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SCIENTIFIC OPINION

Safety of soy leghemoglobin from genetically modified *Komagataella phaffii* as a food additive

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Abstract

The EFSA Panel on Food Additive and Flavourings (FAF Panel) provides a scientific opinion on the safety of soy leghemoglobin from genetically modified *Komagataella phaffii* as a food additive in accordance with Regulation (EC) No 1331/2008. The proposed food additive, LegH Prep, is intended to be used as a colour in meat analogue products. The yeast *Komagataella phaffii* strain MXY0541 has been genetically modified to produce soy leghemoglobin; the safety of the genetic modification is under assessment by the EFSA GMO Panel (EFSA-GMO-NL-2019-162). The amount of haem iron provided by soy leghemoglobin from its proposed uses in meat analogue products is comparable to that provided by similar amounts of different types of meat. The exposure to iron from the proposed food additive, both at the mean and 95th percentile exposure, will be below the 'safe levels of intake' established by the NDA Panel for all population groups. Considering that the components of the proposed food additive will be digested to small peptide, amino acids and haem B; the recipient (non GM) strain qualifies for qualified presumption of safety status; no genotoxicity concern has been identified and no adverse effects have been identified at the highest dose tested in the available toxicological studies, the Panel concluded that there was no need to set a numerical acceptable daily intake (ADI) and that the food additive does not raise a safety concern at the proposed use in food category 12.9 and maximum use level. The Panel concluded that the use of soy leghemoglobin from genetically modified *Komagataella phaffii* MXY0541 as a new food additive does not raise a safety concern at the proposed use and use level. This safety evaluation of the proposed food additive remains provisional subject to the ongoing safety assessment of the genetic modification of the production strain by the GMO Panel (EFSA-GMO-NL-2019-162).

KEYWORDS

food colour, genetically modified *Komagataella phaffii*, meat analogue products, soy leghemoglobin

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SUMMARY

The European Commission requests the European Food Safety Authority to provide a scientific opinion on the safety of soy leghemoglobin from genetically modified *Komagataella phaffii*, formerly *Pichia pastoris*, as a food additive in accordance with Regulation (EC) No 1331/2008, establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

The current opinion pertains exclusively to the safety assessment of the proposed food additive soy leghemoglobin, performed in accordance with Regulation (EC) No 1333/2008.

The proposed food additive, 'LegH Prep', is a liquid preparation containing soy leghemoglobin from genetically modified yeast *K. phaffii*, other compounds derived from the fermentation process, and added ingredients. LegH Prep is intended to be used as a colour in meat analogue products. Soy leghemoglobin is a small holoprotein containing a haem group responsible for the colour of the food additive.

The source of the additive is the yeast *K. phaffii* strain MXY0541, which has been genetically modified to produce the soy leghemoglobin by introducing the heterologous gene LGB2 from soy (*Glycine max*) and by upregulating the native haem biosynthetic pathway. No antimicrobial resistance genes have been introduced into the production strain. The safety of the genetic modification is currently under assessment by the EFSA GMO Panel (EFSA-GMO-NL-2019-162).

The recipient strain of *K. phaffii* has qualified presumption of safety (QPS) status with the qualification 'for production purpose only'. No viable cells of the production strain were detected (see Section 3.1.3.3). Therefore, no concern is expected from yeast metabolites produced during the fermentation process.

The production process involves the fermentation of the production strain of *K. phaffii*, followed by cells lysis and removal of insoluble material. The resulting concentrate is thermally stabilised and formulated with stabilisers, then stored as a frozen liquid.

Analytical data provided by the applicant suggest compliance with proposed specifications. However, the applicant proposed that the authorisation should not be linked to any specific production strain, to accommodate future improvements to the strain. The Panel considered the proposal inadequate, noting that the authorisation of the proposed food additive should be linked to the specified production strain MXY0541 used to support the safety evaluation and any modification of the production strain would need a safety assessment for the resulting modified production strain. Therefore, the Panel recommends the introduction of the production strain MXY0541 in the *Definition* of the specifications for the proposed food additive.

Based on the structural similarities between soy leghemoglobin, animal myoglobins and other plant haemoglobins, the Panel considered that degradation products deriving from the cooking process and the interaction of LegH Prep with food components are expected to be similar to those from animal myoglobins and plant haemoglobins. Therefore, no safety concern was identified with respect to degradation during food processing or interaction with food components.

The applicant provided typical and maximum use levels for the proposed food additive, as described in the specifications, for a single food category (FC 12.9 'Protein products, excluding products covered in category 1.8'). The use levels were expressed in terms of soy leghemoglobin (functional component). The use levels for the whole preparation (i.e. LegH Prep) were also provided, based on the concentration of soy leghemoglobin in the whole preparation.

The applicant provided an estimate of exposure to the proposed food additive based on the output obtained using the FAIM tool at the proposed maximum use levels provided, in compliance with the requirement. However, the Panel considered that the FAIM tool is not appropriate in this case since the food category available in FAIM (FC 12.9) is broader than the requested use by the applicant, which is restricted to 'only meat imitates'. For this reason, EFSA estimated the exposure by using the DietEx tool, rather than the FAIM tool. The DietEx estimation has been performed using the two FoodEx2 codes 'Bovine, minced meat' and 'Bovine and pig, minced meat', used as surrogate of consumption of meat imitates, because information on consumption of meat imitates was not available in the FoodEx2 nomenclature.

At the proposed maximum use levels, the mean exposure to soy leghemoglobin from the use of LegH Prep as a food additive was up to 7.3 mg/kg body weight (bw) per day in toddlers and children. The 95th percentile of exposure to soy leghemoglobin was up to 32.9 mg/kg bw per day in toddlers. At the proposed typical use levels, the mean exposure to soy leghemoglobin from the use of LegH Prep as a food additive was up to 4.1 mg/kg bw per day in toddlers and children. The 95th percentile of exposure to soy leghemoglobin was up to 18.5 mg/kg bw per day in toddlers.

Two FoodEx2 codes were used as surrogates of consumption of meat imitates for estimating the exposure to soy leghemoglobin. The Panel recognised that this assumption is likely to result in an overestimation of the exposure. In addition, it was assumed that 100% of the foods in these FoodEx2 codes would contain LegH Prep at the maximum/typical proposed use levels, which is also an overestimation. The Panel noted that highest exposures are observed for infants and toddlers because, for the estimation of the dietary exposure to the proposed food additive, the consumption of minced meat (primary meat-based food consumed by infants and toddlers) is used as a surrogate for the consumption of meat imitates. Overall, the Panel considered that the uncertainties identified resulted in an overestimation of the exposure to LegH Prep at the maximum/typical proposed use levels in European countries considered in the EFSA Comprehensive database.

The Panel calculated the potential exposure to toxic elements (i.e. iAs, Pb, Cd, Hg) by combining levels of the toxic elements in the proposed food additive with the highest estimated intakes of the food additive itself. The anticipated exposure was then compared to reference points (for iAs, Pb) or health-based guidance values (for Cd and Hg). The presence of Pb, Cd and Hg in the proposed food additive would not give rise to concern. In contrast, for As, the calculated MOE values were considered to be insufficient. Taking into account the calculations performed by the Panel, and the fact that this is

not the only potential dietary source of toxic elements, the Panel considered that the maximum limits in the specifications for toxic elements should be established based on actual concentration of these impurities in the proposed food additive.

The Panel compared the amount of haem iron provided by soy leghemoglobin from its proposed uses in meat analogue products to that provided by an equivalent amount of a corresponding meat product. The Panel estimated that 100 g of the meat analogue will contain ~ [redacted] mg of haem iron, considering that one molecule of soy leghemoglobin contains one atom of haem iron and the maximum proposed use level of the soy leghemoglobin in meat analogue products (0.8%). This amount of haem iron is comparable to that provided by similar amounts of different types of meat (Lombardi-Boccia et al., 2002).

The level of iron in the proposed food additive (3850–8150 mg/kg soy leghemoglobin) combined with the estimated intakes of the proposed food additive (Table 4) could result in an exposure to iron deriving from the consumption of foods where the proposed food additive is intended to be used under the proposed conditions of use. Exposure to iron by population group resulting from the consumption of the proposed food additive at the maximum and typical proposed use levels is displayed in Table 9. The ranges reflect the variable iron content (ranging 3850–8150 mg/kg soy leghemoglobin) reported for the proposed food additive. At the maximum proposed use levels, the highest mean and highest 95th percentile exposure to the proposed food additive among the different population groups is 7.3 mg soy leghemoglobin/kg bw per day for toddlers and children, and 32.9 mg soy leghemoglobin/kg bw per day in toddlers, respectively. The resulting potential exposure to iron would be 0.03–0.06 mg/kg bw day at the mean exposure and 0.13–0.27 mg/kg bw day at the 95th percentile.

The NDA Panel established 'safe levels of intake' of iron from all dietary sources, including fortified foods and food supplements, for adults, adolescents and children. For children less than 1 year of age, safe levels of supplemental intake are given and apply to iron intakes from food supplements and fortified foods (EFSA NDA Panel, 2024). A safe level of intake for iron of 40 mg/day for adults (including pregnant and lactating women) was established (corresponding to 0.57 mg/kg bw per day). For children and adolescents, safe levels of intakes are between 10 mg/day (1–3 years) (corresponding to 0.83 mg/kg bw per day toddlers, 0.43 mg/kg bw per day children; 0.23 mg/kg bw per day for adolescent 10–14 years old) and 35 mg/day (15–17 years) (corresponding to 0.57 mg/kg bw per day). For infants 7–11 months of age, a safe level of supplemental iron intake was established at of 5 mg/day (corresponding to 1 mg/kg bw per day).

The estimate of anticipated exposure to iron from the proposed food additive, both at the mean and 95th percentile exposure, constitutes a small fraction of the safe levels of intake established by the NDA Panel for all population groups. Therefore, no safety concern was identified for the intake of haem iron based on the consumption of meat analogues containing soy leghemoglobin for the general population.

People suffering of haemochromatosis may pay attention to the iron intake from meat analogues containing soy leghemoglobin to control their iron intake. Therefore, they would need to be informed that this proposed food additive contains a source of iron.

The available information supports that the components of LegH Prep will be digested very rapidly in the gastrointestinal tract following the same pathways as other proteins consumed as part of the normal diet, including myoglobins from animal meat. The digested molecules have well understood metabolic fates following absorption and do not give rise to safety concerns. Based on this reasoning, the Panel considered the data package submitted by the applicant sufficient and did not request Tier II tests (EFSA ANS Panel, 2012).

