt from allergen labelling because they have been processed in a way that makes them safe for soy allergic individuals.

The examination of soy allergic individuals have identified allergen specific immunoglobulin E (IgE) antibodies that bind to proteins found in soybean (Ogawa et al. 1993; Holzhauser et al. 2009; Ito et al. 2011). To date, there have been 42 proteins identified in the seed that react to IgE antibodies (AllergenOnline database[6]) but only a portion of these proteins have been confirmed as clinically relevant to humans (Selb et al. 2017).

Similarity of the leghemoglobin amino acid sequence to known allergens

The applicant provided results of *in silico* analyses comparing the *Pichia*-expressed soy leghemoglobin sequence (Figure 2) to known allergenic proteins listed in the Food Allergy Research and Resource Program (FARRP) dataset, which is available through AllergenOnline[7] (University of Nebraska). At the date of the search, there were 1,956 sequences in the allergen database. This broad ranging database includes all the known allergens from legumes, including soybean and peanut. The methodology followed the approach recommended in the Codex guidelines for foods derived from modern biotechnology (Codex 2009).

The results from the analyses identified no similarity between the leghemoglobin protein and known allergenic proteins. The results from these analyses have been published by Jin et al. (2018).

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[5] www.foodstandards.gov.au/consumer/foodallergies/Pages/Allergen-labelling.aspx
[6] www.allergenonline.org/databasebrowse.shtml
[7] www.allergenonline.org

Structural properties

To assess the susceptibility to enzymatic degradation, *Pichia*-produced soy leghemoglobin (test protein) from production strain MXY0541, was incubated with pepsin at a concentration of 10.6U enzyme/µg protein. The incubation was performed at 37°C for 0-60 m, in a simulated gastric fluid system at pH 2.0 (Thomas et al. 2004; Ofori-Anti et al. 2008). Controls included a no enzyme control (test protein only) and a no protein control (enzyme only), incubated for 0 and 60 m. The extent of digestion was visualised by Coomassie staining of the protein run on a tris-glycine SDS-PAGE gel. An LOD was not determined for this test system but samples from the digestion mixes were analysed by liquid chromatography combined with mass spectrometry and the resulting peptides identified.

The results from the pepsin digestions showed that by 2 m there was no visible soy leghemoglobin remaining in the digestion mix. There was no loss of band intensity in the no enzyme control incubated for 60 m therefore the loss of protein in the reaction mix indicated that leghemoglobin was being fully digested by pepsin. Additional evidence was provided through the analysis of the resulting peptides. A total of 49 different peptide fragments were identified across 3 digestion samples, ranging from 5-16 amino acids in length. The majority of the fragments were between 8-11 amino acids. Analysis of the peptide data confirmed that by 2 m soy leghemoglobin is fully digested by pepsin in a simulated gastric fluid assay. These data support the results obtained for the soy leghemoglobin from the MXY0291 production strain (Jin et al. 2018).

In order to assess thermal stability, the applicant provided results from a melt curve analysis. The test sample was the leghemoglobin in the LegH Prep. Control samples were horse myoglobin and chicken egg lysozyme. The data shows that leghemoglobin denatures at 64°C. This indicates that at standard grilling and cooking temperatures, the leghemoglobin protein will be fully denatured.

The applicant also provided results examining acid stability by looking at the dissociation of haem by UV-visual spectroscopy. Examination of the Soret region at pH 7 and pH 2, showed the absorbance decreased at the acidic pH. This reduced absorbance demonstrates the release of the haem prosthetic group due to denaturation of the protein. These data indicate that at an acidic pH that would be found in the stomach, leghemoglobin is susceptible to acidic denaturation.

Specific serum screening

Serum screening is an *in vitro* test that allows examination of cross-reactivity of the soy-allergen IgE antibodies from an allergic patient to the leghemoglobin protein. This test requires collection of sera from a range of patients, which is an invasive technique. FSANZ does not consider there would be any additional value in undertaking serum screening given the findings from the weight of evidence approach outlined above. These studies, showing soy leghemoglobin shares no amino acid sequence similarity to known allergens and is readily digested by pepsin, indicates a low likelihood of cross-reactivity between the protein and IgE antibodies of allergic individuals. Moreover, there is an absence of evidence linking haem-proteins with allergenic reactions in humans.

2.3.3 Safety of the expressed leghemoglobin protein – potential toxicity

To assess potential toxicity, a weight of evidence approach is employed (Codex, 2009) that considers:

a) similarity of the new protein amino acid sequence to known toxins
b) information on the stability of the protein to enzyme degradation
c) animal toxicity studies if the protein shares significant similarity to known toxins or resistance to proteolysis or if it is not similar to proteins that have previously been consumed safely in food

Similarity of the leghemoglobin amino acid sequence to known toxins

The full length sequence for soy leghemoglobin (Figure 2) was compared to sequences in the NCBI-Entrez database designated as "toxin" or "toxic" . No results were returned when using the keyword "toxic" and an *E* score set to less than 10. Two proteins were returned using the keyword "toxin", with sequence identity of 35% and an *E* score of less than 0.1 however, the alignments of these proteins to leghemoglobin was short (31%). Subsequent analysis identified that the homologous proteins are from toxin-producing bacteria but that the proteins themselves are not toxins. It was concluded that the soy leghemoglobin protein does not share similarity to known protein toxins (Jin et al. 2018).

Stability of the protein to enzyme degradation

An analysis of the stability of the protein to enzyme degradation has already been provided in the *Potential Allergenicity* discussion (Section 2.3.2).

Animal Toxicity studies

A range of toxicity assessments were performed by the Applicant and the analyses are provided in Section 2.4.

2.3.4 Safety of the native *Pichia* proteins present in the LegH Prep

The LegH Prep that is the focus of this study, exists as a partially purified cell lysate containing both the novel soy leghemoglobin and native *Pichia* proteins. A comparison of different batches of LegH from MXY0291 and MXY0541, using Coomassie stained protein gels, found several *Pichia* proteins with similar molecular masses that were consistently present. FSANZ notes that not all preparations contained the same proteins, nor proteins at the same levels. This is expected because the proteins that are present in cells would be dependent on the cell cycle stage of each cell, at the time the fermentation run was completed.

The identity of the *Pichia* proteins consistently present in the preparation was determined by mass spectrometry. The amino acid sequences of these proteins were then subjected to bioinformatic analyses, looking for similarity to known toxins and allergens. The data indicates there are no similarities between the proteins identified and known toxins or allergens. These data pertaining to the *Pichia* proteins identified in the MXY0291 preparations were also included in the publication by Jin et al. (2018).

2.3.5 Conclusion

Biochemical analysis of the novel soy leghemoglobin showed the protein expressed was equivalent to that found in the host plant. The molecular mass of the protein was as expected, it was present as a holoprotein and had not undergone post-translational

modification, such as glycosylation. The protein was also shown to be denatured when exposed to heat and acidic conditions.

No safety concerns were identified regarding the potential allergenicity or toxicity of soy leghemoglobin or the *Pichia* proteins. There were no significant similarities between the protein and known allergens or toxins. All the novel proteins were also shown to be susceptible to pepsin digestion and were denatured at standard cooking temperatures and in acidic conditions that mimic the stomach environment.

2.4 Toxicological assessment of LegH Prep

The applicant submitted reports of dietary toxicity studies in rats and *in vitro* genotoxicity studies. The LegH Prep used in these studies was produced using the initial MXY0291 production strain. This test item is considered to be relevant to the assessment of LegH Prep produced by the MXY0541 strain because the expressed soy leghemoglobin protein is equivalent in both strains, several of the Pichia proteins expressed in LegH Prep from MXY0541 are also present in LegH Prep from MXY0291, and the composition of LegH Prep from each strain meets the same specifications.

The Joint Food and Agriculture Organization (FAO)/World Health Organisation (WHO) Expert Committee on Food Additives (JECFA) has previously considered the safety of an enzyme preparation produced by a genetically modified form of *P. pastoris*, phospholipase C, in 2008 (WHO 2009). JECFA noted that *P. pastoris* is a methylotropic yeast which is not known to be associated with disease in humans or animals. The enzyme preparation was found to be free of antimicrobial activity and mycotoxins, was not genotoxic *in vitro* or *in vivo*, and did not cause adverse effects in a 13-week oral toxicity study in rats at doses up to 2000 mg/kg bw/day (1672 mg/kg bw/day on a TOS basis).

2.4.1 Toxicity studies in animals

The applicant submitted reports of three repeated dose dietary toxicity studies with LegH Prep conducted in rats. The results of these studies were also reported in the publication by Fraser et al. (2018). The LegH Prep test article used in all three studies was freeze-dried to enable an increased concentration of soy leghemoglobin in the feed, and to facilitate homogenous mixing with the diet.

14-day dietary toxicity/palatability study in rats (Product Safety Labs 2016a) Regulatory status: Non-GLP; conducted in accordance with OECD TG 407 (2008) and US FDA Redbook 2000, IV. C. 4. a. (2007a)

CRL Sprague Dawley CD® IGS rats (6/sex/group), aged 7 – 8 weeks, were administered freeze-dried LegH Prep (47.6% soy leghemoglobin, Lot no. PP-PGM2-16-081-302) in the diet at concentrations targeting soy leghemoglobin doses of 0, 125, 250 or 500 mg/kg bw/day for 14 days. Stability of the test substance and homogeneity in the diet were evaluated and confirmed. Clinical signs were monitored daily and weekly detailed observations were performed. Body weight and food consumption were monitored at intervals throughout the study. At study termination blood samples were collected for haematology and animals were killed and subjected to a gross necropsy. The liver and spleen were weighed and the liver, spleen and femur bone marrow were subjected to histopathological examination.

No mortalities occurred during the course of the study. In-life clinical signs were limited to discolouration of the urine in 6/6 males and 5/6 females administered the high dose on study day 5 only. This finding was not considered to be treatment related or of toxicological significance in the absence of a correlation with clinical haematology. Mean weekly body

weights and body weight gains in all treated groups were similar to those of controls. Food consumption and food efficiency were similar in all groups, with the exception of significantly lower food consumption between study days 0 – 14 in females given the low and mid-doses. Absolute and relative (to body weight) weights of the liver and spleen were similar to those of controls. Numbers of white blood cells, neutrophils and lymphocytes in males were >25% lower than those of controls, but no dose response was observed at the lower doses and the study authors considered there were no changes in haematology parameters attributable to administration of LegH Prep. No treatment related macroscopic or microscopic changes were observed in the liver, spleen and bone marrow. It was concluded that doses of at least 500 mg/kg bw/day soy leghemoglobin would be tolerated by rats in studies of longer duration.

