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SCIENTIFIC OPINION

Assessment of soy leghemoglobin produced from genetically modified *Komagataella phaffii*, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2019-162)

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Abstract

Genetically modified *Komagataella phaffii* strain MXY0541 was developed to produce soy leghemoglobin by introducing the *LGB2* coding sequence encoding leghemoglobin from soybean (*Glycine max*). The molecular characterisation data and bioinformatic analyses do not raise any safety concerns. The safety of soy leghemoglobin as a food additive has already been assessed by the EFSA FAF Panel (EFSA-Q-2022-00031). The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of soy leghemoglobin protein as expressed in *K. phaffii*, and finds no evidence that the genetic modification would change its overall allergenicity. The GMO Panel concludes that the LegH Prep derived from genetically modified *K. phaffii* strain MXY0541 is safe for human consumption with regard to the effects of the genetic modification. No environmental impact from the use of this product is expected regarding the recombinant DNA sequences possibly remaining in the product. The GMO Panel concludes that LegH Prep from genetically modified *K. phaffii* strain MXY0541 is safe with respect to potential effects on human health and the environment at the proposed use and use level as far as the impact of the genetic modification is concerned. The overall conclusion is that the genetic modification does not lead to safety issues.

KEYWORDS

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

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1 | INTRODUCTION

The application EFSA-GMO-NL-2019-162 is for the authorisation of soy leghemoglobin produced from genetically modified *Pichia pastoris* [current name *Komagataella phaffii*] for use in food in the European Union.

1.1 | Background

On 15 October 2019, the European Food Safety Authority (EFSA) received from the Competent Authority of The Netherlands application EFSA-GMO-NL-2019-162 for authorisation of "the use of soy leghemoglobin (the liquid preparation is referred to as "LegH Prep") produced from genetically modified *Pichia pastoris* (*P. pastoris*) [current name *Komagataella phaffii*] as a flavouring ("meaty taste") in meat analogue products that will be marketed in the European Union (EU)", (Unique Identifier IF-KPØ541-7), submitted by Impossible Foods Inc. (hereafter referred to as 'the applicant') according to Regulation (EC) No 1829/2003.[1] Following receipt of application EFSA-GMO-NL-2019-162, EFSA informed EU Member States (MS) and the European Commission (EC), and made the application available to them. Simultaneously, EFSA published the summary of the application.[2]

EFSA checked the application for compliance with the relevant requirements of Regulation (EC) No 1829/2003, with the EFSA guidance documents, and, when needed, asked the applicant to supplement the initial application. On 15 December 2021, EFSA declared the application valid.

From validity date, EFSA and the Panel on Genetically Modified Organisms of the European Food Safety Authority (referred to hereafter as 'GMO Panel') endeavoured to respect a time limit of six months to issue a scientific opinion on application EFSA-GMO-NL-2019-162. Such time limit was extended whenever EFSA and/or the GMO Panel requested supplementary information to the applicant. In accordance with Regulation (EC) No 1829/2003, any supplementary information provided by the applicant during the risk assessment was made available to the EU Member States and the European Commission (for further details, see the Section 5).

After consultation with the EC and in accordance with Regulation (EC) No 1829/2003, EFSA consulted optionally the nominated risk assessment bodies of EU Member States, including national Competent Authorities within the meaning of Directive 2001/18/EC.[3] The EU Member States had three months to make their opinion known on application EFSA-GMO-NL-2019-162.

1.2 | Terms of Reference as provided by the Requestor

According to Article 3 (1)c of Regulation (EC) No 1829/2003, EFSA and its GMO Panel were requested to carry out a scientific risk assessment of soy leghemoglobin in the context of the scope as defined in application EFSA-GMO-NL-2019-162.

According to Regulation (EC) No 1829/2003, this scientific opinion is to be seen as the report requested under Article 6(6) of that Regulation and in accordance with Article 6(5). In addition to the present scientific opinion, EFSA was also asked to report on the particulars listed under Articles 6(5) of Regulation (EC) No 1829/2003, but not to give an opinion on them because they pertain to risk management.[4]

1.3 | Additional information

The EFSA FAF Panel has evaluated the safety of soy leghemoglobin from genetically modified *K. phaffii (*formerly *P. pastoris)* as a food additive under a mandate from the EC (EFSA-Q-2022-00031)[5] in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings[6] (EFSA FAF Panel, 2024).