Legh Prep was tested in a bacterial reverse mutation assay and two cytogenetic assays in human lymphocytes: a micronucleus test and a chromosomal aberration test. All these tests showed negative results. In the micronucleus and in the chromosomal aberration assays, the final maximum concentrations of soy leghemoglobin were below OECD indications (only in the chromosomal aberration assay the test item tested without S9 reached the cytotoxicity level recommended by OECD 473). Therefore, the relevance of the MN assay results is limited. However, the Panel considered that there was no need to request further information since the recipient (non GM) strain qualifies for QPS status for production purposes, and therefore, no concern is expected from yeast metabolites produced during the fermentation process. Since soy leghemoglobin is constituted by a proteinaceous part, it is not expected to be mutagenic.

Legh Prep has been tested in a number of subchronic toxicity studies. In two 28-day studies, one with a 14-day predosing oestrous cycle determination, there were no adverse effects up to the highest dose tested (750 mg/kg bw per day of soy leghemoglobin).

In a 90-day study, a small increase in thyroid hormones in high-dose males was not considered adverse. In high-dose males, decreased thymus weights were observed; however, the decrease did not correlate with any histopathology findings in the thymus. The Panel considered that the no observed adverse effect level (NOAEL) for LegH Prep was at the highest dose tested 4820 and 5931 mg/kg bw per day for males and females, respectively, which correspond to the dose of 2328 mg/kg bw per day for males and 2865 mg/kg bw per day for females of soy leghemoglobin.

Regarding allergenicity, the Panel considered that it is unlikely that adverse reactions would occur after ingestion of soy leghemoglobin products following the specification and production practices described by the applicant.

Considering that (i) the components of the proposed food additive will be digested to small peptide, amino acids and haem B, (ii) the recipient (non-GM) strain qualifies for QPS status, (iii) no genotoxicity concern has been identified and iv) no adverse effects have been identified at the highest dose tested (4820 mg/kg bw per day) in a 90-day study, the Panel concluded that there was no need to set a numerical ADI and that the food additive does not raise a safety concern at the proposed use in food category 12.9 and maximum use level.

Based on the available data, the Panel concluded that the use of soy leghemoglobin from genetically modified *Komagataella phaffii* (strain MXY0541) as a new food additive does not raise a safety concern at the proposed use and use level.

This safety evaluation of the proposed food additive remains provisional subject to the ongoing safety assessment of the genetic modification of the production strain by the GMO Panel (EFSA-GMO-NL-2019-162).

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the European Commission

1.1.1 | Background as provided by the European Commission

The use of food additives is regulated under the European Parliament and Council Regulation (EC) No 1333/2008 on food additives.[1] Only food additives that are included in the Union list, in particular in Annex II to that regulation, may be placed on the market and used in foods under the conditions of use specified therein. Moreover, food additives shall comply with the specifications as referred to in Article 14 of that Regulation and laid down in Commission Regulation (EU) No 231/2012.[2]

An application has been introduced for the authorisation of the use of soy leghemoglobin from genetically modified *Pichia pastoris*[3] yeast as a colour in meat analogue products, i.e. in the food category 12.9 'Protein products, excluding products covered in category 1.8' of Annex II to Regulation (EC) No 1333/2008.

1.1.2 | Terms of Reference as provided by the European Commission

The European Commission requests the European Food Safety Authority to perform a risk assessment to provide a scientific opinion on the safety of the proposed use of soy leghemoglobin from genetically modified *Pichia pastoris* yeast as a food additive, in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.[4]

1.2 | Interpretation of the Terms of Reference (if appropriate)

Recital (12) of Regulation (EC) No 1333/2008 indicates that '*A food additive which falls within the scope of Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed should be authorised in accordance with that Regulation as well as under this Regulation*.'

Therefore, according to Article 3 (1)c of Regulation (EC) No 1829/2003,[5] EFSA and its GMO Panel received a mandate to carry out a scientific risk assessment of soy leghemoglobin from genetically modified *Komagataella phaffii* (formerly called *Pichia pastoris*) in the context of its scope as defined in application EFSA-GMO-NL-2019-162.[6] The assessment of the safety of the genetic modification is still ongoing at the time of development of the current output.

The current opinion pertains exclusively to the safety assessment of the proposed food additive soy leghemoglobin, performed in accordance with Regulation (EC) No 1333/2008.

1.3 | Additional information

1.3.1 | Information on existing evaluations and authorisations

The EFSA GMO Panel is currently evaluating the safety of the genetic modification to produce Soy leghemoglobin from genetically modified *K. phaffii* (formerly called *Pichia pastoris*), following a request submitted in 2019 (EFSA-GMO-NL-2019-162).

Soy leghemoglobin preparation from a strain of *K. phaffii* was notified in the US as generally recognised as safe (GRAS) in 2017 (FDA, 2017). Soy leghemoglobin was finally authorised as a colour additive in the US in 2019, as provided by a Rule by the Health and Human Services Department, and the Food and Drug Administration (FDA, 2019), which included soy leghemoglobin into the Listing of colour additives exempt from certification.[7]

In 2020, Health Canada assessed the safety of Soy leghemoglobin preparation as an ingredient in all simulated meat and poultry products (Health Canada, 2020). The proposed ingredient, falling into the definition of novel food according to Canadian Food and Drug Regulations, was considered safe for human consumption, as long as the level of the functional component (i.e. leghemoglobin) is below 0.8% (which is comparable to the myoglobin content of beef (i.e. 0.8%–1.8%).

In 2020, Food Standards Australia New Zealand (FSANZ, 2020) assessed the safety of soy leghemoglobin, concluding that it raised no public health and safety concerns associated with its use in meat analogue products at the proposed maximum use level of 0.8% in raw product (as it is the lower end of the myoglobin content of red meat [0.8%–1.8%]).

[1]Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16.

[2]Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, p. 1.

[3]Currently named *Komagataella phaffii (K. phaffii)*.

[4]Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1.

[5]Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1.

[6]EFSA question available on OpenEFSA portal: https://open.efsa.europa.eu/questions/EFSA-Q-2019-00651?search=2019+162.

[7]21 CFR Part 73. https://www.ecfr.gov/current/title-21/part-73.

After a safety evaluation conducted in 2020, Singapore Food Agency (SFA) considered soy leghemoglobin to be safe and authorised its use in meat analogues at a level not exceeding 0.45% (w/w) (Singapore Food Agency (SFA), 2020), as provided in Regulation 28 of Singapore's Food Regulations.[8]

2 | DATA AND METHODOLOGIES

2.1 | Data

The present evaluation is based on the data submitted in the original application dossier (Documentation provided to EFSA No. 1) and additional information submitted by the applicant during the assessment process following requests by EFSA in August 2022 (Documentation provided to EFSA No. 2), in December 2022 (Documentation provided to EFSA No. 3), in July 2023 (Documentation provided to EFSA No. 4), in November 2023 (Documentation provided to EFSA No. 5) and in April 2024 (Documentation provided to EFSA No. 6).

2.2 | Methodologies

This opinion was formulated following the principles described in the EFSA Guidance of the Scientific Committee on transparency with regard to scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing Guidance documents from the EFSA Scientific Committee.

The current 'Guidance for submission for food additive evaluation' (EFSA ANS Panel, 2012), 'Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use' (EFSA GMO Panel, 2011a) and the 'Scientific Guidance for the submission of dossiers on Food Enzymes' (EFSA CEP Panel, 2021) have been followed by the FAF Panel for evaluating the proposed food additive.

3 | ASSESSMENT

3.1 | Technical data

3.1.1 | Identity of the substance

The proposed food additive, 'LegH Prep', is a liquid preparation containing soy leghemoglobin from genetically modified yeast *K. phaffii*, other compounds derived from the fermentation process, and added ingredients. LegH Prep is presented by the applicant as a liquid, reddish-brown concentrate, soluble in aqueous solvents. The proposed food additive is intended to be used as a colour in meat analogue products (Documentation provided to EFSA No. 1).

The functional component of LegH Prep is soy leghemoglobin from genetically modified *K. phaffii* yeast. Soy leghemoglobin is a small holoprotein (16 kDa) consisting of a polypeptide chain containing haem group and is responsible for the colour of the product. In the interest of simplicity, the Panel will use the term 'soy leghemoglobin' when referring to 'soy leghemoglobin protein'. In nature, soy leghemoglobin is expressed within the nitrogen-fixing root nodule of the soybean plant (*Glycine max*), and it exists in symbiotic relationship with haem-producing bacteria within the root nodules (O'Brian et al., 1987). According to Whittaker et al. (1981), four major types of soy leghemoglobin may be found in soybean nodules: Lba, Lbc_1, Lbc_2 and Lbc_3. The applicant states that, to produce LegH Prep, soy leghemoglobin type Lbc_2 is used. Soy leghemoglobin is identified under the CAS number 2411761-31-2 (Documentation provided to EFSA No. 1).

The applicant analysed the three-dimensional structure of soy leghemoglobin using X-ray crystallography and compared it with other plant haemoglobins and animal myoglobins. According to the applicant, the results support the statement that soy leghemoglobin is similar in tertiary structure to plant haemoglobins and animal myoglobins (Documentation provided to EFSA No. 1). The Panel agrees with this conclusion.

3.1.2 | Proposed specifications

The specifications for LegH Prep proposed by the applicant and revised by the Panel are presented in Table 1.

Column B reports the specifications as proposed by the applicant (Documentation provided to EFSA No. 3 and 6). Based on the available technical data, the Panel proposed some modifications to the proposed specifications to clarify the identity of the proposed food additive (Column C).

[8]Sale of Food Act (chapter 283) food (amendment no. 3) Regulations 2020. https://www.sfa.gov.sg/docs/default-source/legislation/sale-of-food-act/food-(amendment-no-3)-regulations-2020.pdf.

TABLE 1 Product specifications as proposed by the applicant (Column B) and proposed revision by the Panel (Column C).