28-day dietary toxicity study in rats (Product Safety Labs 2017a) Regulatory status: GLP; conducted in compliance with OECD TG 407 (2008) and US FDA Redbook 2000, IV. C. 4. a. (2007a)

CRL Sprague Dawley CD® IGS rats (10/sex/group), aged 7 – 8 weeks, were administered freeze-dried LegH Prep (48.82% soy leghemoglobin; Lot no. PP-PGM2-16/088-301; Total Organic Solids [TOS] content 92.5%) via the diet at concentrations targeting doses of 0, 512, 1024 or 1536 mg/kg bw/day LegH Prep for 28 days. These doses corresponded to 0, 474, 947 and 1421 mg/kg bw/day on a TOS basis. Stability of the test substance and homogeneity in the diet was evaluated and confirmed. Body weight and food consumption were monitored at intervals throughout the study. Clinical signs were monitored daily, with detailed observations made weekly. Ophthalmologic evaluations were performed in all animals before study initiation and again on study day 23. Blood and urine samples were collected for haematology, blood coagulation, clinical chemistry and urinalysis during the final week of the study. At study termination, animals were killed and subjected to gross necropsy. Organs and tissues were collected and weighed. Histopathological examination was performed on organs and tissues from animals in the control and high dose groups. Based on observations in the uterus, female reproductive organs from animals in all four groups underwent histopathology. Apeer review of the histopathology of the female reproductive organs was also performed.

No mortalities were observed, and there were no clinical signs attributable to administration of the test item. Mean body weights, body weight gains, food consumption and food efficiency from study days 0 – 28 were similar in all groups. A transient significant reduction in mean daily body weight gain (p < 0.01) and mean food efficiency (p < 0.01) was found between days 7 – 14 in females administered LegH Preparation at 512 mg/kg bw/day.Significant increases in food consumption (p < 0.05-0.01) in males given 1024 mg/kg bw/day and 1536 mg/kg bw/day were observed on days 7 – 14 and 7 -10, respectively. These findings were not considered to be of toxicological relevance given that overall body weight gains and food efficiency over the course of the study were similar to controls and there was no dose-response. Absolute and relative organ weights in all treated groups were similar to controls, with the exception of decreased absolute and relative (to body weight and brain weight) uterus weights in treated females, which were statistically significant (p < 0.05-0.01) in the low and high dose groups. No treatment-related changes in ophthalmology, haematology, clinical chemistry or urinalysis parameters were observed. A small number of statistically significant changes in haematology (Table 2) and clinical chemistry (Table 3) values were observed, but these were not considered to be treatment-related as they were of a small magnitude, did not show a dose-response and were only seen in one sex. Changes in coagulation parameters were limited to a slight increase in activated partial thromboplastin time in males given 1024 and 1536 mg/kg bw/day compared with controls (24.9, 23.9 and 20.2 seconds, respectively, p < 0.05). This change was not considered adverse as the increase did not show a dose-response, the magnitude was slight and there were no correlated pathological or clinical changes.

Table 2: **Haematology values for which statistically significant changes were observed**

Parameter	0 mg/kg bw/day	512 mg/kg bw/day	1024 mg/kg bw/day	1536 mg/kg bw/day
		Females		
Red blood cells (x10^6/μL)	7.59 ± 0.24	8.01 ± 0.38*	7.86 ± 0.24	7.63 ± 0.3
Haemoglobin (g/dL)	15.3 ± 0.5	16.2 ± 0.5*	15.7 ± 0.4	15.5 ± 0.6
Haematocrit (%)	43.6 ± 1.2	45.9 ± 1.2	44.7 ± 1.3	44.0 ± 1.7
Absolute basophil count (x10^3/μL)	0.04 ± 0.01	0.07 ± 0.03*	0.06 ± 0.03	0.05 ± 0.04
Absolute Reticulocyte count (x10^3/μL)	205.8 ± 33.9	182.4 ± 32.9	169.1 ± 30.9*	184.2 ± 33.7

*$p < 0.05$, Dunnett's test

Table 3: **Clinical chemistry values for which statistically significant changes were observed**

Parameter	0 mg/kg bw/day	512 mg/kg bw/day	1024 mg/kg bw/day	1536 mg/kg bw/day
		Males		
Albumin (g/dL)	3.1 ± 0.1	3.2 ± 0.1	3.3 ± 0.1*	3.2 ± 0.1
Potassium (mmol/L)	5.03 ± 0.25	5.19 ± 0.26	5.55 ± 0.61*	5.10 ± 0.25
		Females		
Alkaline phosphatase (U/L)	137 ± 16	107 ± 19*	121 ± 29	108 ± 25*
Glucose (mg/dL)	118 ± 15	103 ± 10*	104 ± 10*	110 ± 14
Globulin (g/dL)	2.9 ± 0.1	3.1 ± 0.2	3.1 ± 0.2*	3.0 ± 0.1
Calcium (mg/dL)	10.5 ± 0.3	10.9 ± 0.3*	11.0 ± 0.3*	10.7 ± 0.4
Chloride (mmol/L)	102.6 ± 1.2	101.3 ± 1.4*	101.1 ± 1.0*	102.1 ± 1.1

*$p < 0.05$, Dunnett's test

No macroscopic or microscopic findings related to administration of the test substance were observed. The presence of fluid-filled uteri, typically associated with the proestrus/estrus stage of the oestrous cycle, was decreased in treated female rats compared with controls. The incidence of animals in metestrus was not significantly different between groups. Histopathological examination of the female reproductive organs found a decreased incidence of dilated uterine lumens in low and high dose rats compared with controls. Uteri were dilated in 4/10 control females, which was consistent with estrus. There were no females with dilated uterine lumens in the low and high dose groups, and 2/10 in the mid-dose group, which correlated with lower incidences of animals in the proestrus/estrus stage of the estrus cycle compared with controls. Rats given the low or high dose tended to be in the metestrus stage of the oestrous cycle, which correlated with the lower uterine weights observed in these groups. This was considered to be an unusual distribution, but the presence of both new and old corpora lutea in females from all groups indicated that females were cycling normally and there were no treatment-related effects on the estrus cycle. The pathology peer review of reproductive organs from all female animals concurred with this conclusion.

The no observed adverse effect level (NOAEL) in this study was 1536 mg/kg bw/day LegH Prep, the highest dose tested. Based on a TOS content of 92.5%, this corresponds to 1421 mg/kg bw/day TOS.

Investigative 28-day dietary study in female rats with a 14-day pre-dosing oestrous cycle determination (Product Safety Labs 2017b) Regulatory status: Non-GLP; conducted in accordance with OECD TG 407 (2008) and US FDA Redbook 2000, IV. C. 4. a. (2007a)

Female CRL Sprague Dawley CD® IGS rats (15/group), aged 7 – 8 weeks, were examined for the potential effects of LegH Prep on the oestrous cycle and reproductive organ histopathology. Prior to dosing, the oestrous cycle was determined in all rats for 14 days (study days 0-13) by vaginal lavage. On study day 14, animals were administered diets containing target doses of 0, 512, 1024 or 1536 mg/kg bw/day LegH Prep (48.82% soy leghemoglobin; Lot no. PP-PGM2-16/088-301) for 28 days. These doses corresponded to 0, 474, 947 and 1421 mg/kg bw/day on a TOS basis. Homogenous distribution of the test substance in the diet was confirmed. Clinical signs were monitored daily, with detailed clinical observations made weekly during the test substance exposure period. Body weight, body weight gain, food consumption and food efficiency were monitored periodically throughout the study. Oestrous cycling was evaluated by vaginal lavage on study days 29-42 and at termination on day 43. At the end of the study, animals were killed and the uterus and ovaries (with oviducts) from all animals were weighed. Oestrous cycle stage at study termination was also determined in all animals by blinded evaluation of the anterior portion of the vagina, the cervix, the uterine bifurcation and horns, the oviducts and the ovary. Reproductive organs and tissues (ovaries, oviducts, uterus, cervix and anterior-most portion of the vagina) from the control and high dose groups were evaluated microscopically.

No mortalities occurred during the course of the study, and no treatment-related clinical signs were observed. Body weight, body weight gain, food consumption and food efficiency were similar to controls in all treated groups. The mean number of oestrous cycles for rats in the treatment groups were similar to controls both prior to and during test substance administration. No test substance-related changes in organ weights were observed. Microscopic analysis of the oestrous cycle at study termination found that all animals were cycling normally with the exception of one animal given the low dose, which appeared to have a prolonged estrus. This finding was considered to be incidental because of the lack of similar findings in animals at the higher dose levels. No treatment-related macroscopic or microscopic changes were observed in the organs and tissues examined.

It was concluded that under the conditions of this study administration of LegH Prep at doses up to 1536 mg/kg bw/day did not affect the oestrous cycle of female Sprague Dawley rats. Based on a TOS content of 92.5%, this corresponds to 1421 mg/kg bw/day TOS.

2.4.2 Genotoxicity studies

Two *in vitro* genotoxicity studies were submitted, a bacterial reverse mutation assay (Ames test) and a chromosomal aberration test in human lymphocytes. These studies used the standard liquid formulation of LegH Prep. The results of these studies were also included in the publication by Fraser *et al.* (2018).

Bacterial reverse mutation assay (Product Safety Labs 2016b) Regulatory status: GLP; conducted in compliance with OECD TG 471 (1997), US FDA Redbook 2000, IV. C. 1. a. (2007b) and Commission Regulation (EC) No 440/2008 B.13/14

The test article for this study was LegH Prep (Batch No. PP-PGM2-16-015-101; purity 6.74% soy leghemoglobin[8]) and the vehicle control was water. Test systems for this assay were *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100, and *Escherichia coli* strain WP2 *uvr*A. For assays conducted without S9 mix for metabolic activation, positive control articles were sodium azide for TA 1535 and TA 100, ICR 191 Acridine for TA157, daunomycin for TA98 and methyl methane sulfonate for WP2 uvrA. For assays conducted with addition of S9 mix for metabolic activation, 2-aminoanthracene in DMSO was used as the positive control for all test strains. The main test was conducted in triplicate with and without metabolic activation using the plate incorporation method, and results were confirmed by a second test using the pre-incubation method. Concentrations of LegH Prep in both tests were 23.384, 74, 233.84, 740, 2338.4, 7400, 23,384 and 74,000 µg/plate, corresponding to soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580 and 5000 µg/plate.

For the plate incorporation test, test solution (100 µL), 500 µL S9 mix or substitution buffer, 100 µL bacterial solution and 2.0 mL of overlay agar were mixed and poured on minimal agar plates. After the agar set, plates were incubated for approximately 65 hours at 37°C and then assessed for numbers of revertant colonies and effects on the growth of the bacterial lawn. For the confirmatory pre-incubation test, the same volumes of test solution, S9 mix or substitution buffer and bacterial solution were incubated under agitation for approximately 30 minutes at 37°C prior to mixing with the overlay agar, pouring onto the plates and proceeding as described for the plate incorporation test.

The test article showed no evidence of precipitation or toxicity, and there was no increase in revertant colonies compared with vehicle controls, with or without S9 mix in either test. A substantial increase in revertant colonies was observed in the presence of the positive controls, confirming the validity of the test system.

It was concluded that LegH Prep was not mutagenic under the conditions of the study.