[1]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. 1–23.

[2]https://open.efsa.europa.eu/questions/EFSA-Q-2019-00651.

[3]Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 12.3.2001, p. 1–38.

[4]https://open.efsa.europa.eu/study-inventory/EFSA-Q-2019-00651.

[5]https://open.efsa.europa.eu/questions/EFSA-Q-2022-00031.

[6]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. 1–6.

2 | DATA AND METHODOLOGIES

2.1 | Data

The GMO Panel based its scientific assessment of the genetic modification on the valid application EFSA-GMO-NL-2019-162, additional information provided by the applicant during the risk assessment, relevant scientific comments submitted by the Member States and relevant peer-reviewed scientific publications.

2.2 | Methodologies

The GMO Panel conducted its assessment in line with the principles described in Regulation (EU) No 1829/2003 and the applicable guidelines 'Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use' (EFSA GMO Panel, 2011). In addition, the following were taken into account: 'Scientific Guidance for the submission of dossiers on Food Enzymes' (EFSA CEP Panel, 2021), 'Guidance on allergenicity assessment of genetically modified plants' (EFSA GMO Panel, 2017), the EFSA Guidance of the Scientific Committee on transparency in the scientific aspects of risk assessment (EFSA, 2009) and following the relevant existing Guidance documents from the EFSA Scientific Committee, and explanatory notes and statements (EFSA, 2010, 2021; EFSA GMO Panel, 2018) for the risk assessment of GM plants.

For this application, in the context of the contracts [EOI/EFSA/SCIENCE/2020/01 – CT02GMO ISA 90-day] and [OC/EFSA/GMO/2020/01 sequencing quality check] the contractors performed preparatory work for the evaluation of the applicant's data.

3 | ASSESSMENT

3.1 | Characteristics of the parental microorganism

3.1.1 | Scientific name, taxonomy and other names

The parental strain is the *K. phaffii* Bg11 strain. As confirmed by the Qualified Presumption of Safety (QPS) Opinion 2020 (EFSA BIOHAZ Panel, 2020), the names *K. phaffii* and *P. pastoris* cannot be used interchangeably. Within the genus *Komagataella, K. phaffii* is a different species to *K. pastoris* and only the latter corresponds to the old name *P. pastoris*. It was communicated to the Applicant in Additional Information request (3) that the name *K. phaffii* should be used.

3.1.2 | Description of its history of use

K. phaffii has QPS status with qualifications. QPS applies for 'production purposes only' and the qualification 'for production purpose only' implies the absence of viable cells of the production organism in the final product and can also be applied for food and feed products based on microbial biomass (QPS Panel Statement part 7 (EFSA BIOHAZ Panel, 2018)).

3.1.3 | History of previous genetic modifications

The recipient strain is the *K. phaffii* Bg11 strain, a derivative of the strain Bg10, which is a derivative of the parental strain NRRL Y-11430. The *K. phaffii* Bg11 strain was obtained by genetic modification, deleting the alcohol dehydrogenase (*Aox1*) gene using double homologous recombination resulting in a decreased ability to metabolise methanol.

Bg11 is a commercial strain that grows more slowly on methanol-containing induction media, which is a desirable trait for industrial fermentation. Bg11 strain does not contain any of the background vector sequences and antibiotic resistance genes employed during homologous recombination.

3.2 | Characteristics of the origin of the inserted sequences (donor organism)

3.2.1 | DNA from defined donor organism

The only heterologous sequence introduced to the production strain is the *LGB2* coding sequence for leghemoglobin from the soybean plant (*Glycine max*), which was synthesised and codon optimised for expression in *K. phaffii*. The other introduced sequences are native of *K. phaffii*, which has the QPS status (QPS Panel Statement part 7 (EFSA BIOHAZ Panel, 2018)) and therefore are not considered to raise safety concerns.

3.3 | Description of the genetic modification

The purpose of the genetic modification is the production of the soy leghemoglobin protein in the *K. phaffii* genetically modified production strain MXY0541.