A. Parameter	B. Specification as proposed by the applicant		C. Specification as revised by the panel
Definition	LegH Prep is a mixture obtained from the cellular lysate of a genetically modified strain of *Komagataella phaffii* expressing the unmodified soy leghemoglobin protein from *K. phaffii* yeast. LegH Prep is produced via fermentation of the yeast *K. phaffii*. The *K. phaffii* production strain contains a series of genetic modifications to allow it to express the soy leghemoglobin protein. Following fermentation, the cells are lysed, and the soy leghemoglobin is concentrated by physical means, including ultrafiltration and other suitable processes The soy leghemoglobin is delivered in a preparation (LegH Prep) that is standardised to contain the desired level of soy leghemoglobin on a wet weight basis and a soy leghemoglobin protein purity content of at least 65% of the total protein fraction. The remainder of the protein fraction in LegH Prep is accounted for by residual proteins from the *K. phaffii* production strain. No viable cells of the production strain shall be detected in LegH Prep		LegH Prep is a mixture obtained from the cellular lysate of the genetically modified strain (MXY0541) of *Komagataella phaffii* expressing the unmodified soy leghemoglobin from *K. phaffii* yeast. LegH Prep is produced via fermentation by the yeast *K. phaffii*. The *K. phaffii* production strain contains a series of genetic modifications to allow it to express the soy leghemoglobin. Following fermentation, the cells are lysed, and the soy leghemoglobin is concentrated by physical means (e.g. ultrafiltration) The soy leghemoglobin is marketed in a preparation (LegH Prep) that is standardised to contain the desired level of soy leghemoglobin on a wet weight basis and a soy leghemoglobin content of at least 65% of the total protein fraction. The remainder of the protein fraction in LegH Prep is accounted for by residual proteins from the *K. phaffii* production strain. No viable cells of the production strain shall be detected in LegH Prep
Trivial name	Leghemoglobin C2 (Lbc2) from genetically modified strain of *K. phaffii* expressing the unmodified soy LegH2 protein		Unchanged
CAS number	2411761-31-2		Unchanged
Molecular weight	16 kDa		Unchanged
Assay	≥ 4% soy leghemoglobin protein		≥ 4% soy leghemoglobin
Description	Aqueous preparation that imparts a reddish brown colour		Reddish brown liquid concentrate
Identification			
Appearance	Liquid		Unchanged
Colour	Reddish brown		Considered unnecessary by the Panel
Soy leghemoglobin protein purity	≥ 65%[a]		Unchanged
Purity			
Moisture	≤ 90%		Unchanged
Solids	≤ 26%		Unchanged
Ash	≤4%		Unchanged
Mercury	< 1.25	mg/kg soy leghemoglobin protein	Maximum limit to be lowered on the basis of the information provided in the dossier and on the considerations of the Panel (Table 8)
Cadmium	< 5	mg/kg soy leghemoglobin protein	Maximum limit to be lowered on the basis of the information provided in the dossier and on the considerations of the Panel (Table 8)
Arsenic	< 1.25	mg/kg soy leghemoglobin protein	Maximum limit to be lowered on the basis of the information provided in the dossier and on the considerations of the Panel (Table 8)
Lead	< 10	mg/kg soy leghemoglobin protein	Maximum limit to be lowered on the basis of the information provided in the dossier and on the considerations of the Panel (Table 8)
Microbiological criteria			
Salmonella spp.	Absent by test		Negative per 25 g
Escherichia coli EHEC (inclusive of O157:H7)	Absent by test		Negative per 25 g
Listeria monocytogenes	Absent by test		Negative per 25 g

[a]The purity of the soy leghemoglobin protein is calculated as the concentration of soy leghemoglobin protein divided by the concentration of the total protein in LegH Prep preparation (Documentation provided to EFSA No. 2).

The applicant provided certificates of analysis for five batches of LegH Prep, showing that LegH Prep proposed to be used as a food additive is manufactured in compliance with the proposed specifications as given in Table 1 (Documentation provided to EFSA No. 1).

Analyses included composition, toxic elements and microbial counts. Content of the functional component (i.e. soy leghemoglobin) in LegH Prep was analysed according to a method developed by the applicant that used ultrahigh-performance liquid chromatography (UPLC) UV VIS and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Based on the analytical results for more than five samples, coming from three different production strains (i.e. the final production strain plus two additional strains, closely related to the final production strain), moisture ranged from 79.7% to 87.5% w/w, carbohydrates ranged from 0.99% to 4.1% and fats accounted for up to 1.7% of the total weight. The content of soy leghemoglobin ranged from 4.5 to 6.7%, corresponding to 68%–82% of the total protein fraction. The remaining protein fraction consists of residual yeast proteins. According to the applicant, no exogenous proteins are added, and no soy leghemoglobins other than Lbc_2 type are present. The total protein content in LegH Prep, calculated by the Kjeldahl method, was analysed in five additional batches [redacted] (Documentation provided to EFSA No. 2).

The applicant proposed not to set an upper limit for the content of soy leghemoglobin in LegH Prep, provided that the amount of LegH Prep added to foods is such that the levels of soy leghemoglobin in foods are maintained below the proposed maximum use levels. The Panel agreed with this proposal.

The Panel considered that the food additive should be better described as a reddish brown liquid concentrate.

As regards toxic elements, the applicant provided analytical data for levels of arsenic (As), lead (Pb), cadmium (Cd) and mercury (Hg) for five independent batches of LegH Prep (Documentation provided to EFSA No. 1). Analyses were conducted using validated methods. For the five batches of LegH Prep, As ranged from 0.01 to 0.04 mg/kg, while Pb, Cd and Hg were reported as below the limit of quantification (LOQ) of 0.01, 0.001 and 0.005 mg/kg, respectively. These values were expressed per kg of whole preparation (i.e. LegH Prep).

Further to an EFSA request, the applicant proposed maximum limits for such toxic elements based on soy leghemoglobin content (i.e. the functional component) (see Table 1). The Panel converted the reported analytical data, originally expressed per kg of whole preparation (i.e. LegH Prep), to the amount of each impurity expressed per kg of functional component (i.e. soy leghemoglobin). Considering the conservative scenario of 4% soy leghemoglobin, the reported levels for As were 0.25–1 mg/kg, while Pb, Cd and Hg were reported as below the LOQ of 0.25, 0.025 and 0.125 mg/kg, expressed per kg soy leghemoglobin, respectively. The Panel noted that there is a wide discrepancy between the reported data and these proposed maximum limits which are significantly higher than the reported levels. The anticipated impact of the proposed specification limits and of the reported analytical data on the potential exposure to these toxic elements due to the proposed use of LegH Prep as a food additive is described in Section 3.3.3 (Tables 7 and 8).

Additional information was submitted on the concentrations of other elements, including iron, in eight batches of LegH Prep (Documentation provided to EFSA No. 2). Iron ranged from 154 to 326 mg/kg, expressed as mg per kg of whole preparation (i.e. LegH Prep).

Iron concentration can be expressed on the basis of the functional component (i.e. soy leghemoglobin). The Panel considered a conservative scenario of 4% soy leghemoglobin out of the whole preparation, resulting in an iron content of 3850–8150 mg/kg soy leghemoglobin based on the measured concentration of iron in the preparation. The potential exposure to iron from the use of the proposed food additive is described in Section 3.3.4.

The applicant proposed that the authorisation should not be linked to any specific production strain, to accommodate future improvements to the strain. However, LegH Prep was obtained from cellular lysate of a specific *K. phaffii* production strain (MXY0541), responsible for the synthesis of soy leghemoglobin. For this reason, the Panel considered this proposal to be inadequate, noting that the current assessment of the safety of LegH Prep as a food additive is linked to the specified production strain (MXY0541) and any modification of the production strain would need a safety assessment for the resulting production strain. The Panel recommends the European Commission to introduce the production strain MXY0541 in the *Definition* of the specifications.

The Panel noted that, according to the proposed definition in the specifications provided by the applicant, no viable cells should be detected in the final product (i.e. LegH Prep). The Panel further noted that the analyses were conducted according to the applicable conditions and controls as described in section 1.3.4.1 of the guidance document published by the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids in 2021 (EFSA CEP Panel, 2021).

3.1.3 | Manufacturing process

The applicant reported information on the production process of LegH Prep (Documentation provided to EFSA No. 1). The first step is the fermentation, using as inoculum the yeast production strain *K. phaffii*, MXY0541 genetically modified to synthetise soy leghemoglobin. Frozen cell banks of the production organism are stored at −80°C in 20% v/v glycerol, which according to the applicant meets the EU specifications for E 422 outlined in Regulation (EU) No 231/2012.[9] Following fermentation, *K. phaffii* MXY0541 cells in the fermentation broth are lysed by physical means (e.g. mechanical shearing, high pressure homogenisation). The lysate is then centrifugated to remove insoluble material and concentrated (e.g. by ultrafiltration). During the concentration step, water content is adjusted to meet the proposed specifications (Documentation

[9]Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, p. 1.

provided to EFSA No. 2). The resulting concentrate undergoes heat treatment to ensure microbial stability and is then formulated with other ingredients. The resulting product is finally stored as a frozen liquid.

When describing the manufacturing process, the applicant stated that the LegH Prep is standardised to contain up to 9% soy leghemoglobin (i.e. the functional component) on a wet weight basis. However, in response to the Panel's request for additional information, the applicant clarified that this value was not intended to imply an upper limit of soy leghemoglobin concentration, but rather to provide an expected typical value (Documentation provided to EFSA No. 2).

Additional details were provided by the applicant on the heating step (Documentation provided to EFSA No. 2), specifying heating conditions and supporting that the heating process does not impact protein integrity.

3.1.3.1 | *Undesirable substances from the production process*

According to the applicant, no undesirable substances are expected in LegH Prep as a carryover from the production process. The production organism, the yeast *K. phaffii*, has been designated as QPS (EFSA BIOHAZ Panel, 2018), and it has been genetically modified to produce soy leghemoglobin by introducing the heterologous gene LGB2 from soy (*Glycine max*) and by upregulating the native haem biosynthetic pathway (Documentation provided to EFSA No. 1). The applicant stated that components in the fermentation media are mostly consumed by the yeast during growth. Analyses conducted by the applicant on five batches of LegH Prep (Documentation provided to EFSA No. 2) demonstrated that no detectable levels of methanol (a component in the fermentation media) are present, and that the concentration of antifoaming agent in the final product is low enough not to represent a concern. The presence of iodine and nitrates has been investigated by the applicant and analytical data for five batches were provided. [redacted] (Documentation provided to EFSA No. 2).

3.1.3.2 | *Production organism*

The source of the additive is the yeast *K. phaffii* strain MXY0541, which has been genetically modified to produce the soy leghemoglobin by introducing the heterologous gene LGB2 from soy (*Glycine max*) (Documentation provided to EFSA No. 1). No antimicrobial resistance genes have been introduced in the production strain. The safety of the genetic modification is currently under assessment by the EFSA GMO Panel (EFSA-GMO-NL-2019-162).

The recipient strain of *K. phaffii* has QPS status with the qualification 'for production purpose only'. No viable cells of the production strain were detected (see Section 3.1.3.3). Therefore, no concern is expected from yeast metabolites produced during the fermentation process.

3.1.3.3 | *Absence of viable cells from the production strain*

The absence of viable cells of the production strain *K. phaffii* MXY0541 in the product was demonstrated in nine independent batches. One millilitre of product was suspended in 9 mL of phosphate buffer and passed through a 0.45 μm pore size filter disc. Filters were placed onto selective agar plates and incubated at 30°C for 6 days. No colonies of the production strain were produced. A positive control was included (Documentation provided to EFSA No. 3). The Panel further noted that the analyses were conducted according to the applicable conditions and controls as described in section 1.3.4.1 of the guidance document published by the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids in 2021 (EFSA CEP Panel, 2021).

3.1.4 | Methods of analysis in food

The applicant developed a method based on UPLC combined with a UV detector to detect and quantify the amount of soy leghemoglobin in LegH Prep and in extracts of a final food product (meat analogue burger) (Documentation provided to EFSA No. 1).

For the validation of the method, the concentration of soy leghemoglobin was quantified in the final preparation using soy leghemoglobin as standard.