Chromosomal aberration assay in cultured human lymphocytes (Eurofins 2017) Regulatory status: GLP; conducted in compliance with OECD TG 473 (2014) and Commission Regulation (EC) No 440/2008 B.10

The test article for this study was LegH Prep (Batch No. PP-PGM2-16-015-101; purity 6.74% soy leghemoglobin[8]) and the vehicle control was cell culture medium. The test system comprised human peripheral blood lymphocytes collected from a healthy non-smoking donor with no known recent exposure to genotoxic chemicals and radiation. Lymphocytes were pre-cultured in the presence of the mitogen phytohaemagglutinin for 48 hours prior to the start of exposure. Following a preliminary cytotoxicity test two experiments were conducted. In

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[8] The study sponsor provided the test laboratory with information indicating that the test article contained 6.74% soy leghemoglobin and this value was used to calculate study concentrations. Product Safety Laboratories prepared a GLP certificate of analysis and found soy leghemoglobin at 6.68%. This value was considered to be within an acceptable margin of error.

experiment I cells were exposed to the test substance in the presence or absence of metabolic activation (S9 mix) for 4 hours, then washed and cultured in fresh medium for a further 20 hours. In experiment II cells were exposed to the test substance for 24 hours in the absence of S9. Cells were cultured in duplicate. Parallel cultures of the negative controls and highest concentrations were treated in the presence of BrdU in order to determine the proliferation index. Positive controls were cyclophosphamide and ethylmethanesulfonate in the presence and absence of metabolic activation, respectively. Two hours prior to harvest, Colcemid® was added to the cultures to arrest cells in metaphase. At harvest cells were stained and 300 metaphases per concentration were evaluated for the presence of chromosomal aberrations.

Concentrations were reported as the concentration of soy leghemoglobin. In experiment I without S9, mitotic indices lower than 70% of control were observed at 1000 µg/mL (69%), 2500 µg/mL (56%) and 5000 µg/mL (54%). In experiment I with S9, no reductions in mitotic index below 70% of control were observed. In experiment II, mitotic indices below 70% of control were seen at 500 µg/mL (69%), 1000 µg/mL (53%), 2000 µg/mL (26%), 3000 µg/mL (13%), 4000 µg/mL (38%) and 5000 µg/mL (42%). Concentrations evaluated for chromosomal aberrations were 500, 1000, 2500 and 5000 µg/mL in experiment I with and without S9. Concentrations of 100, 200, 500 and 1000 µg/mL were evaluated in experiment II, due to the substantial cytotoxicity at concentrations ≥ 2000 µg/mL. No precipitation was observed in experiment I. Precipitation was observed at concentrations ≥ 500 µg/mL in experiment II, but this did not affect the evaluation of aberration rates.

No significant increases in the percentage of cells with chromosomal aberrations compared with controls were observed. In experiment I, with and without S9, some concentrations had aberration rates slightly higher than the laboratory's historical negative control ranges, but these were not considered to be relevant due to the negative control also being slightly higher than the historical negative control range and/or a lack of a dose-response. No significant increases in the number of polyploid cells were observed. A distinct increase in the percentage of structurally aberrant cells was observed in all the positive control cultures, confirming the validity of the assay.

It was concluded that LegH Prep was not clastogenic in human lymphocytes.

2.4.3 Conclusion

LegH Prep was not genotoxic *in vitro*, and did not cause adverse effects in short-term toxicity studies in rats. The NOAEL in a 28-day repeated dose dietary toxicity study in rats was the highest dose tested, 1536 mg/kg bw/day or 1421 mg/kg bw/day on a TOS basis.

2.5 Nutritional impact

2.5.1 Introduction

Dietary iron is an essential micronutrient that is required for a range of cell functions. Iron can be found in several proteins including haemoglobin which transports oxygen for cell respiration; myoglobin which acts as an oxygen reserve in muscle cells; cytochromes which are involved in metabolism; transferrin, an iron transport protein; and ferritin and haemosiderin that store iron in the body. Iron can exist in two interchangeable oxidation states that allow it to reversibly bind to ligands such as oxygen, nitrogen and sulphur.

The iron content of the body is highly conserved, with only a small amount being lost each day in the absence of bleeding (Bothwell et al. 1979). The estimated average requirement (EAR) for iron is 6 mg/day for adult males, 8 mg/day for women to age 50 and 5 mg/day thereafter, and 4–8 mg/day for children aged 1–18 years. The EAR during pregnancy is

23 mg/day (age 14–18 years) and 22 mg/day (age 19–50 years) (NHMRC and MoH 2006).

The two main forms of dietary iron are haem and non-haem iron. Iron from animal tissue including meat, fish and poultry may be haem or non-haem, with haem iron contributing approximately 55–70% of the total iron content (Lombardi-Boccia et al. 2002). Most iron from plant sources, including cereals and legumes, is non-haem iron. Although plants do contain small quantities of haem iron they are not considered to be a major source of haem iron in the diet.

2.5.2 Bioavailability of iron from soy leghemoglobin

As discussed previously (Section 2.2 and 2.3.2) soy leghemoglobin is structurally similar to myoglobin; is completely digested by pepsin; and the haem moiety of leghemoglobin is released at pH 2, which is within the pH range in the stomach. The applicant provided data indicating that soy leghemoglobin and equine myoglobin denature at 64°C and 69°C, respectively. Published studies have reported that most of the myoglobin (approximately 85%) in beef samples denatures at 70°C (Trout 1989; Hunt et al. 1999). Therefore soy leghemoglobin has similar thermal stability properties to bovine myoglobin.

Several factors contribute to the bioavailability of iron including other dietary constituents of a meal, the form of iron (e.g haem or non-haem), physiological iron status, exercise, and age. Iron absorption is regulated by the body, increasing in individuals that are iron deficient and decreasing in cases of iron overload.

The bioavailability of non-haem iron can be decreased by dietary factors such as phytates from grains and legumes, tannins from spices, fruits, vegetables or tea, calcium, and phosphates (Hurrell and Egli 2010), with phytate being the primary inhibitor (Hallberg et al. 1987). The insolubility of complexes formed between iron, phytate and proteins is considered to be the major reason for phytate inhibition of iron absorption (Champagne 1988). However, the presence of meat, fish, poultry or ascorbic acid (vitamin C) enhances the bioavailability of non-haem iron and have been found to counteract the effect of inhibitors such as phytates and tannins (Lopez and Martos 2004).

In general, haem iron is more bioavailable than non-haem iron. It has been estimated that 15–25% of haem iron is absorbed compared to 5–12% of non-haem iron (Hallberg 1983), and that although haem iron contributes 10–15% of the total dietary iron intake of omnivores, it represents at least 40% of the total absorbed iron (Björn-Rasmussen et al.1974).

Similar to non-haem iron, other dietary components can affect the absorption of haem iron. For example, calcium has been shown to inhibit haem iron absorption at intake levels greater than 40 mg (Hallberg 1998), while the presence of meat enhances haem iron absorption (Carpenter and Mahony 1992). The reason for the improved bioavailability of both haem and non-haem iron due to the presence of meat may be due to the binding of peptides formed from digestion of meat which prevent the formation of insoluble iron aggregates that are poorly absorbed (Lopez and Martos 2004). The interaction of haem with peptides produced from the proteolytic digestion of globin is also thought to prevent the formation of insoluble haem polymers (Hooda et al. 2014).

The applicant provided a published study to support the bioequivalence of haem iron from soy leghemoglobin and bovine haemoglobin (Proulx and Reddy 2006). This in vitro study used Caco-2 cells to compare the iron bioavailability of crude and purified soy leghemoglobin compared to bovine haemoglobin. Caco-2 cells are a human intestinal adenocarcinoma cell line that exhibit enterocyte-like properties and have been previously used for non-haem iron bioavailability studies (Au and Reddy 2000; Yun et al. 2004).

Experiments were undertaken with and without a food matrix. The change in ferritin concentration ("ferritin response") was used as an index of iron bioavailability (Glahn et al. 1998). In the food matrix experiment, corn tortillas were prepared using unfortified tortilla flour and either soy root nodule (SRN) extract, partially purified soy leghemoglobin (LHbA), bovine haemoglobin (BHb) or ferrous sulphate. Tortillas were cooked and lyophilised. Gastric and intestinal digestion was then simulated for all samples. The non-food matrix test solutions contained either SRN, purified soy leghemoglobin (LHbD), BHb or ferrous sulphate. Caco-2 cells were incubated with test solutions for 24 h, lysed and ferritin concentration was calculated as percentages compared to ferrous sulphate.

The results of the non-food matrix experiment found that the ferritin response for BHb was approximately 2-fold higher than all other samples. No statistically significant difference was observed between the ferritin responses for SRN, LHbD and ferrous sulphate ($P > 0.05$).

For the food matrix experiment, the LHbA and BHb enriched tortillas exhibited similar ferritin responses that were $27 \pm 6\%$ and $33 \pm 10\%$ higher, respectively, than the ferrous sulphate response ($P < 0.05$). The ferritin response for SRN was 19% lower than for ferrous sulphate, although it was not statistically significant ($P > 0.05$). Heat treatment had no effect on the ferritin response with either LHbA or BHb.

The results of this in vitro study provide supporting evidence that the bioavailabilities of soy leghemoglobin and bovine hemoglobin are likely to be similar, however no in vivo studies of bioavailability were provided in the application. FSANZ searched Pubmed using the search terms "((leghemoglobin or leghaemoglobin) and (bioavailability or absorption))" and no additional publications on the bioavailability of haem iron from soy leghemoglobin were located.

Bioavailability of haem iron from soy leghemoglobin in an Impossible Foods meat analogue product

As previously discussed, soy leghemoglobin is a haem protein with structural similarities to animal myoglobins. In general haem iron is more bioavailable than non-haem iron, however it is usually consumed as part of a meal containing meat and the meat component has been shown to enhance the bioavailability of haem iron.

The evidence is conflicting on the effect of plant proteins similar to those that would be present in the proposed Impossible Foods meat analogue products on haem iron bioavailability and there is limited research in this area. Some studies have found that partial substitution of meat with soy protein increased the bioavailability of haem iron (Lynch et al. 1985) while others have reported that the absorption of haem iron from meat-free meals is half that of haem in a meal with meat when protein content is constant (Hallberg et al. 1979). A more recent study found that the addition of cereal, pea or lentil protein to haem iron did not affect iron absorption but soy protein had an inhibitory effect. However the relevance of this study is unclear as the haem iron used in the study was dissociated from globin and pure haem iron has low solubility at low gastric pH (Vaghefi et al. 2002; Weinborn et al. 2015).

2.5.3 Conclusion

Approximately 15–25% of haem iron in the diet is absorbed compared to 5–12% of non-haem iron. Iron absorption is regulated by the body, increasing in individuals that are iron deficient and decreasing in cases of iron overload.

Data provided in the application indicates that soy leghemoglobin has similar physicochemical properties to animal myoglobins. No relevant in vivo studies on bioavailability were available, however an in vitro study provides supporting evidence that the

bioavailabilities of soy leghemoglobin and bovine haemoglobin are likely to be similar.

Haem iron from soy leghemoglobin is expected to have similar bioavailability to haem iron from mammalian haem proteins (e.g. myoglobin present in muscle tissue). The absence of meat proteins in the proposed meat analogue product may decrease haem iron bioavailability. However, because the proposed meat analogue products have a higher total iron content relative to comparison meat products, FSANZ considers that meat analogue products containing soy leghemoglobin do not present a nutritional disadvantage to consumers in Australia and New Zealand.

2.6 Dietary intake assessment

2.6.1 Approach to estimating intakes of LegH Prep and iron

Dietary intake assessments require concentration data for the chemical of interest in food, and consumption data for those foods collected through national nutrition surveys.