3.3.1 | Information relating to the genetic modification

To express the soy leghemoglobin protein, several transformation steps involving different expression constructs were applied to the Bg11 recipient strain (named [redacted] by the applicant). A series of modifications starting from Bg11 strain led to the optimised production strain (MXY0541) that produces higher yields of soy leghemoglobin protein. The transformation steps and the DNA constructs used to achieve the final production strain are listed below.

Generation of strain [redacted]

Modifications to upregulate the haem biosynthetic pathway of Bg11.

Genes that code for [redacted] enzymes of the *Komagataella* haem biosynthesis pathway were amplified from the *Komagataella* genome and cloned into two plasmids: [redacted] and [redacted]. The two [redacted] plasmids were transformed by electroporation into the recipient strain Bg11. The two plasmids have [redacted] to [redacted] and [redacted] between two [redacted]. The [redacted] were [redacted] from the [redacted] by [redacted], followed by [redacted] of the [redacted]. Gene [redacted] to [redacted] is [redacted]. After the [redacted], [redacted] was [redacted] in order to [redacted]. The resulting [redacted] were [redacted] and the absence of resistance genes was confirmed by PCR.

Constructs used to upregulate the haem biosynthetic pathway.

- The plasmid [redacted] was used to express [redacted] genes coding for [redacted] of the haem pathway: [redacted] from *K. phaffii*. In all these cassettes, the genes are under the control of the promoter of [redacted] and the [redacted] terminator [redacted], both from *K. phaffii*. The [redacted] vector also contains an expression cassette for the [redacted] and an expression cassette for [redacted]. These two cassettes are flanked by [redacted].
- The plasmid [redacted] was used to express [redacted] genes coding for [redacted] of the haem pathway: [redacted] from *K. phaffii*. In all these cassettes, the genes are under the control of the promoter of [redacted] and the [redacted] terminator [redacted], both from *K. phaffii*. The [redacted] vector also contains an expression cassette for the [redacted] and an expression cassette for [redacted]. These two cassettes are flanked by [redacted].
- The episomal plasmid [redacted] was used to express the [redacted]. It contains two expression cassettes containing the following genetic elements:
  - the [redacted] expression cassette, consisting of the [redacted] promoter [redacted], the [redacted] gene [redacted] and the [redacted] terminator from *K. phaffii*,
  - the [redacted] expression cassette, consisting of [redacted] promoter [redacted], the gene [redacted] and the [redacted] terminator [redacted]

  All the vectors contain elements necessary for the maintenance and selection of the plasmid in bacteria.

Generation of strain [redacted]

Modification to [redacted].

The [redacted] cassette and the plasmid [redacted] containing the [redacted] were co-transformed into strain [redacted]. Colony PCR was used to screen transformants with the cassette integrated at the [redacted]. The [redacted] [redacted].

Constructs used to overexpress [redacted].

A linear cassette was targeted to the [redacted] to overexpress the [redacted]. The gene coding for the [redacted] is under the control of [redacted] promoter and the [redacted] terminator. The linear cassette carries a

[redacted] and the [redacted]. All the genetic elements derive from *K. phaffii*.

The [redacted] empty vector was used for selection. It contains the [redacted] expression cassette consisting of the [redacted] promoter [redacted], the gene [redacted] and the [redacted] terminator [redacted].

The [redacted].

Generation of strain MXY0291

This strain was constructed to achieve overexpression of LGB2 gene.

The *LGB2* codon sequence from soybean (*G. max*) was codon-optimised for expression in *K. phaffii*. The gene included in the linear cassette [redacted] was introduced in strain [redacted] along with the vector [redacted]. Colony PCR was used to screen transformants with the cassette integrated at [redacted]. The [redacted]. PCR was used to confirm the removal of [redacted] plasmid. The resulting strain MXY0291 contains [redacted] *LGB2* codon sequence. Protein expression and qPCR were used to characterise strain MXY0291.

Construct used to express the soy Leghemoglobin protein.

A linear cassette was used to overexpress the soy Leghemoglobin protein. The *LGB2* codon sequence coding for the soy Leghemoglobin is under the control of [redacted] promoter and the [redacted] terminator. The linear cassette carries a [redacted] and the [redacted]. Both, the promoter and the terminator derive from *K. phaffii*.