The quantification of soy leghemoglobin in the meat analogue matrix was carried out by the validated method developed by the applicant. Recovery studies were conducted in the product under different storage conditions and prepared with several concentrations of either fresh or 5-month-old LegH Prep. The samples were prepared by incubating 2 g of the burger (previously ground in a freezer/mill) in the extraction buffer under mechanical agitation, centrifuged, filtered and stored refrigerated or frozen before UPLC analysis. For the analysis, the standards and samples were injected. Only the standards were injected in replicates of five. The method performance at a 95% confidence level was LOD of 0.02 mg/mL and LOQ of 0.08 mg/mL. The upper limit for quantification was 2 mg/mL.

The recoveries of soy leghemoglobin from the product prepared at 0.8% (proposed maximum use level) at 0.4% and 1.2% soy leghemoglobin (0.5 and 1.5 times the proposed maximum use level) were in the range of 85–115%. When the product was prepared at 0.55% and at the proposed maximum use level (0.8%) with 5-month-old leghemoglobin, and stored at 4°C for 9 days, the recoveries were also within the same range of 85%–115%. The frozen storage study at –20°C for 6 months, using fresh LegH Prep at 0.55% in the product, resulted in the same recovery range (85%–115%) as in the previous experiments.

3.1.5 | Stability of the substance, and reaction and fate in food

The applicant studied the stability of LegH Prep as such and when incorporated into a meat analogue product (Documentation provided to EFSA No. 1).

Eleven different batches of LegH Prep as such were tested for stability as a frozen liquid at −20°C for up to 26 months. The stability was determined in terms of soy leghemoglobin concentration and purity. pH was also monitored. Results indicated that the concentration of soy leghemoglobin remained stable during the storage.

The stability of LegH Prep incorporated into a meat analogue was tested during storage for up to 9 days at 4°C, and after 6 months at −20°C. For each test, two different batches of LegH Prep were used. Stability was investigated by the % recovery of soy leghemoglobin from the meat analogue. Results indicated that the % of recovery soy leghemoglobin slightly decreased from the beginning of the storage (98%–105%) respect to the end of refrigerated storage test after 9 days (85%–92%) and after 6 months of storage at −20°C (85.1%–87.6%).

According to the applicant, soy leghemoglobin contains a haem B group, similar to animal myoglobins and other plant haemoglobins. As in animal-based meat, the haem B group is responsible for the red colour of the uncooked product, which is due to the iron-bound oxygen within the haem B molecule. The cooking process causes the oxidation of iron, and thus, the oxygen molecule is released, resulting in the loss of red colour. Similar to other globin proteins, when soy leghemoglobin is heated as in cooking above approximately 62°C or exposed to a low pH environment, as in the human stomach it denatures, and the haem B group is released, playing a major role in generating the flavours and aromas characteristic of cooked meat (Documentation provided to EFSA No.1).

The applicant provided additional information on the interactions of LegH Prep with food (Documentation provided to EFSA No. 2). The applicant stated that based on the structural similarities between soy leghemoglobin, animal myoglobins and other plant haemoglobins, the degradation products are expected to be similar, as well as the interaction of LegH Prep with food components. The Panel agrees with this statement.

3.2 | Proposed uses and use levels

The applicant submitted the proposed typical and maximum use levels for the proposed food additive, as described in the specifications, for a single food category, according to food categories in Part D of Annex II of Regulation (EC) No 1333/2008. The use levels were expressed in terms of soy leghemoglobin (i.e. the functional component). The use levels for the whole preparation (i.e. LegH Prep) were also provided (Table 2). Use levels for LegH Prep were provided considering the concentration of soy leghemoglobin in the whole preparation (i.e. 4%–6.74%, reflecting the specifications) (Documentation provided to EFSA No. 1 and 2).

TABLE 2 Proposed typical and maximum use levels of the food additive, expressed in terms of both mg of soy leghemoglobin per kg of final food product and mg of LegH Prep per kg of final food product.

Food category number	Food category name	Restrictions	Expressed as	[redacted]	Proposed maximum use levels (mg/kg)
12.9	Protein products, excluding products covered in category 1.8	Only meat imitates	Soy leghemoglobin	[redacted]	8000
			LegH Prep	[redacted]	118,700–200,000

3.3 | Exposure data

3.3.1 | Food consumption data used for exposure assessment

EFSA comprehensive European food consumption database

Since 2010, the EFSA Comprehensive European Food Consumption Database has been populated with national data on food consumption at a detailed level. Competent authorities in the European countries provide EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. EFSA Guidance on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011)). The version of the Comprehensive database taken into account in this assessment was published in December 2022.[10]

The food consumption data gathered by EFSA were collected by different methodologies and thus direct country-to-country comparisons may not be appropriate. Depending on the food category and the level of detail used for exposure calculations, uncertainties could be introduced owing to possible subjects' underreporting and/or misreporting of the consumption amounts. Nevertheless, the EFSA Comprehensive Database includes the currently best available food consumption data across Europe.

[10] https://www.efsa.europa.eu/en/data-report/food-consumption-data#the-efsa-comprehensive-european-food-consumption-database.

Food consumption data from infants, toddlers, children, adolescents, adults and the elderly were used in the exposure assessment. For the present assessment performed with DietEx, food consumption data were available from 43 different dietary surveys carried out in 22 European countries (Table 3).

TABLE 3 Population groups considered for the exposure estimates of soy leghemoglobin from genetically modified *Komagataella phaffii*.

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From more than 12 weeks up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain
Children	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

Consumption records were codified according to the FoodEx2 classification system, which is the nomenclature used in DietEx (EFSA, 2015). Nomenclature from the FoodEx2 classification system was linked to the food categorization system (FCS) as presented in Annex II of Regulation (EC) No 1333/2008, part D, which is the nomenclature used in FAIM, to perform the exposure assessments. In practice, the FoodEx2 food codes were matched to the FCS food categories.

Food categories considered for the exposure assessment of soy leghemoglobin from genetically modified *Komagataella phaffii* using DietEx

The food categories for which the use of soy leghemoglobin from genetically modified *K. phaffii* is proposed were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx2 classification system), at the most detailed level possible (up to FoodEx2 Level 7) (EFSA, 2015).

Exposure estimations were performed using the DietEx tool by selecting the two FoodEx2 codes 'Bovine, minced meat' and 'Bovine and pig, minced meat', used as surrogates for the consumption of meat imitates, as the consumption database does not contain eating occasions of the FoodEx2 code 'meat imitates'. This assumption is likely to result in an overestimation of the exposure.

3.3.2 | Exposure to soy leghemoglobin from genetically modified *Komagataella phaffii*

Estimate of exposure based on the food additives intake model (FAIM) tool

The applicant provided an estimate of exposure to the proposed food additive based on the output obtained using the FAIM tool at the proposed maximum use levels provided (Documentation provided to EFSA No. 1), which is compliant with the requirement.

However, the Panel considered that the FAIM tool is not appropriate in this case since the food category available in FAIM (FC 12.9 'Protein products, excluding products covered in category 1.8') is broader than the requested use by the applicant, which is restricted to 'only meat imitates'.

For this reason, EFSA estimated the exposure by using the DietEx tool, rather than the FAIM tool.

Estimate of exposure based on DietEx tool

Exposure estimations were performed by the Panel using the DietEx tool,[11] selecting the two FoodEx2 codes 'Bovine, minced meat' and 'Bovine and pig, minced meat' (See Appendix A and B for detailed exposure calculations). The proposed maximum and typical use levels refer to the technologically active substance of the food additive i.e. soy leghemoglobin as proposed by the applicant (see Section 3.2).

[11] https://www.efsa.europa.eu/en/science/tools-and-resources/dietex.

TABLE 4 Summary of dietary exposure to soy leghemoglobin from the use of LegH Prep as a food additive at the proposed maximum/typical use levels in six population groups, estimated by EFSA using the DietEx tool (minimum–maximum across the dietary surveys in mg/kg bw per day).

	Infants (12 weeks-11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
Proposed maximum use level exposure assessment scenario (mg/kg bw per day)						
Mean	0.0[a]–1.4	0.0[a]–7.3	0.0[a]–7.3	0.0[a]–4.6	0.0[a]–3.0	0.0[a]–1.8
95th percentile	0.0[b]–11.5	0.0[b]–32.9	0.0[b]–28.0	0.0[b]–18.0	0.0[b]–13.2	0.0[b]–11.7
Proposed typical use level exposure assessment scenario (mg/kg bw per day)						
Mean	0.0[a]–0.8	0.0[a]–4.1	0.0[a]–4.1	0.0[a]–2.6	0.0[a]–1.7	0.0[a]–1.0
95th percentile	0.0[b]–6.5	0.0[b]–18.5	0.0[b]–15.8	0.0[b]–10.1	0.0[b]–7.4	0.0[b]–6.6

[a]The low end of the range equals to 0.0 mg/kg since certain surveys showed no eating events for the chosen food categories.
[b]95th percentile is null when there are less than 5% consumers.

At the proposed maximum use levels, the mean exposure to soy leghemoglobin from the use of LegH Prep as a food additive resulted up to 7.3 mg/kg bw per day in toddlers and children. The 95th percentile of exposure to soy leghemoglobin was up to 32.9 mg/kg bw per day in toddlers.

At the proposed typical use levels, the mean exposure to soy leghemoglobin from the use of LegH Prep as a food additive resulted up to 4.1 mg/kg bw per day in toddlers and children. The 95th percentile of exposure to soy leghemoglobin was up to 18.5 mg/kg bw per day in toddlers.

Main food categories contributing to exposure to soy leghemoglobin from genetically modified *Komagataella phaffii*

Among the two FoodEx2 codes of 'Bovine, minced meat' and 'Bovine and pig, minced meat', the 'Bovine, minced meat' is contributing more in most of the dietary surveys (See Appendix C).

Uncertainty analysis

In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and summarised in Table 5.

TABLE 5 Qualitative evaluation of influence on uncertainties on the dietary exposure estimate.

Sources of uncertainties	Direction[a]
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Methodology used to estimate high percentiles (95th) long-term (chronic) exposure based on data from food consumption surveys covering only a few days	+
Correspondence of proposed use levels to the food items in the EFSA Comprehensive Database: uncertainties to which types of food the levels refer	+/–
Food categories selected for the exposure assessment: use of two foods to account for the consumption of meat imitates	+/–
Concentration data: Proposed maximum/typical use levels considered applicable to all foods within the entire foodEx2 code, whereas most probably not all eating occasions belonging to a proposed foodEx2 code will contain LegH Prep as a food additive	+

[a]+, uncertainty with potential to cause overestimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

LegH Prep is requested to be authorised in meat imitates within the food category 12.9 'Protein products, excluding products covered in category 1.8'. Two FoodEx2 codes were considered for estimating the exposure to this substance because information on consumption of meat imitates was not available. However, the Panel recognised that this assumption is likely to result in an overestimation of the exposure. In addition, it was assumed that 100% of the foods in these FoodEx2 codes would contain LegH Prep at the maximum/typical proposed use levels which is an overestimation. The Panel noted that highest exposures are observed for infants and toddlers because, for the estimation of the dietary exposure to the proposed food additive, the consumption of minced meat (main meat-based food consumed by infants and toddlers) is used as a surrogate for the consumption of meat imitates.