Intakes were estimated in two ways. Firstly using the maximum concentration of 0.8% soy leghemoglobin requested by the applicant, and secondly using a more refined estimate based on the likely use levels of soy leghemoglobin (0.45% for beef analogue products and 0.25% for pork analogue products). Additionally, as soy leghemoglobin is a source of iron, potential increases in population iron intakes from consumption of meat analogue products containing soy leghemoglobin have also been estimated.

The data used in this assessment include:
- food consumption data from the available Australian and New Zealand national nutrition surveys (Australian Bureau of Statistics [ABS], 2014; Ministry of Health [MoH] 2005; MoH 2012)
- published (baseline) iron intakes derived from the Australian and New Zealand national nutrition surveys (ABS 2015a; ABS 2015b; MoH 2003; University of Otago and MoH 2011)
- iron concentrations in foods from the food composition datasets from the Australian and New Zealand national nutrition surveys (FSANZ 2016a; MoH 2005; MoH 2012).

Further details of FSANZ's approach to conducting the dietary intake assessment are at Appendix 1. This includes details of the:
- food consumption data used in the assessment (A1.1)
- concentration data used in the assessment (A1.2)
- assumptions and limitations of the dietary intake assessment (A1.3).

A detailed discussion of the FSANZ methodology and approach to conducting dietary intake assessments is set out in *Principles and Practices of Dietary Exposure Assessments for Food Regulatory Purposes* (FSANZ, 2009).

2.6.2 Estimated intakes of LegH Prep and iron

The applicant has requested permission to add soy leghemoglobin in the form of LegH Prep to meat analogue products at levels of not more that 0.8% soy leghemoglobin. The applicant has stated that the LegH Prep will be added to a meat analogue product at levels needed to recreate the flavour and aroma of meat, for example 0.45% soy leghemoglobin for beef analogue products and 0.25% soy leghemoglobin for pork analogue products.

There are no existing permissions for use of LegH Prep and soy leghemoglobin in foods on the market in Australia and New Zealand, therefore the estimated intakes of LegH Prep are

based only on intake from meat analogue products containing soy leghemoglobin at the levels proposed in the application. As there are existing sources of iron currently in the food supply, the estimated intakes for iron take into account the baseline intakes of iron from existing sources (including foods, beverages and supplements), as well as the amount that could be contributed by foods containing soy leghemoglobin at the levels proposed in the application.

It was assumed for the dietary intake assessment that consumers may choose to eat meat analogue products containing soy leghemoglobin in the same amounts they currently consume minced meat and poultry products, and vegetarian meat alternatives. The foods included in each of these food groups, which have been used to derive the consumption amounts for the assessment, are detailed in Appendix 1: Table A1.1. The mean and 90th percentile consumption amounts of these food groups, based on the most recent national nutrition survey data for Australia and New Zealand, are listed in Appendix 1 (Table A1.2 and Table A1.3).

FSANZ have conducted the dietary intake assessment of LegH Prep and iron using two scenarios:

- '*Maximum proposed use level*': all meat analogue products consumed contain the maximum proposed concentration of soy leghemoglobin at 0.8%.
- '*Likely use level*': beef, lamb and unspecified meat analogue products contain 0.45% soy leghemoglobin, and pork and poultry analogue products contain 0.25% soy leghemoglobin.

The estimated intakes of LegH Prep and iron based on the *'likely use level'* scenario more accurately reflect estimates of dietary intake over a long period of time or lifetime, reflecting chronic dietary exposure.

The population groups assessed are listed in Table 4 and Table 5. In regards to LegH Prep, the toxicological assessment did not identify any population sub-groups for which there were specific safety considerations. Therefore the dietary intake assessment for LegH Prep was conducted for the general Australian and New Zealand populations based on the dietary survey data available. The population groups used for the iron intake assessment align with the baseline published iron intake data available for Australia and New Zealand. For Australia, the population groups used for the dietary intake assessment are the same as the Nutrient Reference Value (NRV)[9] age groups. The New Zealand survey age groups and the NRV age groups do not match because results from the New Zealand surveys were not reported exactly according to the NRV age group cut offs.

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[9] Nutrient reference values, Australia and New Zealand: https://www.nrv.gov.au/

Table 4: **Population groups used in the dietary intake assessment for LegH Prep**

Country	Nutrition survey	Age group	No. respondents (Day 1 only)	No. respondents (Day 1 and 2)
Australia	2011-12 NNPAS	2 years and above	Not used	7735
New Zealand	2002 NZ CNS	5 – 14 years	3275	n/a
	2008 NZ ANS	15 years and above	4721	n/a

Table 5: **Population groups used in the dietary intake assessment for iron**

Country	Survey	Population surveyed	Age groups analysed	Sex groups analysed
Australia	2011–12 NNPAS	2 years and above	2–3 years 4–8 years 9–13 years 14–18 years 19–30 years 31–50 years 51–70 years 71 years and over	Male and Female
New Zealand	2002 NZ CNS	5-14 years	5-6 years 7-10 years 11-14 years	Male and Female
	2008 NZ ANS	15 years and above	15–18 years 19–30 years 31–50 years 51–70 years 71 years and over	Male and Female

For the dietary intake assessment of iron, FSANZ first compared the baseline intakes of iron from food and beverages for meat-eaters with that of non-meat eaters in order to determine whether non-meat eaters should be considered as a separate population group (Appendix 2: Table A2.1). For the following reasons it was determined that it was not necessary to consider non-meat eaters as a separate population group to meat-eaters:

- the baseline mean iron intake of non-meat eaters was below that of meat eaters, and the purpose of estimating iron intakes was to consider potential increases in population iron intakes and whether these were above safe levels;
- the two scenarios used for the assessment are conservative and considered protective for estimating the iron intakes for meat eaters and non-meat eaters alike.

Estimated intake of LegH Prep

Dietary intake assessment results were calculated based on 'consumers only', that is, those people in the national nutrition survey who reported consuming minced meat and poultry products, and/or vegetarian meat alternatives. Population statistics (mean and 90th percentile) were derived from each individual's intake of soy leghemoglobin then converted to the corresponding amount of LegH Prep, based on the commercial preparation of LegH Prep containing up to 9% soy leghemoglobin, and having a TOS content of 22%. The TOS encompasses the protein component and other organic material derived from the protein source and manufacturing process.

The estimated intake of LegH Prep for the Australian and New Zealand population is shown in Table 6.

Table 6: **Estimated dietary intake of LegH Prep for Australia and New Zealand**

Country	*Population (consumers only)*	*Mean intake (g/day TOS)*		*P90 intake (g/day TOS)*		*Mean intake (mg/kg bw/day TOS)*		*P90 intake (mg/kg bw/day TOS)*	
		Maximum proposed use level	*Likely use level*	*Maximum proposed use level*	*Likely use level*	*Maximum proposed use level*	*Likely use level*	*Maximum proposed use level*	*Likely use level*
Australia[*]	2 years and above	1.2	0.7	2.7	1.5	20.4	11.0	45.4	24.3
New Zealand[^]	5-14 years	2.2	1.1	4.3	2.4	59.9	31.8	123.6	67.5
	15 years and above	2.3	1.2	5.0	2.8	29.3	15.5	65.1	35.3

[*] 2011-12 Australian National Nutrition and Physical Activity Survey. Based on consumption data from consumers with two days of data only.
[^] 2002 New Zealand National Children's Nutrition Survey and the 2008–09 New Zealand Adult Nutrition Survey. Based on day 1 consumption data for consumers only.

Iron content of meat analogue products containing soy leghemoglobin

Soy leghemoglobin is a source of iron which, if approved, will be added as an ingredient in meat analogue products that may already contain naturally occurring iron from other ingredients. The total amount of iron in a representative meat analogue product (i.e. the Impossible Burger) containing the likely use level of 0.45% soy leghemoglobin was provided in Table D.3-1, Section D of the application. In this same table the applicant compared the average amount of iron in the Impossible Burger to the typical amount of iron in raw minced meat and unprepared veggie or soy burgers, based on composition data from the USDA Food Composition Databases. Table 7 shows the amount of iron in the Impossible Burger and in minced meat and poultry products and other vegetarian meat alternatives, based on Australian and New Zealand food composition data (FSANZ 2016a; New Zealand Institute for Plant & Food Research Limited [PFR] and MoH 2019). As the amount of haem and non-haem iron contributing to the total iron content is not available in the food composition databases for Australia and New Zealand only total iron content is included in the table. The bioavailability of haem and non-haem iron is discussed within this document in section 2.5 Nutritional impact.

Table 7: **Amount of iron in meat analogue products containing soy leghemoglobin compared to minced meat and poultry products and other vegetarian meat alternatives**

Country	Food	Iron content (mg/100g)
Not applicable	Impossible Burger with 0.45% soy leghemoglobin (raw form)*	3.7
	Impossible Burger with 0.8% soy leghemoglobin (raw form)**	4.9
Australia^	Beef, mince, >10% fat, raw	1.45
	Lamb, mince, raw	1.77
	Pork, mince, raw	0.93
	Chicken, mince, raw	0.7
	Sausage, vegetarian style, added Fe, Zn and vitamin B12, raw	3.5
	Sausage, vegetarian style, unfortified, raw	2.5
	Tofu (soy bean curd), firm, as purchased	2.9
New Zealand$^\Sigma$	Beef mince, standard, 10-20% fat, raw	1.96
	Lamb, mince, standard, raw	1.04
	Pork, mince, raw	2.05
	Chicken, mince, raw, premium, Tegal	0.32
	Tofu, soy bean curd, regular, firm, raw	2.00
	Tempeh	2.70

*The expected amount of iron from soy leghemoglobin at use level 0.45% (see A1.2) in addition to the average amount of non-haem iron in 100 g of the Impossible Burger (2.1 mg) calculated from the non-haem iron content reported in Table E.1.1-1, Section E of the application.
** The expected amount of iron from soy leghemoglobin at the proposed maximum level of 0.8% soy leghemoglobin (see A1.2) in addition to the average amount of non-haem iron in 100 g of the Impossible Burger calculated from the non-haem iron content reported in Table E.1.1-1, Section E of the application.
^ AUSNUT 2011-13 Food Nutrient Database (FSANZ 2016a).
$^\Sigma$ New Zealand Food Composition Database (PFR and MoH 2019).

Estimated intakes of iron with contribution from soy leghemoglobin

The estimated mean and high intakes of iron with the additional contribution from soy leghemoglobin are summarised in Table 8 (Australia) and Table 9 (New Zealand). The food composition datasets from the Australian and New Zealand national nutrition surveys do not separate the amount of haem and non-haem iron in food and beverages, therefore only total iron was estimated.

The estimated amount of additional iron contributed by soy leghemoglobin (mean and 90th percentile) was derived from each individual's intake of soy leghemoglobin then converted to the corresponding amount of iron, based on 0.35% of the mass of the protein being iron (see Appendix 1: Section A1.2). The range of meat analogue products that would contain soy leghemoglobin, should the permission to add it be approved is unknown, therefore it was assumed that the amount of iron contributed by ingredients other than the soy leghemoglobin would be within the range of iron in minced meat and poultry products, and vegetarian meat alternative products currently on the market in Australia and New Zealand. In reality there would be variation in the actual amount of iron contributed by other plant-based ingredients in products containing soy leghemoglobin, however, this was unable to be taken into account for this assessment.