The [redacted] is described above.

Generation of strain [redacted]

Modification to [redacted].

The strain [redacted], a linear cassette containing the [redacted] promoter and the [redacted] gene was introduced in strain [redacted] along with vector [redacted] used for selection, [redacted]. The [redacted].

Constructs used to [redacted].

- A linear cassette was used to [redacted]. The gene is under the control of the [redacted] promoter. Both, the gene and the promoter derive from *K. phaffii*.
- The [redacted] is described above.

Generation of strain [redacted]

This strain was constructed to achieve [redacted] of *LGB2* gene.

The [redacted] soy Leghemoglobin codon sequence (codon optimised for expression in *K. phaffii)* was introduced as [redacted]. In the resulting strain, [redacted], the [redacted] was demonstrated with PCR and genome sequencing. The [redacted].

Construct used to [redacted] the soy leghemoglobin.

A linear cassette was used to express the soy leghemoglobin. The *LGB2* codon sequence coding for soy leghemoglobin is under the control of [redacted] promoter and the [redacted] terminator. Both the promoter and the terminator derive from *K. phaffii*.

The [redacted] is described above.

Generation of production strain MXY0541

This strain was constructed to achieve [redacted] of *LGB2* gene.

Modification to [redacted] the *LGB2* gene. [redacted] soy leghemoglobin *LGB2* coding sequence (codon optimised for expression in *K. Phaffii)* was introduced in the strain [redacted], by [redacted] of the [redacted] using the [redacted] vector. In the resulting strain, MXY0541, the newly introduced *LGB2* codon sequence was [redacted].

Construct used to [redacted] the soy leghemoglobin (*LGB2* gene).

A linear cassette was used to introduce the *LGB2* codon sequence (from *G. max*) under the control of [redacted] promoter and the [redacted] terminator (both from *K. phaffii)*.

The DNA sequencing of the MXY0541 strain was carried out by WGS and demonstrated the presence of *LGB2* sequence variants as below detailed. Three separate modifications were characterised: Modification (1) containing [redacted] of the [redacted]; Modification (2) containing [redacted] of the [redacted]; Modification (3) containing [redacted] of the [redacted] and [redacted] native to *K. phaffii*.

The determination of the exact number of leghemoglobin genes in the tandem repeats is not considered relevant for the safety of the food product.

The absence of the resistance genes to [redacted] and [redacted] from the genome of the production strain was demonstrated by PCR analysis and by the inability of the production strain to grow in the presence of these antimicrobials.

The quality of the sequencing methodology and corresponding datasets was assessed by the EFSA GMO Panel and complies to the requirements listed in the EFSA Technical Note (2018) [Contract OC/EFSA/GMO/2020/01] and those of the EFSA statement (2021)[7] for the coverage of the three *LGB2* variants in MXY0541 strain.

3.4 | Information relating to the GMM

The production strain is the genetically modified yeast strain *K. phaffii* MXY0541 and was deposited in the DSMZ-German Collection of Microorganisms and Cell cultures (Germany). The applicant determined the taxonomic identity of the strain based on multigene phylogenetics analysis using the following genes: actin, *RPB2, RPS25A, TEF2, TEF4* to distinguish between the *Komagataella* species. The analysis included the type strain of *K. phaffii* NRRLY-7556. Based on the evidence provided it was concluded that the strain MXY0541 is *K. phaffii*.

3.4.1 | Structure and amount of any vector and/or donor nucleic acid remaining in the GMM

The MXY0541 strain contains three cassettes designed to express the *LGB2* gene in three loci in the genome, one in the [redacted] locus, one in the [redacted] locus and one in the [redacted] locus. The WGS data analysis demonstrated that none of the sequences (antibiotic resistance and other backbone sequences) used to introduce the haem biosynthetic enzymes, or the plasmids used in the co-transformation were present in the genome assembly or as extra-chromosomal plasmids. The removal of the antibiotic resistance genes was also confirmed by PCR with primer pairs specific to the [redacted] and the [redacted] resistance genes, and to the gene providing resistance to the [redacted].