Overall, the Panel considered that the uncertainties identified resulted in an overestimation of the exposure to LegH Prep at the maximum/typical proposed use levels in European countries considered in the EFSA Comprehensive database.

3.3.3 | Anticipated exposure to toxic elements from the use of the proposed food additive

The applicant provided maximum limits, expressed per kg of functional component (i.e. soy leghemoglobin), for As (1.25 mg/kg), Pb (10 mg/kg), Cd (5 mg/kg) and Hg (1.25 mg/kg) in the proposed food additive (Documentation provided to EFSA

No. 3 and 6), for the purpose of defining appropriate specifications (Table 1). As noted already, the actual data provided for the five batches of the proposed food additive were much lower than the maximum limits proposed in the specifications.

Levels of toxic elements in the proposed food additive combined with the highest estimated intakes of the additive itself (mean and the 95th percentile resulting from the exposure assessment using DietEx for the maximum use level scenario among the different population groups, i.e. 7.3 mg soy leghemoglobin/kg bw per day for toddlers and children, and 32.9 mg soy leghemoglobin/kg bw per day in toddlers, respectively) presented in Table 4, could result in an exposure which can be compared with the following reference points (RP) or health-based guidance values (HBGV) for the toxic elements (Table 6). It is considered that any mercury or arsenic in the food additive correspond to the elements in the inorganic form rather than organic forms. Consequently, for the comparison, the HBGV for inorganic mercury and the RP for inorganic arsenic are used (Table 6).

TABLE 6 Reference points/health-based guidance value for toxic elements potentially present in the proposed food additive.

Element HBGV/RP (μg/kg bw/d or/w)	Basis/reference
Inorganic arsenic (iAs)/0.06 μg/kg bw per day (BMDL05)	The reference point is based on a benchmark dose lower confidence limit (BMDL05) of 0.06 μg/kg bw per day identified for skin cancer. The reference point is considered to cover lung cancer, bladder cancer, skin lesions, ischaemic heart disease, chronic kidney disease, respiratory disease, spontaneous abortion, stillbirth, infant mortality and neurodevelopmental effects. An MOE of 1 would correspond to the exposure level that is associated with a 5% increase relative to the background incidence for skin cancer, based on the available data. An MOE of 1 raises a health concern Because there are no precedents in EFSA for identification of an MOE of low concern, when using a BMDL derived from human cancer data the CONTAM Panel decided not to determine a value for an MOE of low concern EFSA CONTAM Panel (2024)
Lead (Pb)/0.5 μg/kg bw per day ($BMDL_{01}$)	The reference point is based on a study demonstrating perturbation of intellectual development in children with the critical response size of 1 point reduction in IQ. The EFSA CONTAM Panel mentioned that a 1 point reduction in IQ is related to a 4.5% increase in the risk of failure to graduate from high school and that a 1 point reduction in IQ in children can be associated with a decrease of later productivity of about 2%. A risk cannot be excluded if the exposure exceeds the $BMDL_{01}$ (MOE lower than 1) EFSA CONTAM Panel (2010)
Mercury (Hg)/4 μg/kg bw per week (TWI)	The HBGV was set using kidney weight changes in male rats as the pivotal effect. Based on the $BMDL_{10}$ of 0.06 mg/kg bw per day, expressed as mercury, and an uncertainty factor of 100 to account for inter- and intraspecies differences, with conversion to a weekly basis and rounding to one significant figure, a TWI for inorganic mercury of 4 μg/kg bw per week, expressed as mercury was established. EFSA CONTAM Panel (2012)
Cadmium (Cd)/2.5 μg/kg bw per week (TWI)	The derivation of the reference point is based on a meta-analysis to evaluate the dose–response relationship between selected urinary cadmium and urinary beta-2-microglobulin as the biomarker of tubular damage recognised as the most useful biomarker in relation to tubular effects. A group-based $BMDL_5$ of 4 μg Cd/g creatinine for humans was derived. A chemical-specific adjustment factor of 3.9 was applied to account for human variability in urinary cadmium within each dose-subgroup in the analysis resulting in a reference point of 1.0 μg Cd per g creatinine. In order to remain below 1 μg Cd/g creatinine in urine in 95% of the population by age 50, the average daily dietary cadmium intake should not exceed 0.36 μg Cd/kg bw, corresponding to a weekly dietary intake of 2.5 μg Cd/kg bw EFSA CONTAM Panel (2009)

Abbreviations: $BMDL_{01}$, benchmark dose (lower confidence limit); HBGV, Health-based guidance value; MOE, margin of exposure; RP, Reference point; TWI, Tolerable Weekly Intake.

The risk assessment of the undesirable impurities helps inform whether there could be a possible health concern if these impurities would be present in the proposed food additive according to two different concentration scenarios: (i) at the maximum limit values in the food additive as proposed by the applicant in the specifications (Table 7); or (ii) if the limits were established based on the analytical data reported for five batches of the proposed food additive (Table 8). In this second scenario, the rounded up highest measured level of the toxic element was considered (1 mg/kg for As). In the absence of any measured value(s), the reported LOQs were considered (0.25 mg/kg for Pb, 0.125 mg/kg for Hg, 0.025 mg/kg for Cd) and a factor of 5 was applied to allow flexibility with respect to representativeness, homogeneity and differing analytical methods. All limits are expressed per kg of functional component (i.e. soy leghemoglobin). The analytical data reported by the applicant have been converted by the Panel to reflect the concentration of toxic elements per kg of functional component (i.e. soy leghemoglobin) (see Section 3.1.2).

The Panel emphasised that the choice of the maximum limits for toxic elements in the specifications is in the remit of the risk management. The numbers used here were merely taken to support the risk assessment of these toxic elements as presented below.

TABLE 7 Risk assessment for toxic elements using the maximum limits provided in the specifications as proposed by the applicant (Documentation provided to EFSA No. 3 and 6).

Exposure to soy leghemoglobin (mg/kg bw/day)	MOE for iAs at 1.25 mg/kg[c]	MOE for Pb at 10 mg/kg[c]	% of the TWI for Cd at 5 mg/kg[c]	% of the TWI for Hg at 1.25 mg/kg[c]
7.3[a]	6.6	6.8	10.2	1.6
32.9[b]	1.5	1.5	46.1	7.2

Abbreviation: iAs, inorganic arsenic.

[a]Highest mean exposure level among the different population groups (proposed maximum use level exposure assessment scenario).

[b]Highest 95th percentile exposure level among the different population groups (proposed maximum use level exposure assessment scenario).

[c]Kg of functional component (i.e. soy leghemoglobin).

TABLE 8 Risk assessment for toxic elements using the analytical data provided by the applicant and expressed per kg of functional component (i.e. soy leghemoglobin) (Documentation provided to EFSA No. 1). The rounded up highest measured value of the level of the toxic element was used (this was only possible for arsenic) or, in the absence of any measured value(s), the reported LOQ and by applying a factor of 5.

Exposure to soy leghemoglobin (mg/kg bw/day)	MOE for iAs at 1 mg/kg[c]	MOE for Pb at 1.25 mg/kg[c]	% of the TWI for Cd at 0.125 mg/kg[c]	% of the TWI for Hg at 0.625 mg/kg[c]
7.3[a]	8.2	54.8	0.3	0.8
32.9[b]	1.8	12.2	1.2	3.6

Abbreviation: iAs, inorganic arsenic.

[a]Highest mean exposure level among the different population groups (proposed maximum use level exposure assessment scenario).

[b]Highest 95th percentile exposure level among the different population groups (proposed maximum use level exposure assessment scenario).

[c]Kg of functional component (i.e. soy leghemoglobin).

The resulting figures in Tables 7 and 8 show that the presence of Pb, Cd and Hg in the proposed food additive either at the proposed maximum limits or at the values selected by the Panel would not give rise to concern. In contrast, for As, the calculated MOE values were considered to be insufficient, irrespective of whether these were calculated from the proposed maximum limit in the specifications (Table 7) or from the value selected by the Panel (Table 8). Taking into account the calculations performed by the Panel, and the fact that this is not the only potential dietary source of toxic elements, the Panel considered that the maximum limits in the specifications for toxic elements should be established based on actual concentration of these impurities in the proposed food additive.

3.3.4 | Anticipated exposure to iron from the use of the proposed food additive

The level of iron in the proposed food additive (3850–8150 mg/kg soy leghemoglobin) combined with the estimated intakes of the proposed food additive (Table 4) could result in an exposure to iron deriving from the consumption of foods where the proposed food additive is intended to be used under the proposed conditions of use. Exposure to iron by population group resulting from the consumption of the proposed food additive at the maximum and typical proposed use levels is displayed in Table 9. The ranges reflect the variable iron content (ranging 3850–8150 mg/kg soy leghemoglobin) reported for the proposed food additive. At the maximum proposed use levels of the proposed food additive, the highest mean estimate among the different population groups was identified in toddlers and children (7.3 mg soy leghemoglobin/kg bw per day), and the highest 95th percentile estimate was identified in toddlers (32.9 mg soy leghemoglobin/kg bw per day). Hence, the resulting potential exposure to iron would be 0.03–0.06 mg/kg bw day at the mean exposure in toddlers and children, and 0.13–0.27 mg/kg bw day at the 95th percentile in toddlers.

TABLE 9 Exposure to iron (mg/kg bw per day) deriving from the consumption of the proposed food additive by population group. 'Safe levels of intake' of iron taken from EFSA NDA Panel (2024) are also reported.

	Infants (12 weeks to 11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
Exposure to iron (mg/kg bw per day) deriving from the consumption of the proposed food additive by population group at the proposed maximum use level						
Mean[a]	0.01–0.01	0.03–0.06	0.03–0.06	0.02–0.04	0.01–0.02	0.01–0.01
95th percentile[a]	0.04–0.09	0.13–0.27	0.11–0.23	0.07–0.15	0.05–0.11	0.05–0.10
Exposure to iron (mg/kg bw per day) deriving from the consumption of the proposed food additive by population group at the proposed typical use level						
Mean[a]	0.00–0.01	0.02–0.03	0.02–0.03	0.01–0.02	0.01–0.01	0.00–0.01
95th percentile[a]	0.03–0.05	0.07–0.15	0.06–0.13	0.04–0.08	0.03–0.06	0.03–0.05
Safe levels of intake per population group (EFSA NDA Panel, 2024) expressed as mg/kg bw[b] day						
	1	0.83	0.43	0.23–0.57	0.57	0.57

[a]Only the upper bound of the range of exposure to the proposed food additive has been considered.