Therefore to estimate the total iron intake, the contribution of iron from soy leghemoglobin was added to baseline mean and high usual intakes of iron from food and beverages (ABS 2015a; MoH 2003; University of Otago and MoH 2011) and an additional amount from dietary supplements (e.g. vitamin and mineral tablets) (ABS 2015b). For the high usual intakes of iron from food and beverages, the highest reported percentile from the published usual intakes of iron from food and beverages were used for each country: 95th percentile intakes for the Australian population and 90th percentile intakes for the New Zealand population. The sources of iron that were combined to derive the estimated total intakes are shown in full in Appendix 2 (Table A2.2 and Table A2.3).

Table 8: **Estimated dietary intake of iron (mg/day) for the Australian population with additional contribution from soy leghemoglobin**

Age group (years)	UL*	Mean total intake¥		High total intakeΩ	
		Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level
Males					
2-3	20	12	12	17	16
4-8	40	13	13	19	18
9-13	40	18	17	25	23
14-18	45	20	18	28	26
19-30	45	23	22	32	30
31-50	45	22	21	31	29
51-70	45	18	17	27	25
71 and over	45	20	19	28	26
Females					
2-3	20	11	11	16	14
4-8	40	12	12	17	16
9-13	40	15	15	22	21
14-18	45	20	20	27	25
19-30	45	23	22	29	28
31-50	45	19	18	25	24
51-70	45	17	16	24	22
71 and over	45	16	16	23	22

*Upper Level of intake for iron based on the Nutrient Reference Values for Australia and New Zealand (NHMRC and MoH 2006).

¥Sum of mean intake from food and beverages, dietary supplements and soy leghemoglobin (ABS 2015a; ABS 2015b).

ΩSum of high intake from food and beverages, mean intake from dietary supplements and high intake from soy leghemoglobin (ABS 2015a; ABS 2015b).

Table 9: **Estimated dietary intake of iron (mg/day) for the New Zealand population with additional contribution from soy leghemoglobin**

Age group (years)	UL*	Mean total intake ¥		High total intake Ω	
		Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level
Males					
5-6	40	16	15	21	19
7-10	40	20	18	26	24
11-14	40-45**	22	21	31	27
15-18	45	22	20	31	27
19-30	45	27	25	39	34
31-50	45	25	23	35	31
51-70	45	20	19	31	27
71 and over	45	20	19	27	25
Females					
5-6	40	14	13	18	16
7-10	40	18	16	23	21
11-14	40-45**	23	22	30	28
15-18	45	23	21	29	26
19-30	45	25	24	31	28
31-50	45	21	20	28	25
51-70	45	18	17	24	21
71 and over	45	18	17	24	22

* Upper Level of intake for iron based on the Nutrient Reference Values for Australia and New Zealand (NHMRC and MoH 2006).
**The UL for 9-13 years olds is 40 mg/day. The UL for 14 year olds is 45 mg/day.
¥Sum of mean intake from food and beverages, dietary supplements and soy leghemoglobin (ABS 2015b; MoH 2003; University of Otago and MoH 2011).
ΩSum of high intake from food and beverages, mean intake from dietary supplements and high intake from soy leghemoglobin (ABS 2015b; MoH 2003; University of Otago and MoH 2011).

2.6.3 Risk characterisation

No hazard was identified in a repeated dose oral toxicity study in rats at doses of up to 1421 mg/kg bw/day LegH Prep TOS, the highest dose tested. Mean and P90 estimated dietary intakes of LegH Prep at the maximum proposed use level were 20 – 60 mg/kg bw/day TOS and 45 – 124 mg/kg bw/day TOS, respectively. Mean and P90 estimated dietary intakes of LegH Prep at the likely use level were 11 – 32 mg/kg bw/day TOS and 24 – 68 mg/kg bw/day TOS, respectively. The estimated intakes of LegH prep for both scenarios are considered to be conservative and over-estimate consumption as it is unlikely that consumers will eat meat analogue products containing soy leghemoglobin in the same amounts or with the same frequency they currently consume minced meat and poultry products, and vegetarian meat alternatives (particularly over a long period of time).

The margins of exposure (MOEs) between the NOAEL of 1421 mg/kg bw/day TOS in the rat oral toxicity study and estimated dietary exposures at the maximum proposed use level ranged between 20 – 70 for mean exposures and between 10 – 30 at the 90th percentile. At likely use levels, MOEs for mean and P90 estimated dietary intakes ranged between 40 – 130 and 20 – 60, respectively. These MOEs are not considered to be of concern given that: a sufficient body of knowledge exists on the safety of the source organism (it is not pathogenic or toxigenic), the soy leghemoglobin and *Pichia* proteins will be digested like other dietary proteins and do not share any significant similarities to known allergens or toxins; and the conservative nature of the dietary exposure assessment which is likely to overestimate intakes over a long period of time.

As shown in Table 7, the total amount of iron in a representative meat analogue product containing soy leghemoglobin at 0.8% is higher than the amount of iron in minced meat and poultry and other vegetarian meat alternatives on the market in Australia and New Zealand. The amount of iron in the Impossible Burger (3.7 mg/100 g) at the likely use level of 0.45% is similar to the amount of iron in vegetarian style sausages with added iron (3.5 mg/100 g).

The Upper Level of Intake (UL) is a NRV that defines the highest average daily nutrient intake level likely to pose no adverse health effects to almost all individuals in the general population. As intake increases above the UL, the potential risk of adverse effects increases (NHMRC and MoH 2006). At the current baseline levels of iron intake from food and beverages for the Australian and New Zealand populations there are no age/sex groups which exceed the UL for iron at the highest reported percentile of iron intake. With the addition of iron from soy leghemoglobin to baseline iron intakes from food and beverages, and from dietary supplements, the estimated high intake of iron within all population age/sex groups for both Australia and New Zealand were still below the respective ULs for both scenarios. The estimated iron intakes are considered to be conservative and an overestimation of actual iron intakes for the following reasons:

- While the proportion of Australians with an intake of iron from dietary supplements was less than 14% in the most recent survey (ABS 2015b), the mean intake of iron from supplements from this proportion of consumers was used to derive the estimated mean and high intakes for each age/sex group, and they are not likely to be consumed every day over a lifetime.
- It is unlikely that consumers will eat meat analogue products containing soy leghemoglobin in the same amounts they currently consume minced meat and poultry products, and other vegetarian meat alternatives, particularly over a long period of time or a lifetime.

As the estimated iron intakes fall below the UL for iron for each age/sex group, for both the *maximum proposed use level* and *likely use level* scenarios, it is unlikely that consumption of meat analogue products containing soy leghemoglobin would pose a risk of iron exceedances to the Australian and New Zealand populations.

2.7 Manufacturing process

2.7.1 Manufacturing process of the LegH Prep

The LegH Prep is manufactured in compliance with cGMP. A schematic overview of the manufacturing process is presented in Figure 3.
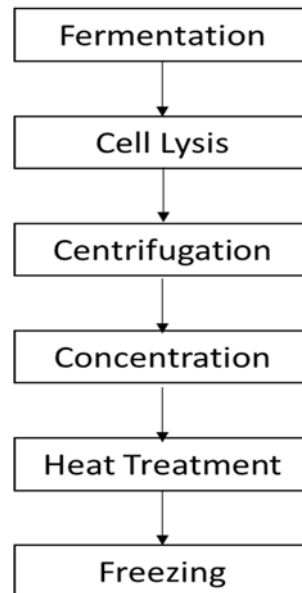

Figure 3 *Schematic overview of the manufacturing process for LegH Prep*

The production strain MXY0541 is cultured to high biomass in a submerged fed-batch fermentation system. At the end of the fermentation run, the *Pichia* cells are lysed by mechanical shearing or high-pressure homogenisation. The insoluble and soluble fraction of the cells are separated by centrifugation, followed by several steps to remove impurities and to ensure no viable cells remain in the lysate. The lysate is then concentrated, mixed with stabilisers (for example sodium ascorbate and sodium chloride), heat treated and stored at -20°C.

FSANZ supports that the raw materials and processing aids employed in the production of LegH Prep are standard ingredients used in the food/enzyme industry and are suitable for their intended use. Impossible Foods have demonstrated that they have in-process controls in place, under cGMP, to ensure the purity and consistency of the final preparation. LegH Prep is well characterized and meets food-grade specifications, which include acceptable limits on compositional criteria and impurities such as heavy metals and microbiological contaminants.

2.7.2 Analytical methods for detection

Methods for measuring proximate levels in the LegH Prep follow standard methodologies from the AOAC, although internal methods were developed for determining the percentage of and purity of the soy leghemoglobin and using these values. Details of the internal methods were provided as confidential information. Levels of metal impurities are measured following standard methodologies from the US EPA. Microbial presence was determined by standard methods from the AOAC.

2.7.3 Characterisation of the production strain MXY0541

Safety of host and donor organisms

Host organism

The host organism is a commercial strain of *Pichia pastoris*, known as Bg11 A detailed discussion of the history of use of *Pichia* has been addressed in Section 2.2.

Donor organisms

Glycine max

The gene for leghemoglobin was based on the leghemoglobin C2 protein sequence found in soybean (*Glycine max*).

Soy is known as one of the major food allergens, due to the presence of a range of proteins in the mature seed that have been associated with anti-nutrients and allergenicity (OECD 2012). Due to this allergenicity, many soy products require allergen labelling in Australia and New Zealand. Although soybean is known to express allergenic proteins, there is no evidence to suggest that soy leghemoglobin is an allergenic protein, especially as haem-containing proteins are found in common food sources, especially red meat. A more detailed assessment of allergenicity concerns are addressed in Section 2.3.2.

Pichia pastoris

Several endogenous genes and regulatory elements were re-introduced into the host organism, *Pichia pastoris*. The genes include Mxr1 and the enzymes involved in the haem biosynthesis pathway. Mxr1, a transcription factor, drives expression of genes associated with the AOX1 promoter (pAOX1). The regulatory elements include the promoters and terminators from AOX1, AOX2 and DAS2, which are commonly used for the expression of recombinant proteins in *Pichia*. A detailed discussion of the history of use of *Pichia* has been addressed in Section 2.2.

Nature and characterisation of the genetic modification

Characterisation of the genetic modification is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. This characterisation addresses three main aspects:

- A detailed description of the DNA sequences introduced to the host genome and the method used to transform the host
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Expression of leghemoglobin from *Glycine max* in *Pichia pastoris*

In order to express the leghemoglobin C2 protein in *P. pastoris*, three expression cassettes were developed. Each expression cassette was designed to target three distinct loci in the *Pichia* genome using homologous recombination. Each expression cassette contained the leghemoglobin gene flanked by regulatory elements belonging to the targeted gene.

The gene for leghemoglobin was chemically synthesised based on the sequence from soybean (*G. max*). Chemical synthesis was used to enable codon use to be optimised for

expression of the plant protein in yeast. The primary amino acid sequence of the expressed protein is the same as that found in soybean (P02236[10]).