The recipient strain from which the production organism was derived belongs to *K. phaffii*, which is considered by EFSA to be suitable for the QPS approach to safety assessment when used for production purposes (EFSA BIOHAZ Panel, 2018). The production strain was identified as *K. phaffii* and the traits introduced raise no safety concerns (see conclusions of the present assessment in Section 4). Therefore, the QPS status is extended to the MXY0541 production strain (EFSA BIOHAZ Panel, 2024).

3.4.2 | Stability of the genetic traits in the GMM

Stability of the genetic traits was demonstrated in accordance with the requirements of the GMM Guidance (EFSA GMO Panel, 2011). The applicant analysed by multiplex PCR three independent production lots of the strain MXY0541. The stability of the intended genetic traits at the end-of-product lots was confirmed.

3.4.3 | Rate and level of expression of the new genetic material and activity of the expressed proteins

The rate of expression is not relevant for this assessment. The level of expression is relevant as to the final concentration of soy leghemoglobin protein in the LegH Prep which is in the final product, as described in Section 3.7.3.

3.4.4 | Description of identification and detection techniques

The applicant provided a qualitative PCR-based method to detect and demonstrate the presence of DNA coding for the leghemoglobin produced in *K. phaffii* MXY0541. The [redacted] is [redacted] which [redacted] of the [redacted].

[7]EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. EFSA Journal 2021;19(7) https://doi.org/10.2903/j.efsa.2021.6506.

3.4.5 | Conclusions of the molecular characterisation

K. phaffii MXY0541 is a derivative of the *K. phaffii* Bg11 strain, which has QPS status. The heterologous *LGB2* coding sequence is expressed in the *K. phaffii* MXY0541 strain for soy leghemoglobin production. The heterologous expression of soy leghemoglobin in *K. phaffii* MXY0541 strain is not considered to pose any concern. Other changes introduced into the MXY0541 production strain to improve the biosynthesis of soy leghemoglobin were aimed at the upregulation of the native *K. phaffii* haem biosynthesis pathway, and that of [redacted], a [redacted] of the native *K. phaffii* [redacted]. All these modifications are based on the introduction of *K. phaffii* endogenous DNA sequences and are not deemed to be a cause of concern either. In conclusion, the genetic modifications of the optimised production *K. phaffii* MXY0541 strain do not raise any safety concern.

3.5 | Information relating to the production process

The Section 3.1.3 'Manufacturing process' in the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024) details this information.

3.6 | Information relating to the product preparation process

The soy leghemoglobin protein is delivered in a liquid preparation (LegH Prep) that is standardised to contain ≥4% soy leghemoglobin protein on a wet weight basis and a soy leghemoglobin protein purity of ≥65%. The remainder of the protein fraction in the LegH Prep is accounted for by residual proteins from the production strain.

3.6.1 | Demonstration of the absence of the GMM in the product

The absence of viable cells of the production strain MXY0541 in the product was demonstrated in nine samples, each one from independent batches. All applicable conditions and controls as described in Section 1.3.4.1 of the EFSA CEP Panel Guidance (2021) were taken into consideration and tested. No production strain colonies were detected, demonstrating absence of viable cells of strain MXY0541 in the tested batches.

3.6.2 | Information on the possible presence of recombinant DNA

The presence of recombinant DNA that is not native to *K. phaffii*, was revealed by qualitative PCR. The applicant extracted the DNA from three batches, but only tested one of them, which was found to contain recombinant DNA. Based on this evidence, the EFSA GMO Panel concluded that there is presence of recombinant DNA in the product batches.

3.7 | Description of the product

3.7.1 | Designation of the product

The proposed food additive, 'LegH Prep' is a liquid preparation containing soy leghemoglobin protein 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. LegH Prep is produced by the strain MXY0541 and is further detailed in Sections 3.1.1 'Identity of the substance' and 3.1.2 'Proposed specification' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024).

3.7.2 | Intended use and mode of action

As described by the applicant, the primary purpose of adding soy leghemoglobin to meat analogue products is to recreate the flavour and aroma of the animal-derived counterpart when cooked. This flavouring effect is achieved through denaturation of soy leghemoglobin and release of the haem B moiety during the preparation process of the meat analogue products. The intended use of the product is further detailed in Sections 3.1.5 'Stability of the substance and reaction and fate in food' and 3.2 'Proposed uses and use levels' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024).