[b]Default body weight: Infants, 5 kg; Toddlers, 12 kg; Children, 23.1 kg; Adolescents (10–14 years), 43.4 kg; Adolescents (14–18 years), 61.3 kg; Adults and Elderly, 70 kg. EFSA Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data (EFSA Scientific Committee, 2012).

The NDA Panel established 'safe levels of intake' of iron from all dietary sources, including fortified foods and food supplements, for adults, adolescents and children. For children less than 1 year of age, safe levels of supplemental intake are given and apply to iron intakes from food supplements and fortified foods (EFSA NDA Panel, 2024). A safe level of intake for iron of 40 mg/day for adults (including pregnant and lactating women) was established (corresponding to 0.57 mg/kg bw per day). For children and adolescents, safe levels of intakes are between 10 mg/day (1–3 years) (corresponding to 0.83 mg/kg bw per day toddlers, 0.43 mg/kg bw per day children; 0.23 mg/kg bw per day for adolescent 10–14 years old) and 35 mg/day (15–17 years) (corresponding to 0.57 mg/kg bw per day). For infants 7–11 months of age, a safe level of supplemental iron intake was established at of 5 mg/day (corresponding to 1 mg/kg bw per day).

3.4 | Biological and toxicological data

3.4.1 | Absorption, distribution, metabolism and excretion

To establish whether the proposed food additive or its breakdown products are absorbed from the gastrointestinal tract, the applicant used theoretical considerations to characterise the absorption of LegH Prep components and provided data from in vitro pepsin digestibility assays at pH 2 in simulated gastric fluid (Documentation provided to EFSA No. 1).

The in vitro studies showed rapid digestion (within 2 min) of soy leghemoglobin and of *K. phaffii* proteins present in LegH Prep (Jin et al., 2018; Reyes et al., 2021). The products of protein digestion are small peptides and amino acids along with haem B released from soy leghemoglobin, and thus considered equivalent to those also present in the usual diet (e.g. myoglobins from animal meat and other haemoglobins from plants).

The available information supports that the components of LegH Prep will be digested in the gastrointestinal tract following the same pathways as other proteins consumed as part of the normal diet, including myoglobins from animal meat. The digested molecules have well understood metabolic fates following absorption and do not give rise to safety concerns. These conclusions apply equally to the food additive as such or following denaturation by cooking.

3.4.2 | Acute toxicity

No data on acute toxicity were submitted by the applicant.

3.4.3 | Genotoxicity

The potential of a soy leghemoglobin to induce gene mutations was tested in a bacterial reverse mutation assay in *S*. Typhimurium strains TA1535, TA1537, TA98 and TA100 and in E. coli WP2 uvrA, according to OECD test guideline (TG) 471 (OECD, 2020) and in compliance with good laboratory practice (GLP) (Documentation provided to EFSA No. 1). LegH Prep containing 6.74% soy leghemoglobin was tested up to a maximum concentration of 74,000 µg/plate, corresponding to a soy leghemoglobin concentration of 5000 µg/plate, in both the absence and presence of metabolic activation (chemically induced rat liver S9 mix). In the first experiment, the plate incorporation method was used. The results were then confirmed using the pre-incubation modification method.

No signs of precipitation or cytotoxicity were noticed. No increase in the number of revertant colonies was observed in any experimental condition. Positive control substances induced the expected increases in revertant colonies, confirming the sensitivity of the test.

LegH Prep containing 16.7% soy leghemoglobin was tested for the ability to induce micronuclei in human lymphocytes, in compliance with OECD TG 487 (OECD, 2016a) and with GLP principles (Documentation provided to EFSA No. 1). Two separate experiments were performed: In the first experiment, a short-term exposure (4 hours) was applied with and without metabolic activation (S9 mix); in the second experiment, a 44-hour continuous treatment was used only without metabolic activation. The timing of the continuous exposure was compatible with a duration of twice a normal cell cycle, as recommended by OECD TG 487 (OECD, 2016a). The maximum concentrations tested were 250 and 650 µg/mL in the short-term treatment with and without S9, respectively, and 750 µg/mL in the long-term treatment without S9. The maximum concentrations were selected based on precipitation and caused only moderate cytotoxicity (relative total growth ≥ 70%). At least 2000 binucleated cells per concentration were analysed.

No biologically relevant or statistically significant increase of the micronucleus frequency was noted after treatment with the test item in any experimental condition. The positive control substances induced distinct and statistically significant increases of the micronucleus frequency, demonstrating the validity of the assay. However, it should be noted that the test item contained only 16.7% soy leghemoglobin and that, due to precipitation, a rather low amount of soy leghemoglobin was tested in this study.

The potential of soy leghemoglobin to induce structural chromosome aberrations was tested in human lymphocytes, in line with OECD TG 473 (OECD, 2016b) and GLP principles (Documentation provided to EFSA No. 1). The test item was a batch of LegH Prep containing 6.74% soy leghemoglobin. Two separate experiments were performed: In the first experiment, a short-term exposure of 4 h, followed by a recovery period of 24 h, was applied with and without metabolic activation

(S9 mix); in the second experiment, a 24-h treatment, followed by a 24-h recovery time, was used only without metabolic activation.

The maximum concentrations tested were 5000 μg/mL in the short-term treatment with and without S9 and 1000 μg/mL in the long-term treatment without S9. In the long-term treatment, higher concentrations could not be tested, due to precipitation. Evident cytotoxicity was observed in the two experiments without metabolic activation: 54% relative mitotic index after the 4-h treatment (concentration 5000 μg/mL), 53% relative mitotic index after the 24-hour treatment (concentration 1000 μg/mL). No cytotoxicity was reported with metabolic activation.

No statistically significant increase in the chromosomal aberration rates was noted after treatment with the test item in any experimental condition. A total of 300 cells were evaluated for each test item concentration, 200 for the positive controls. The positive control substances induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the efficiency of the test system. It should be noted that the test item contained only 6.74% soy leghemoglobin; however, evident cytotoxicity (> 50%) was reported in the absence of S9, while no relevant reduction of the mitotic index was observed in the presence of S9.

LegH Prep was tested in a bacterial reverse mutation assay and two cytogenetic assays in human lymphocytes: a micronucleus test and a chromosomal aberration test. All these tests showed negative results. The bacterial test was conducted with very high amounts of test item; therefore, the actual concentration of soy leghemoglobin in the maximum tested dose corresponded to 5000 μg/plate, as recommended by OECD TG 471 (OECD, 2020). In the micronucleus, the maximum concentrations of soy leghemoglobin were below OECD indications (only in the chromosomal aberration assay the test item tested without S9 reached the cytotoxicity level recommended by OECD TG 473 [OECD, 2016b]). Therefore, the relevance of the results of the MN assay is limited. However, the Panel considered that there was no need to request further information since the potential genotoxicity of the proposed food additive was addressed taking into account that (i) the recipient (non-GM) producer strain is recommended for the QPS status for production purposes; therefore, no concern is expected from yeast metabolites produced during the fermentation process; (ii) soy-leghemoglobin is constituted by a proteinaceous part, that is not expected to be mutagenic, and by haem, that could be associated with the production of potentially genotoxic reactive oxygen species (ROS), due to the presence of iron (IARC, 2018). Considering that iron is an essential element, a normal constituent of the human diet and that the contribution of the additive to the iron exposure is in the same order of magnitude as that from food, the Panel concluded that there is no concern for potential genotoxicity associated with the exposure to soy leghemoglobin/haem iron.

3.4.4 | Subchronic toxicity

A 14-day dietary toxicity study was performed with LegH Prep (47.6% soy leghemoglobin), administered to groups of adult Crl: Sprague–Dawley CD® IGS rats (6/sex/group) to provide target dose levels of 0, 125, 250 and 500 mg/kg bw per day of soy leghemoglobin. No adverse effects (viability, clinical signs, body weight, food consumption, haematology, organ weights, macroscopic and microscopic findings) occurred up to the highest dose tested (Documentation provided to EFSA No. 1).

A 28-day dietary toxicity study was conducted in Crl:SD CD® IGS rats (10/sex/group). LegH Prep (48.82% soy leghemoglobin) was administered at the dose levels of 512, 1024 and 1536 mg/kg bw per day, which correspond to 250, 500 and 750 mg/kg bw per day of soy leghemoglobin as well as a basal diet control (Documentation provided to EFSA No. 1). The study was conducted according to OECD TG 407 (OECD, 2008). There were no mortalities, clinical observations, ophthalmology, body weight, body weight gain, food consumption or food efficiency changes attributable to LegH Prep administration. Decreases in uterine weight were observed in all treated female rats; however, these changes were not dose-dependent and not accompanied with adverse histopathological findings; therefore, the Panel concluded that they were not adverse. In addition, the Panel noted that there were no such changes in the other rat studies. The no observed adverse effect level (NOAEL) was determined to be 750 mg/kg bw per day of soy leghemoglobin, the highest dose tested.

A 28-day study with a 14-day pre-dosing oestrous cycle determination was conducted in Crl: Sprague–Dawley CD® IGS rats (15/group) (Documentation provided to EFSA No. 1). LegH Prep (48.82% of soy leghemoglobin) was administered at the dose levels of 512, 1024 and 1536 mg/kg bw per day, which correspond to 250, 500 and 750 mg/kg bw per day of soy leghemoglobin. No adverse effects occurred at any dose tested. The NOAEL was determined to be 750 mg/kg bw per day of soy leghemoglobin, the highest dose tested.

A 90-day dietary toxicity study in rats with a 28-day recovery period was performed with a LegH Prep (48.3% soy leghemoglobin) according to OECD TG 408 (OECD, 2018) and in compliance with GLP principles (Documentation provided to EFSA No. 3). Although the diet preparation was not done in a GLP accredited facility, the quality systems applied to the diet preparation and the analysis were sufficient to reassure the Panel that the test diet could be used in this study (Documentation provided to EFSA No. 4). Groups of Sprague–Dawley CD IGS rats (10/sex/group for the main study, with an additional 5 animals/sex/group for the 28-day recovery segment with control and high dose only) were fed diets containing 0 (control), 30,000, 60,000 or 90,000 mg/kg diet LegH Prep (concentration of soy LegH Prep added to the diet as a dehydrated powder) which corresponded to a target exposure of 0 mg/kg bw per day (control), 1875 mg/kg bw per day, 3750 mg/kg bw per day and 5625 mg/kg bw per day of LegH Prep. The actual mean overall (Days 0–91) daily intake at the three target concentrations was calculated to be 1637, 3202 and 4820 mg/kg bw per day of LegH Prep for males and 2025, 4128 and 5931 mg/kg bw per day of LegH Prep for females. In this study, there were no mortalities and no treatment-related clinical observations. The Panel noted that, in the absence of findings in clinical observations, no functional observation is

needed, according to OECD TG 408 (OECD, 2018). Body weight, body weight gain, food consumption and feed efficiency were not statistically significantly different between the control groups and treatment groups. With regard to haematological parameters, mean cell haemoglobin (MCH) and mean cell volume (MCV) were slightly increased in high-dose males, but the increase is not considered of toxicological relevance. Regarding clinical chemistry, LDL cholesterol was increased in mid-dose males. In urinalysis parameters, urine ketone was increased in mid- and high-dose males. These changes were not dose-dependent, within the natural variability. Therefore, the Panel considered these changes not treatment-related or adverse. Increases in thyroid-related hormones, i.e. TSH (in high-dose males +/– recovery), T4 (in high-dose males + recovery; decrease in high-dose females) and T3 (in all treated males) were not correlated with microscopic changes in thyroid, not consistent between males and females, and the magnitude of the effects were not biological relevant and therefore not considered adverse. In high-dose males, decreased thymus weights were observed (approx. 24%), i.e. in absolute (low and high dose), relative to body weight (low and high dose) and relative to brain weights. These decreases did not correlate with any histopathology findings in the thymus. No treatment-related histological findings were reported.