The expression cassettes were introduced separately into the host using electroporation of a linearised fragment containing just the expression cassette sequences. As there was no selection marker present within the leghemoglobin expression cassettes, co-transformation was performed using a plasmid containing an antibiotic resistance gene. After selection of transformants, plasmid curing was used to remove the antibiotic resistance plasmid.

To identify positive transformants, PCR analysis was used as an initial screening tool. When the final production strain MXY0541 was chosen, whole genome analysis using short-read DNA sequencing was performed using a Pac-Bio system. The production organism MXY0541 contains all three leghemoglobin transformation events. The production organism MXY0291 that was granted GRAS status in the US (US FDA 2018; GRN737) contains only one of the transformation events.

Locus 1: FSANZ's analysis of a range of data from MXY0541 indicates:
1) inserts containing the leghemoglobin gene expression cassette are present at the insertion locus. The inserts contain multiple tandem repeats of the expression cassette
2) the sequence for the leghemoglobin gene is as expected, with no mutations or rearrangements
3) the flanking regions on either side of the inserted gene contain the expected regulatory sequences.

Locus 2: FSANZ's analysis of the PCR and sequencing data from MXY0541 indicates:
1) several tandem repeats of the expression cassette at the insertion locus
2) the gene copy number cannot be fully determined. The actual copy number could not be completely delineated using whole genome sequencing because of the repetitive nature of the sequence in the tandem repeats and the short-read DNA sequencing employed
3) the insert has replaced the target gene of locus 2
4) the sequence located at the 5' end of the gene contains the expected regulatory sequences.

FSANZ further identified that the sequence located at the 3' end of the gene includes the terminator sequence of the target gene for locus 2 but this sequence is in the complement direction and may not encode a functional polyadenylation site. This could reduce the stability and transport of the mRNA transcript and thus translation efficiency, but would not impact safety.

FSANZ also identified base pair differences in some of the leghemoglobin gene inserts, where the sequence data was of high quality. These differences could result in amino acid changes and expression of truncated proteins. However, as already noted, it is unlikely these proteins would be translated because of the non-functional polyadenylation sequence. These changes would therefore not impact safety.

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[10] www.uniprot.org/uniprot/P02236

Locus 3: FSANZ's analysis of the PCR and sequencing data from MXY0541 indicates:

1) there are several tandem repeats of the expression cassette
2) the sequence for leghemoglobin gene is as expected, with no mutations or rearrangements
3) the sequence located at the 3' end of the gene contains the expected regulatory sequences
4) the target gene at locus 3 has not been disrupted.

FSANZ further identified that the sequence located at the 5' end of the leghemoglobin gene did not contain the expected promoter for the target gene. Instead, this region contained the promoter sequence from another gene located upstream of the locus in the *Pichia* genome and included a partial sequence of the neighbouring protein coding gene and terminator sequence. This would explain why the target gene at locus 3 was not disrupted, as the flanking sequences in this expression cassette would not have allowed for targeted homologous recombination. The insertion was also not located at the locus but instead, preceded the gene from which the alternate promoter was obtained. However, the expression cassette that was inserted contains a functional promoter and terminator, flanking a gene containing no mutations and the insertion does not interrupt any other genes.

Overexpression of the endogenous haem co-factor

Haem is an important co-factor required for the oxygen-transporting role of leghemoglobin and provides the dietary source of iron. It is also a required co-factor for the function of several critical haem-proteins in yeast thus haem could become a limiting factor in the production of recombinant leghemoglobin (Franken et al. 2011; Krainer et al. 2015). To solve this potential issue, the applicant chose to increase the level of endogenous haem by introducing the genes encoding the eight enzymes used to synthesis haem. The enzymes have been well characterised and the biosynthesis pathway is the same in yeast, plants and animals (reviewed in Layer et al. 2010; Franken et al. 2011).

Expression cassettes were generated to introduce haem enzyme genes, targeted to a single locus. The haeme enzyme genes and associated regulatory sequences were all sourced from the host organism. The expression cassettes also contained antibiotic resistance genes sourced from non-host organisms, flanked by Cre-Lox sequences (Dale and Ow 1991). The expression cassettes were transformed into the host as linearised fragments of DNA, free of vector backbone sequences, using electroporation. After selection of positive transformants, the antibiotic resistance genes were excised from the transformed cells using the Cre-Lox system.

Characterisation of the inserted haem synthesis pathway genes were performed by PCR and whole genome sequencing. The characterisation identified that at least one functional copy of every enzyme was inserted at the locus. For all enzymes, there is at least one copy where the DNA sequences are as expected.

During the initial PCR screen, it was observed that one of the enzyme genes existed in both full and truncated forms. A subsequent expression cassette was designed to replace the truncated versions but the reinsertion of the expression cassette did not repair the truncated gene variants. Furthermore, some mutations were created in the recombination event. FSANZ noted that the replacement expression cassette only contained a promoter sequence and gene coding region with no terminator sequence thus it would be highly unlikely any transcripts would be stable or translated. In addition, peptide mapping focusing on the specific enzyme product identified only the full length protein with the expected peptide sequence, confirming that the product is as expected. A full insert of this enzyme was achieved in the original transformation event targeting the locus.

Overexpression of the endogenous transcription factor Mxr1

A common regulatory element used for the expression of recombinant proteins in *Pichia* is the promoter for the alcohol oxidase gene (pAOX1). This promoter was used for several of the recombinant haem enzyme genes and for one of the expression cassettes for leghemoglobin. To ensure sufficient activation of all the genes driven by pAOX1, the applicant chose to increase expression of the main transcription factor for pAOX1, known as Mxr1 (Lin-Cereghino et al. 2006).

A single expression cassette was generated, containing the Mxr1 gene from the host, flanked by the *Pichia* AOX1 promoter and terminator sequences. FSANZ noted that in GRN737 (US FDA 2018) the terminator sequence was declared as tFDH, however from the DNA sequence provided to FSANZ, we can confirm that the terminator sequence is tAOX1. As there were no selective marker genes included in the expression cassette, a plasmid containing an antibiotic resistance gene was co-transformed. After selection of positive transformants, plasmid curing was used to generate transformants no longer containing the antibiotic resistance plasmid.

The applicant noted that due to the cloning strategy employed for Mxr1, six extra N-terminal amino acids are encoded in the final protein. Analysis of the final leghemoglobin product using mass spectrometry showed that the protein engineered Mxr1 protein was not present.

Characterisation of the inserted Mxr1 insert confirmed a single copy is present in the locus and there are no mutations or rearrangements.

Absence of the selective marker genes: To confirm absence of the antibiotic resistance genes used to select positive transformants, which were subsequently removed by either cre-lox excision or plasmid curing, results from three methods were provided: growth on selective agar; PCR targeting the antibiotic resistance gene; and whole genome sequencing. Results obtained from the final production strain MXY0541 showed absence of all the antibiotic resistance genes used. These analyses confirmed that both the curing and cre-lox excision processes were successful.

Inheritance and genetic stability of the inserted DNA: Genetic stability and inheritance of the introduced genes was confirmed by analysis of cultures at the end of a standard fermentation run (150-200 generations). PCR analysis targeting all the inserted genes showed that all the inserts were present and at the expected sizes, confirming inheritance and stability.

Presence of novel DNA in the final product: A genomic DNA preparation of the final leghemoglobin preparation was performed and quantified using NanoDrop spectrophotometry. The data shows that the amount of DNA present in the LegH Prep is approximately 300 mg/l. As the amount of LegH Prep added to the burgers will range from 5-10 ml, the amount of DNA would be approximately 1.5-3 mg (i.e. 0.3mg/ml). This DNA will contain the novel DNA that encodes the recombinant soy leghemoglobin.

2.7.4 Conclusion

Several expression cassettes were generated to introduce ten genes into *P. pastoris* in order to express soy leghemoglobin. The data provided by the applicant demonstrated that for every gene introduced, there is at least one full copy of the insert that has been integrated into the host. In some situations, more than one copy of a gene has been inserted. In one situation, a truncated version of a single gene has been inserted multiple times. All the gene inserts were shown to be stably integrated over several generations. Data was also provided confirming successful removal of the antibiotic selection markers used during the

transformant process. The conclusion from this assessment is the production strain MXY0541 poses no safety concerns.

3   Risk assessment summary and conclusion

FSANZ has conducted a comprehensive assessment following the internationally recognised risk analysis framework. The assessment was based on a weight of evidence approach, combining information and scientific evidence provided by the applicant with independent sources.

In conducting the risk assessment of the soy leghemoglobin and the LegH Prep, a number of criteria have been addressed, including the safety of the *P. pastoris* host strain, novel proteins, toxicity of the LegH Prep and a nutritional and dietary intake assessment. The safety assessment of the source organism and novel proteins concluded there were no public health and safety concerns. The source organism is a well characterised yeast with a recognised safe history of use for the production of food enzymes. It is neither pathogenic nor toxigenic.

The novel soy leghemoglobin was shown to be equivalent to that expressed in soybean and was shown to be expressed as a holoprotein. Analyses of the potential allergenicity or toxicity of all the novel proteins, including soy leghemoglobin and the *Pichia* proteins, did not identify any significant similarities to known allergens or toxins. The proteins were shown to be susceptible to pepsin digestion and were denatured at standard cooking temperatures and in acidic conditions that mimic the stomach environment. The shelf life and specifications of the LegH Prep are also appropriate for addition to meat analogue products.

The applicant submitted *in vitro* genotoxicity studies in bacterial and mammalian cells and an oral toxicity study in rats. These studies are intended to confirm the outcome of the compositional and bioinformatic analysis conducted as a part of the safety assessment. No hazard was identified in the submitted studies. LegH Prep was not genotoxic *in vitro* and did not cause adverse effects in short-term toxicity studies in rats. The NOAEL of freeze-dried LegH Prep in a 28-day dietary toxicity study in rats was 1536 mg/kg bw/day, the highest dose tested. This dose corresponds to 1421 mg/kg bw/day TOS.

Mean and P90 estimated dietary intakes of LegH Prep at the maximum proposed use level were 20 – 60 mg/kg bw/day TOS and 45 – 124 mg/kg bw/day TOS, respectively. Mean and P90 estimated dietary intakes of LegH Prep at the likely use level were 11 – 32 mg/kg bw/day TOS and 24 – 68 mg/kg bw/day TOS, respectively. The estimated intakes of LegH Prep for both scenarios are considered to be conservative and over-estimate consumption as it is unlikely that consumers will eat meat analogue products containing soy leghemoglobin in the same amounts or with the same frequency they currently consume minced meat and poultry products, and vegetarian meat alternatives (particularly over a long period of time).

The MOEs between the NOAEL of 1421 mg/kg bw/day TOS in the rat oral toxicity study and estimated dietary exposures at the maximum proposed use level ranged between 20 – 70 for mean exposures and between 10 – 30 at the 90th percentile. At likely use levels, MOEs for mean and P90 estimated dietary intakes ranged between 40 – 130 and 20 – 60, respectively. These MOEs are not considered to be of concern given that: a sufficient body of knowledge exists on the safety of the source organism, the soy leghemoglobin and *Pichia* proteins will be digested like other other dietary proteins and do not share any significant similarities to known allergens or toxins; and the conservative nature of the dietary exposure assessment which is likely to overestimate intakes over a long period of time.