3.7.3 | Composition

The reporting of the composition of LegH Prep is detailed in Section 3.1.2 'Proposed specification' of the EFSA FAF Panel Opinion, with Specifications as revised by the Panel in column C of Table 1 (EFSA FAF Panel, 2024). Based on analytical results from three batches coming from the MXY0541 strain, the content of soy leghemoglobin ranged from 4.5% to 4.8%, corresponding from 68% to 77% of the total protein fraction.

3.8 | Considerations of the GMM and/or its product for human health

3.8.1 | Toxicology

The heterologous *LGB2* coding sequence introduced into the *K. phaffii* production strain encodes for the soy leghemoglobin protein. The strategy to assess the toxicological impact of any changes related to the genetic modification of *K. phaffii* focuses on the assessment of the newly expressed soy leghemoglobin protein in strain MXY0541.

3.8.1.1 | *Evaluation of protein newly expressed by the introduced genes*

The soy leghemoglobin protein newly expressed in the genetically modified *K. phaffii* (strain MXY0541) was never assessed before by the GMO Panel. A weight-of-evidence approach was followed by the GMO Panel to assess the toxicological profile of this soy leghemoglobin protein as present in the LegH Prep, taking into account all of the information relevant for its assessment, including molecular characterisation of the genetic modification, updated bioinformatic analyses for similarity to toxins, history of safe use for consumption as food, in vitro studies and in vivo toxicity studies.

Bioinformatic analysis

Bioinformatic analyses performed by the applicant on the amino acid sequences of the soy leghemoglobin protein in strain MXY0541, revealed no significant similarities to known toxins.

History of safe use for consumption as food

Information on the source organism of the gene

The only heterologous sequence introduced into the *K. phaffii* production strain is the *LGB2* coding sequence encoding for the soy leghemoglobin protein. In soybean plant, this gene is only expressed within root nodules colonised by nitrogen-fixing bacteria (O'Brian et al., 1987).

Information on structure, function and mode of action of the soy leghemoglobin protein

The three-dimensional structure of haemoglobin proteins is highly conserved. In all cases, the globin structural fold involves eight alpha helical segments that harbour a haem co-factor with the complexed iron in its centre.

Information on identity/homology of soy leghemoglobin protein to other proteins in conventional sources

Leghemoglobin proteins are found predominantly in legume species and function in the nitrogen fixation process to control the free oxygen concentration. The soy leghemoglobin protein is present in the root nodule of the soybean plant, which is not normally consumed by humans. Upon request, the applicant carried out amino acid sequence alignments to compare the primary sequence of soy leghemoglobin with that of other haemoglobin proteins found in plants and animals. These sequence alignments revealed that soy leghemoglobin shares a rather limited primary amino acid sequence identity with haemoglobin proteins that are expressed in edible parts of plants and, hence, occur commonly in the human diet. The highest amino acid sequence identities were found with haemoglobin proteins from rice (44% identity) and maize (40%). A similar situation was also seen for the comparison between soy leghemoglobin and animal myoglobins.

Overall conclusion on the history of safe use for consumption of soy leghemoglobin

The GMO Panel considers the information above as not sufficient to duly document the history of safe use for consumption of the newly expressed soy leghemoglobin protein.

In vitro studies

For the assessment of the soy leghemoglobin protein newly expressed in strain MXY0541, the applicant provided in vitro degradation studies on the resistance to pepsin of the LegH Prep and stability studies to thermal and acidic treatment of the soy leghemoglobin protein. The assessment of these studies is detailed in Sections 3.4.1 'Absorption, distribution, metabolism and excretion' and 3.4.7 'Immunotoxicity, hypersensitivity/allergy' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024).

The GMO panel acknowledges that the evaluation made by the FAF panel covers the stability of the soy LegH Prep, in which the soy leghemoglobin protein is present (EFSA FAF Panel, 2024). No indications of concern were identified by the GMO Panel.