The Panel considered that under the conditions of this study, the NOAEL for LegH Prep was at the highest dose tested, i.e. 4820 and 5931 mg/kg bw per day for males and females, respectively, which corresponds to a dose of soy leghemoglobin of 2328 mg/kg bw per day for males and 2865 mg/kg bw per day for females.

3.4.5 | Reproductive and developmental toxicity

No data on reproductive and developmental toxicity were submitted by the applicant.

3.4.6 | Chronic toxicity, carcinogenicity

No data on chronic toxicity and carcinogenicity were submitted by the applicant.

3.4.7 | Immunotoxicity, hypersensitivity/allergy

The Panel's assessment of the potential risk of allergenicity focus: (i) on the source of the recombinant protein; (ii) on the potential of the newly expressed protein to induce sensitisation or to elicit allergic reactions in already sensitised persons; and (iii) on whether the transformation may have altered the allergenic properties of the modified microorganism. Furthermore, the assessment also takes into account potential adjuvant properties of the newly expressed proteins, which is defined as the ability to enhance an allergic reaction.

Proteins expressed by the introduced genes

A weight-of-evidence approach was followed, taking into account all the information obtained on the newly expressed proteins, as no single piece of information or experimental method yielded sufficient evidence to predict allergenicity (Codex Alimentarius, 2009; EFSA GMO Panel, 2011a, 2011b, 2017).

The LGB2 gene expressing the leghemoglobin protein originates from *Glycine max* (soybean), which is considered a common allergic source by Regulation.[12] In this respect, the applicant confirms that they will comply with requirements set in such Regulation regarding the labelling of this product as 'Contains Soy' (Documentation provided to EFSA No. 1).

Bioinformatic analyses of the amino acid sequences of the soy leghemoglobin protein, using the criterion of > 35% identity in a sliding window of 80 amino acids, revealed no relevant similarities to known allergens. The applicant also made reference to the potential of the alpha-gal syndrome (tick-bite induced allergy to mammalian meat). However, it is not considered a safety concern by the Panel as the antibody response is built against the disaccharide galactose-a-1,3-galactose (a-gal) located on glycoproteins and glycolipids in non-primate mammalian meat (Documentation provided to EFSA No. 1).

The applicant provided a study on the resistance to degradation by pepsin of the 'LegH Prep' in solutions at pH ~ 2 (Documentation submitted to EFSA No. 1). The integrity of the test preparation in samples of the incubation mixture taken at various time points was analysed by SDS–PAGE followed by protein staining. The leghemoglobin protein was degraded by pepsin after 2 minutes of incubation. Furthermore, the applicant identified the minimum temperature of denaturation of the soy leghemoglobin protein as ~ 65°C. While at pH ~ 2, the soy leghemoglobin protein denatures and releases the haem ligand.

In addition, the applicant provided information on the safety of the leghemoglobin protein regarding their potential hazard to cause a coeliac disease response (Documentation submitted to EFSA No. 2). For such assessment, the applicant

[12]Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. OJ L 304, 22.11.2011, p. 18.

followed the principles described in the EFSA GMO Panel guidance document (EFSA GMO Panel, 2017). The assessment of the leghemoglobin protein identified no perfect or relevant partial matches with known coeliac disease peptide sequences.

Evaluation of allergenicity of the LegH Prep

The Panel notes that *K. phaffii* is widely used as a host for expression of recombinant proteins and is included in the list of QPS-recommended biological agents intentionally added to food or feed (EFSA BIOHAZ Panel, 2018). As such, *K. phaffii* has been used to produce food enzymes and pharmaceutical agents (Ahmad et al., 2014; Balamurugan et al., 2007; Brady et al., 2020; Cereghino & Cregg, 2000; Kurtzman, 2009; Reyes et al., 2021; Sørensen, 2010) (Documentation provided to EFSA No. 1 and 2). EFSA has also evaluated the safety of a genetically modified *K. phaffii* for its use in the production of the food enzyme phospholipase C (EFSA CEP Panel, 2019). Moreover, safety evaluations of other protein products (3-phytase, 6-phytase, fumonisin esterase and endo-1,4-β-xylanase) produced from *K. phaffii* have also been carried out by EFSA (EFSA FEEDAP Panel, 2020, 2022a, 2022b, 2023). However, in this case, the intended use was as feed additive and, therefore, human exposure was not considered. According to the applicant, in terms of GRAS notifications, other food ingredients (i.e. a phospholipase C enzyme preparation, a soluble egg-white protein and a myoglobin preparation) using *K. phaffii* as expression system are currently available in the US marketplace and no reports of adverse reactions have been reported (Documentation provided to EFSA No. 1).

The applicant also provided an assessment of allergenicity with the focus on *K. phaffii* residual proteins present in the LegH Prep (Documentation provided to EFSA No. 1). Proteomics analysis was conducted to identify and quantify the residual *K. phaffii* proteins present in the LegH Prep (Jin et al., 2018; Reyes et al., 2021). The applicant identified 17 of the most abundant proteins in strain MXY0291, each of the proteins representing ≥ 1% of the total protein fraction in the batches. Ten of these proteins shared more than 35% identity over 80 amino acids windows with known allergens in the Allergenonline database. Similarly, the applicant identified 11 of the most abundant proteins in strain MXY0541. Four of these were shown to share more than 35% identity over 80 amino acids windows. Some of these matches were against contact or inhalation allergens. Other matches were identified against fish, crustaceans and carrot (Jin et al., 2018; Reyes et al., 2021). The applicant argues that the majority of the matched allergens have only demonstrated their capacity to elicit allergic reactions upon dermal exposure (i.e. contact allergens) or via inhalation. For the remaining food allergens, the applicant considers that there is a low percentage of patients that showed immunoreactivity to these allergens and that no immunoreactivity was observed after heating. Soy leghemoglobin only makes up to 0.8% in the final plant-based meat products and the abundance of each *K. phaffii* protein in that final product is in trace amounts. According to the applicant, these matched allergens are unlikely to pose a major concern for the allergenicity of the LegH Prep as a food ingredient which is subjected to a heat treatment step.

The applicant also provided an assessment of the residual *K. phaffii* proteins mentioned above for their capacity to trigger coeliac disease. The assessment of these proteins revealed one partial match containing the Q-F-P-Q motif and required further investigations. Based on additional considerations on the position and nature of amino acids flanking the motifs (EFSA GMO Panel, 2017), the relevant peptide containing the motif does not raise concern as it fails to mimic gluten sequences.

The applicant provided in vitro degradation studies on the digestibility by pepsin of the LegH Prep in solutions at pH ~ 2. The results demonstrated that the leghemoglobin protein and the associated *K. phaffii* proteins were more than 90% digested within 2 min (Documentation provided to EFSA No. 1).

The Panel considered that: (i) *K. phaffii* is widely used as a host for expression of recombinant proteins and holds a QPS status; (ii) the genetic modification of *K. phaffii* does not lead to an increase in its overall allergenicity; (iii) in vitro degradation studies on the resistance to pepsin of the 'soy leghemoglobin preparation' showed no concerns; and (iv) the stability of the preparation was also tested by thermal and acidic treatment. It is also noted that the intended use of LegH Prep is very specific, i.e. a component within meat analogue products at levels delivering not more than 0.8% soy leghemoglobin and the LegH Prep is to be used cooked. Therefore, in relation to the potential of allergic reactions against *K. phaffii* potential allergens, the Panel is of the opinion that it is unlikely that adverse reactions would occur after ingestion of soy leghemoglobin products following the specification and production practices described by the applicant.

Adjuvanticity

The Panel did not find an indication that the leghemoglobin protein might be an adjuvant.

4 | DISCUSSION

The European Commission requests the EFSA to perform a risk assessment to provide a scientific opinion on the safety of the proposed use of soy leghemoglobin from genetically modified *Komagataella phaffii*, formerly *Pichia pastoris* as a food additive, in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

The source of the additive is the yeast *K. phaffii* strain MXY0541, which has been genetically modified to produce the soy leghemoglobin by introducing the heterologous gene LGB2 from soy (*Glycine max*) and by upregulating the native haem

biosynthetic pathway. No antimicrobial resistance genes have been introduced in the production strain. The recipient strain qualifies for QPS status; therefore, no concern is expected from yeast metabolites produced during the fermentation process. The safety of the genetic modification is currently under assessment by the EFSA GMO Panel (EFSA-GMO-NL-2019-162).

The proposed food additive is a liquid preparation containing soy leghemoglobin (the functional component), along with residual *K. phaffii* proteins and added ingredients. It is referred to as 'LegH Prep' and it is intended to be used as a food colour in meat analogue products.

Soy leghemoglobin is a small holoprotein (16 kDa) consisting of a polypeptide chain containing haem group and is responsible for the colour of the food additive. Soy leghemoglobin is similar in its tertiary structure to plant haemoglobins and animal myoglobins.

The production process begins with the fermentation step, using an inoculum of the production strain MXY0541. Then, the cells are lysed, and insoluble material within the lysate is removed. The concentrate undergoes thermal treatment and is formulated with stabilisers. The resulting product is finally stored as a frozen liquid.

The applicant provided analytical data on five batches of the proposed food additive, showing that LegH Prep is produced according to the proposed specifications. The Panel considered the specifications provided by the applicant sufficient to properly characterise the food additive, with some minor modifications (see Table 1). The Panel noted that there is a wide discrepancy between the reported analytical data and the proposed maximum limits for toxic elements which are significantly higher than the reported concentrations. The Panel considered that the maximum limits in the specifications for toxic elements should be established based on actual concentration of these impurities in the proposed food additive.