The nutrition assessment focused on the relative bioavailability of haem iron from soy

leghemoglobin compared to haem iron from animal sources. Haem iron from soy leghemoglobin is expected to have similar bioavailability to haem iron from mammalian haem proteins (e.g. myoglobin present in muscle tissue). The absence of meat proteins in the proposed meat analogue product may decrease haem iron bioavailability. However, because iron absorption is regulated tightly by the body, and the proposed meat analogue products have higher total iron content relative to comparison foods, any decrease in haem iron bioavailability should not result in a nutritional disadvantage to consumers in Australia and New Zealand.

The estimated intakes of iron (with the additional iron contribution from soy leghemoglobin) for all population age/sex groups assessed for both the Australian and New Zealand populations are below the ULs for iron. The estimated iron intakes in FSANZs assessment, for both the *maximum proposed use level* and *likely use level* scenarios, are considered to be conservative and an overestimation of actual iron intakes. It is unlikely that consumption of meat analogue products containing soy leghemoglobin would pose a risk of iron exceedances to the Australian and New Zealand populations, including at levels up to 0.8% soy leghemoglobin.

As of March 2020, the applicant advised they had sold approximately 100,000,000 quarter-pound (113 g) servings of meat analogue products containing soy leghemoglobin. Its post-marketing surveillance has identified one complaint per 600,000 servings based on the current formulation (released on the market in the US in early 2019), but none of these complaints has been confirmed as an adverse event due to consumption of these products.

The assessment of soy leghemoglobin and the LegH Prep concluded that there are no public health and safety concerns associated with its use in meat analogue products at the proposed level of up to 0.8% soy leghemoglobin.

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Appendix 1: FSANZ Approach to the dietary intake assessment

A dietary intake or exposure assessment is the process of estimating how much of a nutrient or food chemical a population, or population subgroup consumes. Dietary intake of nutrients is estimated by combining food consumption data with food composition data. The process of doing this is called 'dietary modelling'.

Dietary intake = food chemical concentration x food consumption

FSANZ's approach to dietary modelling is based on internationally accepted procedures for estimating intake of nutrients (FSANZ, 2009). Different dietary modelling approaches may be used depending on the assessment, the type of food chemical, the data available and the risk assessment questions to be answered. In the majority of assessments FSANZ uses the food consumption data from each person in the national nutrition surveys to estimate their individual dietary intake. Population summary statistics such as the mean intake or a high percentile intake are derived from the ranked individual person's intakes from the nutrition survey. In some cases, as used in this assessment for estimating total iron intakes, FSANZ will use the usual mean and high percentile usual intakes of nutrients that are published based on nutrition survey data.

An overview of how dietary intake assessments are conducted and their place in the FSANZ Risk Analysis Process is provided on the FSANZ website[11].

FSANZ has a custom-built computer program called 'Harvest' to calculate nutrient intakes and dietary exposures to food chemicals. Harvest was used for this assessment to:
- estimate the dietary intake of LegH Prep
- compare the baseline iron intake of meat eaters to non-meat eaters
- extract the combined consumption amounts for minced meat and poultry products and vegetarian meat alternatives for Australia and New Zealand to estimate population iron intakes with the additional contribution from soy leghemoglobin.

Further detailed information on conducting dietary intake assessments at FSANZ is provided in *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ 2009)[12].

A1.1 Food consumption data used

The most recent food consumption data from national nutrition surveys were used to estimate the combined consumption amounts for minced meat and poultry products or vegetarian meat alternatives for Australia and New Zealand. Dietary intake assessments based on food consumption data from national nutrition surveys provide the best estimation of actual consumption of a food and the resulting estimated dietary intake for the Australian and New Zealand populations. However, it should be noted that national nutrition survey data do have limitations. The national nutrition survey data used for these assessments, the design of these surveys and the key attributes, including survey limitations, are set out below.

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[11] www.foodstandards.gov.au/science/riskanalysis/Pages/default.aspx
[12]
www.foodstandards.gov.au/science/exposure/documents/Principles%20_%20practices%20exposure%20assessment%202009.pdf.

Further information on the national nutrition surveys used to conduct dietary intake assessments is available on the FSANZ website at: http://www.foodstandards.gov.au/science/exposure/Pages/dietaryexposureandin4438.aspx.

2011–12 Australian National Nutrition and Physical Activity Survey (2011–12 NNPAS)

The 2011–12 Australian National Nutrition and Physical Activity Survey (NNPAS) undertaken by the Australian Bureau of Statistics is the most recent food consumption data for Australia. This survey includes dietary patterns of a sample of 12,153 Australians aged 2 years and above. The survey used a 24-hour recall method for all respondents, with 64% of respondents also completing a second 24-hour recall on a second, non-consecutive day. The data were collected from May 2011 to June 2012 (with no enumeration between August and September 2011 due to the Census). Only those respondents who had two days of food consumption data (n=7,735) were used to derive the consumer consumption amounts for meat, poultry and vegetarian meat alternatives. The survey data was weighted for use in all calculations. Consumption and respondent data from the survey were incorporated into the Harvest program from the Confidentialised Unit Record Files (CURF) data set (ABS, 2014).

The baseline iron intakes from food and beverages, and dietary supplements, used for the Australian population in this assessment are those derived from the 2011-12 survey and published by the ABS (ABS, 2015a; ABS, 2015b). The NCI method that is used to estimate usual nutrient intakes was used to estimate the usual mean and high percentile iron intakes from food and beverages. The baseline data on intakes from dietary supplements is based on consumers who had taken iron from supplements the day prior to interview, and excludes pregnant and breastfeeding females. These intakes were used deterministically to estimate iron intakes from the diet in addition to the iron from the addition of soy leghemoglobin to meat analogue products.

2002 New Zealand National Children's Nutrition Survey (2002 NZ NCNS)

The 2002 NZ NCNS was a cross-sectional and nationally representative survey of 3,275 New Zealand children aged 5-14 years. The data were collected during the school year from February to December 2002. The survey used a 24-hour food recall and provided information on food and nutrient intakes, eating patterns, frequently eaten foods, physical activity patterns, dental health, anthropometric measures and nutrition-related clinical measures. It was also the first children's nutrition survey in New Zealand to include a second day diet recall data for about 15% of the respondents, and dietary intake from both foods (including beverages) and dietary supplements. For the consumption amounts for minced meat and poultry products and vegetarian meat alternatives in this assessment, only the first (day 1) 24-hour recall food consumption data for all consumers was used as not enough respondents completed a second 24 hour recall to enable a reliable or representative two day average to be calculated. The survey data were weighted for use in all calculations. Consumption and respondent data from the survey were incorporated into the Harvest program from the Confidentialised Unit Record Files (CURF) data set (MoH, 2005).

Baseline iron intakes from food and beverages used for New Zealand Children aged 5 to 14 years are those that were published with the 2002 NZ CNS (MoH, 2003). They are adjusted intakes derived from the distribution of usual intakes of iron using the PC-SIDE computer software. A search of the published literature found no iron intake data from dietary supplements for the New Zealand population that is equivalent to that published for the Australian 2011-12 NNPAS. It was assumed in this assessment that the average intake of iron from dietary supplements for the New Zealand population (for those who consume supplements) is the same as the Australian population.

2008-9 New Zealand Adult Nutrition Survey (2008-9 NZ ANS)

The 2008-9 NZ ANS provides comprehensive information on the dietary patterns of a sample of 4,721 respondents aged 15 years and above. The survey was conducted on a stratified sample over a 12 month period from October 2008 – October 2009. The survey used a 24-hour recall methodology with 25% of respondents also completing a second 24-hour recall. The information collected in the 2008 NZANS included food and nutrient intakes, dietary supplement use, socio-demographics, nutrition related health, and anthropometric measures. For the consumption amounts for minced meat and poultry products and vegetarian meat alternatives in this assessment, only the first day (day 1) 24-hour recall food consumption data for all consumers was used as not enough respondents completed a second 24 hour recall to enable a reliable or representative two day average to be calculated. The survey data were weighted for use in all calculations. Consumption and respondent data from the survey were incorporated into the Harvest program from the Confidentialised Unit Record Files (CURF) data set (MoH, 2012).

Baseline iron intakes from food and beverages used for New Zealanders 15 years and above are those published with the 2008-9 NZ ANS (University of Otago and MoH, 2011). They are adjusted intakes derived from the distribution of usual intakes of iron using the PC-SIDE computer software.

Food groups and consumption amounts used in the dietary intake assessment

FSANZ dietary intake assessment assumes that consumers may choose to eat meat analogue products containing soy leghemoglobin in the same amounts they currently consume of traditional minced meat and poultry products, and vegetarian meat alternatives. The food groups used to derive the consumption amounts are listed in Table A1.1. The consumption amounts for Australia and New Zealand for consumers of these foods is shown in Table A1.2 and shown for each age/sex group in Table A1.3. The consumption amounts include foods reported as eaten and where used in processed foods or mixed dishes, based on FSANZ recipe database in Harvest.

Table A1.1: **Food groups used to derive consumption amounts for meat analogue products containing soy leghemoglobin**

Food groups	*Foods included in this group*	*Foods not included in this group*
Minced meat products	All minced meat including sausages and luncheon meat.	Meat in whole or cut pieces.
Minced poultry products	All minced poultry including sausages and luncheon meat.	Poultry in whole or cut pieces.
Vegetarian meat alternatives	Vegetable patties, tofu, tempeh, vegetarian sausages and other meat analogues, falafel and nut meat.	Vegetable fritters (e.g. corn fritters).

Table A1.2: **Mean and 90th percentile consumption amounts for minced meat and poultry products and vegetarian meat alternatives for the Australian and New Zealand populations**

Country	Age group (years)	Mean consumption (g/day)	Consumption at 90th percentile (g/day)
Australia*	2 years and above	63	139
New Zealand^	5 – 14 years	111	222
	15 years and above	116	257

* 2011-12 Australian National Nutrition and Physical Activity Survey. Based on consumption data from consumers with two days of data only.
^ 2002 New Zealand National Children's Nutrition Survey and the 2008–09 New Zealand Adult Nutrition Survey. Based on day 1 consumption data only from consumers only.

Table A1.3: **Mean and 90th percentile consumption amounts of minced meat and poultry products and vegetarian meat alternatives by age group and sex**

Country	Sex	Age group (years)	Mean consumption (g/day)	Consumption at 90th percentile (g/day)
Australia*	Male	2-3	34	71
		4-8	50	97
		9-13	63	144
		14-18	93	190
		19-30	80	182
		31-50	75	164
		51-70	72	171
		>= 71	71	156
	Female	2-3	34	92
		4-8	37	82
		9-13	49	102
		14-18	44	98
		19-30	59	124
		31-50	54	123
		51-70	58	140
		>= 71	53	117
New Zealand^	Male	5-6	95	174
		7-10	122	224
		11-14	144	307
		15-18	139	274
		19-30	155	396
		31-50	138	290
		51-70	106	269
		>= 71	94	188
	Female	5-6	84	145
		7-10	98	196
		11-14	97	200
		15-18	116	227
		19-30	107	255
		31-50	99	234
		51-70	91	179
		>= 71	84	197

* 2011-12 Australian National Nutrition and Physical Activity Survey. Based on consumption data from consumers with two days of data only.
^ 2002 New Zealand National Children's Nutrition Survey and the 2008–09 New Zealand Adult Nutrition Survey. Based on day 1 consumption data from consumers only.

A1.2 Concentration data used

LegH Prep

The applicant has proposed a maximum level of 0.8% soy leghemoglobin for use in meat analogue products. The applicant has stated that the likely use levels are 0.45% soy leghemoglobin in beef analogue products and 0.25% soy leghemoglobin in pork analogue products. The dietary intake assessment assumes that these levels apply to the raw product, and that LegH Prep would be further concentrated where moisture loss occurs during cooking. There are no other sources or existing permissions for soy leghemoglobin in foods currently on the market in Australia and New Zealand.

Population statistics (mean and 90th percentile) for soy leghemoglobin intakes were derived from each individual's intake of soy leghemoglobin which were then converted to the corresponding amount of LegH Prep, based on the commercial preparation of LegH Prep containing up to 9% soy leghemoglobin, and having a TOS content of 22%.

Iron

Based on the atomic mass of iron (55.845) and the soy leghemoglobin protein containing the iron (16,131 Da), 0.35% of the mass of the protein is iron. For every gram of soy leghemoglobin added to a meat analogue product it is expected that 3.5 mg of iron has been contributed. If soy leghemoglobin is added to raw meat analogue products at the maximum proposed use level 0.8% (8 g/kg) the expected concentration of iron in these products (raw form) is 28 mg/kg. If soy leghemoglobin is added to raw meat analogue products at the use level of 0.45% (4.5 g/kg) the expected concentration of iron in these products (raw form) is 15.75 mg/kg. If soy leghemoglobin is added to raw meat analogue products at the use level of 0.25% (2.5 g/kg) the expected concentration of iron in these products (raw form) is 8.75mg/kg.

A1.3 Assumptions and limitations of the dietary intake assessment

Where significant uncertainties in the data exist, conservative assumptions were made in the intake assessment to ensure the estimated intakes of LegH Prep and iron were not underestimated. Assumptions made in the dietary intake assessment include:
- Moisture loss from cooking meat analogue products containing soy leghemoglobin is equivalent to the loss from cooking the meat, poultry or vegetarian meat alternatives they are replacing in the assessment.
- In estimating the intakes for the *likely use level* scenario, minced pork and poultry products can be used as a proxy for meat analogue products that may contain soy leghemoglobin at 0.25%. All other minced meat products (including minced beef and lamb products) and vegetarian meat alternatives consumed in the national nutrition survey can be used as a proxy for meat analogue products that may contain soy leghemoglobin at 0.45%.
- Iron intakes that were published for each national nutrition survey reflect the current intakes.
- The iron intake of respondents who did not consume meat or any meat products on day 1 or day 2 of the survey are representative of the iron intake of a 'non-meat eater'.
- The average intake of iron from dietary supplements for the New Zealand population (for those who consume supplements) is the same as the Australian population.
- The iron contributed by ingredients other than the soy leghemoglobin in meat analogue products is within the range of iron in meat, poultry and vegetarian meat alternative products currently on the market in Australia and New Zealand.

- Intakes of iron from added iron for foods with existing permissions in Schedule 17 of the Food Standards Code (for example, breakfast cereals) are captured by the baseline intake data published for each national nutrition survey.

In addition to the specific assumptions made in relation to this dietary intake assessment, there are a number of limitations associated with the nutrition surveys on which this assessment is based. A discussion of these limitations is included in Section 6 of the *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ, 2009).

References

ABS (2014) National Nutrition and Physical Activity Survey, 2011–12, Basic Confidentialised Unit Record Files. Australian Bureau of Statistics, Commonwealth of Australia, Canberra

FSANZ (2009) Principles and practices of dietary exposure assessment for food regulatory purposes. Report prepared by Food Standards Australia New Zealand, Canberra http://www.foodstandards.gov.au/publications/Pages/Principles-and-Practices-of-Dietary.aspx

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MoH (2012) 2008-9 New Zealand Adult Nutrition Survey: National Confidentialised Unit Record File (CURF). Ministry of Health, Wellington

Appendix 2: Estimated dietary intake of iron with additional contribution from soy leghemoglobin

For the dietary intake assessment of iron, FSANZ first compared the baseline intakes of iron from food for meat-eaters with that of non-meat eaters for the Australian and New Zealand population using Harvest. This was undertaken in order to determine whether non-meat eaters should be considered as a separate population group in estimating dietary intakes of iron with the additional contribution from soy leghemoglobin from meat analogue products. The results of this comparison are included in Table A2.1.

Respondents who consumed meat (including poultry, seafood and game) or meat containing mixed dishes or products (e.g. fish sauce) on either day of the nutrition surveys were defined for the purpose of the assessment as 'meat eaters'. Those who did not consume meats or meat products were defined as 'non-meat eaters'. Only the first day of data was used for comparison of the iron intake of meat eaters to non-meat eaters as this allowed for a larger sample size of non-meat eaters to calculate the mean intake. All data were weighted in the calculations.

Table A2.1: **Current mean iron intake of meat eaters and non-meat eaters**

Country	Age group (years)	Mean iron intake (mg/day)	
		Meat eaters	*Non-meat eaters*
Australia*	2 years and above	11	8
New Zealand^	5 – 14 years	11	8
	15 years and above	12	9

*2011-12 Australian National Nutrition and Physical Activity Survey. Based on day 1 consumption data for all respondents.
^ 2002 New Zealand National Children's Nutrition Survey and the 2008–09 New Zealand Adult Nutrition Survey. Based on day 1 consumption data for all respondents.

For all surveys the mean intake of iron for non-meat eaters was below that of meat eaters. As this assessment considered only exceedances of the UL, it was determined that it was not necessary to consider the estimated iron intake of non-meat eaters as a special population group.

The estimated mean and high intakes of iron, including the contribution from soy leghemoglobin, for the Australian and New Zealand population are listed in Table A2.2 and Table A2.3.

Table A2.2: Estimated dietary intake of iron (mg/day) for the Australian population with additional contribution from soy leghemoglobin

Age group (years)	UL[*]	Mean intake from food[€]	High intake from food (P95)[€]	Mean intake from supplements[Σ]	Intake from soy leghemoglobin (mean)[£]		Intake from soy leghemoglobin (P90)[£]		Mean total intake[¥]		High total intake[Ω]	
					Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level
Males												
2-3	20	8	12	3	1	1	2	1	12	12	17	16
4-8	40	9	13	3	1	1	3	2	13	13	19	18
9-13	40	12	17	4	2	1	4	2	18	17	25	23
14-18	45	13	19	4	3	1	5	3	20	18	28	26
19-30	45	13	19	8	2	1	5	3	23	22	32	30
31-50	45	13	19	7	2	1	5	3	22	21	31	29
51-70	45	12	18	4	2	1	5	3	18	17	27	25
71 +	45	12	18	6	2	1	4	2	20	19	28	26
Females												
2-3	20	7	10	3	1	1	3	1	11	11	16	14
4-8	40	8	12	3	1	1	2	1	12	12	17	16
9-13	40	9	14	5	1	1	3	2	15	15	22	21
14-18	45	9	14	10	1	1	3	1	20	20	27	25
19-30	45	9	14	12	2	1	3	2	23	22	29	28
31-50	45	9	14	8	2	1	3	2	19	18	25	24
51-70	45	10	15	5	2	1	4	2	17	16	24	22
71 +	45	9	14	6	1	1	3	2	16	16	23	22

[*]Upper Level for iron based on the Nutrient Reference Values for Australia and New Zealand (NHMRC and MoH 2006).
[€] Intake derived from 2011-12 NNPAS (ABS 2015a). Published usual intakes for Australia report the 95th percentile intake; this has been used for the estimated high intakes from food for Australia.
[Σ] Intake derived from 2011-12 NNPAS (ABS 2015b).
[£] Population statistics (mean and 90th percentile) were derived from each individual's intake of soy leghemoglobin then converted to the corresponding amount of iron.
[¥]Sum of mean intake from food and beverages, dietary supplements and soy leghemoglobin.
[Ω]Sum of high intake from food and beverages, mean intake from dietary supplements and high intake from soy leghemoglobin.

Table A2.3: Estimated intake of iron (mg/day) for the New Zealand population with additional contribution from soy leghemoglobin

Age group (years)	UL*	Mean intake from food €	High intake from food (P90) €	Mean intake from supplements Σ	Intake from soy leghemoglobin (mean) £		Intake from soy leghemoglobin (P90) £		Mean total intake ¥		High total intake Ω	
					Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level	Maximum proposed use level	Likely use level
Males												
5-6	40	10	13	3	3	1	5	3	16	15	21	19
7-10	40	12	16	4	3	2	6	3	20	18	26	24
11-14	40-45**	14	19	4	4	2	9	5	22	21	31	27
15-18	45	14	19	4	4	2	8	4	22	20	31	27
19-30	45	14	20	8	4	2	11	6	27	25	39	34
31-50	45	14	20	7	4	2	8	4	25	23	35	31
51-70	45	13	19	4	3	2	8	4	20	19	31	27
71 +	45	12	16	6	3	1	5	3	20	19	27	25
Females												
5-6	40	9	11	3	2	1	4	2	14	13	18	16
7-10	40	10	13	5	3	1	5	3	18	16	23	21
11-14	40-45**	10	15	10	3	1	6	3	23	22	30	28
15-18	45	9	13	10	3	2	6	3	23	21	29	26
19-30	45	10	12	12	3	2	7	4	25	24	31	28
31-50	45	10	14	8	3	1	7	3	21	20	28	25
51-70	45	10	14	5	3	1	5	3	18	17	24	21
71 +	45	9	13	6	2	1	6	3	18	17	24	22

* Upper Level for iron based on the Nutrient Reference Values for Australia and New Zealand (NHMRC and MoH 2006).

**The UL for 9-13 years olds is 40 mg/day. The UL for 14 year olds is 45 mg/day.

€ Intake derived from the 2002 NZ CNS and 2008-9 NZ ANS (Ministry of Health 2003; University of Otago and MoH 2011). The highest reported intake in these publications is the 90th percentile; this has been used for the estimated high intakes from food for New Zealand.

Σ Australian mean intake for supplements used (ABS 2015b). For 5-6 year olds the average used is for Australians aged 4-8 years. For 7-10 years olds the mean used is for Australians aged 9-13 years. For 11-14 year olds the average used is for Australians aged 14-18 years.

£ Population statistics (mean and 90th percentile) were derived from each individual's intake of soy leghemoglobin then converted to the corresponding amount of iron.

¥ Sum of mean intake from food and beverages, dietary supplements and soy leghemoglobin. As the intakes in the table have been rounded to the nearest whole number the mean total intake for each age group may not always exactly equal the sum of the intakes displayed.

Ω Sum of high intake from food and beverages, mean intake from dietary supplements and high intake from soy leghemoglobin. As the intakes in the table have been rounded to the nearest whole number the high total intake for each age group may not always exactly equal the sum of the intakes displayed.

References

ABS (2015a) Australian health survey: Usual Nutrient Intakes, 2011-12. Table 4:Essential minerals (and caffeine). Australian Bureau of Statistics, Commonwealth of Australia, Canberra

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