The proposed food additive was obtained from the cellular lysate of a specific production strain of *K. phaffii*, responsible for the synthesis of soy leghemoglobin. However, the applicant proposed that the authorisation should not be linked to any specific production strain, to accommodate future improvements to the strain. The Panel considered the proposal inadequate, noting that the authorisation of the proposed food additive should be linked to the specified production strain MXY0541 used to support the safety evaluation and any modification of the production strain would need a safety assessment for the resulting modified production strain. Therefore, the Panel recommends the introduction of the production strain MXY0541 in the *Definition* of the specifications for the proposed food additive.

The absence of undesirable substances from the production process was supported by the analytical data provided by the applicant. The absence of viable cells of the production strain *K. phaffii* MXY0541 in the product was demonstrated in nine independent batches.

The applicant conducted stability tests on the proposed food additive. The results suggest that LegH Prep remains stable under the tested storage conditions. Because of the structural similarities between soy leghemoglobin and other globin proteins, including animal myoglobins, degradation products deriving from the cooking process are expected to be similar.

The applicant provided typical and maximum use levels for the proposed food additive, as described in the specifications, for a single food category (FC 12.9 'Protein products, excluding products covered in category 1.8'). The use levels were expressed in terms of soy leghemoglobin (functional component). The use levels for the whole preparation (i.e. LegH Prep) were also provided. The applicant proposed use levels for LegH Prep considering the concentration of soy leghemoglobin in the whole preparation (i.e. 4%–6.74%, as proposed by the applicant).

The applicant provided an estimate of exposure to the proposed food additive based on the output obtained using the FAIM tool at the proposed maximum use levels provided, in compliance with the requirement. However, the Panel considered that the FAIM tool is not appropriate in this case since the food category available in FAIM (FC 12.9 'Protein products, excluding products covered in category 1.8') is broader than the requested use by the applicant, which is restricted to 'only meat imitates'. For this reason, EFSA estimated the exposure by using the DietEx tool, rather than the FAIM tool. The DietEx estimation has been performed using the two FoodEx2 codes 'Bovine, minced meat' and 'Bovine and pig, minced meat', used as surrogate of consumption of meat imitates, because information on consumption of meat imitates was not available in the FoodEx2 nomenclature.

At the proposed maximum use levels, the mean exposure to soy leghemoglobin from the use of LegH Prep as a food additive was up to 7.3 mg/kg bw per day in toddlers and children. The 95th percentile of exposure to soy leghemoglobin was up to 32.9 mg/kg bw per day in toddlers.

At the proposed typical use levels, the mean exposure to soy leghemoglobin from the use of LegH Prep as a food additive was up to 4.1 mg/kg bw per day in toddlers and children. The 95th percentile of exposure to soy leghemoglobin was up to 18.5 mg/kg bw per day in toddlers.

Two FoodEx2 codes were used as surrogates of consumption of meat imitates for estimating the exposure to soy leghemoglobin. The Panel recognised that this assumption is likely to result in an overestimation of the exposure. In addition, it was assumed that 100% of the foods in these FoodEx2 codes would contain LegH Prep at the maximum/typical proposed use levels, which is also an overestimation. The Panel noted that highest exposures are observed for infants and toddlers because, for the estimation of the dietary exposure to the proposed food additive, the consumption of minced meat (main meat-based food consumed by infants and toddlers) is used as a surrogate for the consumption of meat imitates. Overall, the Panel considered that the uncertainties identified resulted in an overestimation of the exposure to LegH Prep at the maximum/typical proposed use levels in European countries considered in the EFSA Comprehensive database.

The Panel calculated the potential exposure to toxic elements (i.e. iAs, Pb, Cd, Hg) by combining levels of the toxic elements in the proposed food additive with the highest estimated intakes of the food additive itself. The anticipated exposure was then compared to reference points (for iAs, Pb) or health-based guidance values (for Cd and Hg). The presence of

Pb, Cd and Hg in the proposed food additive would not give rise to concern. In contrast, for As, the calculated MOE values were considered to be insufficient.

The Panel compared the amount of haem iron provided by soy leghemoglobin from its proposed uses in meat analogue products to that provided by an equivalent amount of a corresponding meat product. The Panel estimated that 100 grams of the meat analogue will contain ~ [redacted] mg of haem iron, considering that one molecule of soy leghemoglobin contains one atom of haem iron and the maximum proposed use level of the soy leghemoglobin in meat analogue products (0.8%). This amount of haem iron is comparable to that provided by similar amounts of different types of meat (Lombardi-Boccia et al., 2002).

The level of iron in the proposed food additive (3850–8150 mg/kg soy leghemoglobin) combined with the estimated intakes of the proposed food additive (Table 4) could result in an exposure to iron deriving from the consumption of foods where the proposed food additive is intended to be used under the proposed conditions of use (Table 9). The estimate of anticipated exposure to iron from the proposed food additive, both at the mean and 95th percentile exposure, constitutes a small fraction of the safe levels of intake established by the NDA Panel for all population groups. Therefore, no safety concern was identified for the intake of haem iron based on the consumption of meat analogues containing soy leghemoglobin for the general population.

People suffering of haemochromatosis may pay attention to the iron intake from meat analogues containing soy leghemoglobin to control their iron intake. Therefore, they would need to be informed that this proposed food additive contains a source of iron.

The available information supports that the components of LegH Prep will be digested very rapidly in the gastrointestinal tract following the same pathways as other proteins consumed as part of the normal diet, including myoglobins from animal meat. The digested molecules have well understood metabolic fates following absorption and do not give rise to safety concerns. Based on this reasoning, the Panel considered the data package submitted by the applicant sufficient and did not request Tier II tests (EFSA ANS Panel, 2012).

LegH Prep was tested in a bacterial reverse mutation assay and two cytogenetic assays in human lymphocytes: a micronucleus test and a chromosomal aberration test. All these tests showed negative results. In the micronucleus and in the chromosomal aberration assays, the final maximum concentrations of soy leghemoglobin were below OECD indications (only in the chromosomal aberration assay, the test item tested without S9 reached the cytotoxicity level recommended by OECD 473). Therefore, the relevance of the MN assay results is limited. However, the Panel considered that there was no need to request further information since the recipient (non GM) strain qualifies for QPS status for production purposes, and therefore, no concern is expected from yeast metabolites produced during the fermentation process. Since soy leghemoglobin is constituted by a proteinaceous part, it is not expected to be mutagenic.

LegH Prep has been tested in a number of subchronic toxicity studies. In two 28-day studies, one with a 14-day predosing oestrous cycle determination, there were no adverse effects up to the highest dose tested (750 mg/kg bw per day of soy leghemoglobin).

In a 90-day study, a small increase in thyroid hormones in high-dose males was not considered adverse. In high-dose males, decreased thymus weights were observed; however, the decrease did not correlate with any histopathology findings in the thymus. The Panel considered that the NOAEL for LegH Prep was at the highest dose tested 4820 and 5931 mg/kg bw per day for males and females, respectively, which correspond to the dose of 2328 mg/kg bw per day for males and 2865 mg/kg bw per day for females of soy leghemoglobin.

Regarding the potential allergenicity, the Panel considered that: (i) *K. phaffii* is widely used as a host for expression of recombinant proteins and qualifies for QPS status; (ii) the genetic modification of *K. phaffii* does not lead to an increase in its overall allergenicity; (iii) in vitro degradation studies on the resistance to pepsin of the soy leghemoglobin preparation showed no concerns; and (iv) the stability of the preparation was also tested by thermal and acidic treatment. It is also noted that the intended use of the product is very specific, i.e. a component within meat analogue products at levels delivering no more than 0.8% soy leghemoglobin and the product is to be used cooked. Therefore, in relation to the potential of allergic reactions against *K. phaffii* potential allergens, the Panel is of the opinion that it is unlikely that adverse reactions would occur after ingestion of soy leghemoglobin products following the specification and production practices described by the applicant.

Considering that (i) the components of the proposed food additive will be digested to small peptide, amino acids and haem B, (ii) the recipient (non GM) strain qualifies for QPS status, (iii) no genotoxicity concern has been identified and (iv) no adverse effects have been identified at the highest dose tested (4820 mg/kg bw per day) in a 90-day study, the Panel concluded that there was no need to set a numerical ADI and that the food additive does not raise a safety concern at the proposed use in food category 12.9 and maximum use level.

5 | CONCLUSIONS

Based on the available data, the Panel concluded that the use of soy leghemoglobin from genetically modified *Komagataella phaffii* (strain MXY0541) as a new food additive does not raise a safety concern at the proposed use and use level.

This safety evaluation of the proposed food additive remains provisional subject to the ongoing safety assessment of the genetic modification of the production strain by the GMO Panel (EFSA-GMO-NL-2019-162).

6 | DOCUMENTATION AS PROVIDED TO EFSA

1. Application for the approval of soy leghemoglobin from *Pichia Pastoris* yeast as a food additive in the European Union pursuant to European Parliament and Council Directive (EC) 1333/2008, 16 December 2008 on Food Additives. Technical Dossier. Impossible Foods Inc. June 2022.
2. Additional information submitted by the applicant following requests by EFSA. Impossible Foods Inc. November 2022.
3. Additional information submitted by the applicant following requests by EFSA. Impossible Foods Inc. May 2023.
4. Additional information submitted by the applicant following requests by EFSA. Impossible Foods Inc. September 2023.
5. Additional information submitted by the applicant following requests by EFSA. Impossible Foods Inc. February 2024.
6. Additional information submitted by the applicant following requests by EFSA. Impossible Foods Inc. April 2024.

ABBREVIATIONS

AFSSA	Agence Française de Sécurité Sanitaire des Aliments
ANS Panel	Panel on Food Additives and Nutrient Sources added to Food
BIOHAZ Panel	Panel on Biological Hazards
BMDL	Benchmark dose (lower confidence limit)
BW	body weight
CAS	Chemical Abstracts Service
CEP Panel	Panel on Food Contact Materials, Enzymes and Processing Aids
CONTAM Panel	Panel on Contaminants in the Food Chain
FAF Panel	Panel on Food Additives and Flavourings
FAIM	Food Additives Intake Model
FC	food category
FCS	food categorization system
FDA	Food and Drug Administration
FEEDAP Panel	Panel on Additives and Products or Substances used in Animal Feed
FSANZ	Food Standards Australia New Zealand
GLP	good laboratory practice
GM	genetically modified
GMO	genetically modified organisms
GRAS	generally recognised as safe
HBGV	health-based guidance value
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	limit of detection
LOQ	limit of quantification
MCH	mean cell haemoglobin
MCV	mean cell volume
MN	micronucleus
MOE	margin of exposure
NOAEL	no observed adverse effect level
OECD	Organization for Economic Co-operation and Development
QPS	qualified presumption of safety
ROS	reactive oxygen species
RP	reference point
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SFA	Singapore Food Agency
TG	test guideline
TWI	tolerable weekly intake
UPLC	ultra-performance liquid chromatography
USDA	United States Department of Agriculture
UV VIS	ultraviolet visible

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