In vivo toxicity studies

For the assessment of the soy leghemoglobin protein newly expressed in strain MXY0541, the applicant provided a 90-day dietary toxicity study in rats with a 28-day recovery period, performed with a LegH Prep from the commercial production strain MXY0541 (48.3% soy leghemoglobin protein), according to OECD TG 408 (OECD, 2018) and in compliance with GLP principles. The assessment of this study is detailed in Section 3.4.4 'Subchronic toxicity' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024). The GMO panel acknowledges that the evaluation made by the FAF panel covers the safety of the soy LegH Prep from which it is possible to derive the concentration of the soy leghemoglobin protein (48.3% soy leghemoglobin) tested in animals. This allowed the GMO Panel to conclude that no adverse effects were reported in the 90-day study up to the highest doses tested (2352 and 2894 mg/kg body weight per day of soy leghemoglobin protein, respectively in males and females).

Furthermore, three additional toxicity studies in rats, performed with soy LegH Prep from strain MXY0291, were provided by the applicant: a 14-day dietary toxicity study (47.6% soy leghemoglobin protein, corresponding to an upper dose tested of 500 mg/kg bw per day of soy leghemoglobin protein), a 28-day dietary toxicity study (48.8% soy leghemoglobin protein, corresponding to an upper dose tested of 750 mg/kg bw per day of soy leghemoglobin protein) and a 28-day study with a 14-days pre-dosing estrous cycle determination (48.8% of soy leghemoglobin protein, corresponding to an upper dose tested of 750 mg/kg bw per day of soy leghemoglobin protein). The assessment of these studies is detailed in Section 3.4.4 'Subchronic toxicity' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024). The GMO Panel acknowledges that the evaluation by the FAF panel covers the safety of the soy LegH Prep from which it is possible to derive concentration of the soy leghemoglobin protein tested in animals. This allowed the GMO Panel to conclude that no adverse effects were reported in these studies up to the highest doses tested of the soy leghemoglobin protein.

3.8.1.2 | *Evaluation of constituents other than proteins*

The impact of the genetic modification on the constituents other than protein, is the altered haem group expression (see Section 3.3). The resulting altered iron intake is discussed in the Section 3.8.3 on nutrition.

Formation of metal complexes between leghemoglobin and divalent cations may not be limited to iron, as selective interactions between other metals and heme proteins were described in the scientific literature (Basak et al., 2016). The risk assessment on the presence of different divalent Pb, Cd and Hg, as well as As, in the meat analogues containing the LegH Prep is provided in Section 3.3.3 'Anticipated exposure to toxic elements from the use of the proposed food additive' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024).

3.8.1.3 | *Evaluation of the product*

Whereas the Section 3.8.1.1 herein of toxicology covers the safety of the soy leghemoglobin protein, the evaluation of the LegH Prep product is presented in EFSA FAF Panel (2024) and no indications of concern were identified.

3.8.2 | Allergenicity

The Panel's assessment of the potential risk of allergenicity focuses: (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.

3.8.2.1 | *Proteins expressed by the introduced genes*

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

The *LGB2* coding sequence expressing the leghemoglobin protein originates from *G. max* (soybean), which is considered a common allergic source by Regulation.[8] 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'.

The risk assessment of the soy leghemoglobin protein newly expressed by the introduced genes from strain MXY0541 and related information is detailed in Section 3.4.7 'Immunotoxicity, hypersensitivity/allergy' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024). No indications of concern were identified by the GMO Panel.

[8]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.

3.8.2.2 | *Evaluation of allergenicity of the product*

According to the EFSA GMO Panel guidance on the GMMs and their products intended for food and feed use (EFSA GMO Panel, 2011), the possibility of increased overall allergenicity of the GMM and/or its product should be considered (e.g. expression of allergens, including endogenous allergens).

The genetic modifications have been extensively characterised and no indications of concern were identified (see Section 3.3). The GMO Panel considers that the genetic modifications do not raise concerns on allergenicity.

The risk assessment of the LegH Prep from strain MXY0541 is detailed in Section 3.4.7 'Immunotoxicity, hypersensitivity/allergy' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024).

3.8.2.3 | *Adjuvanticity*

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

3.8.3 | Nutritional assessment

As indicated in the GMM guidance (EFSA GMO Panel, 2011), a nutritional intake assessment was conducted based on the consumption of conventional foods likely to be replaced by the meat analogue containing the LegH Prep.

Minerals

Following a request of the GMO Panel, the applicant provided analytical data from three batches of LegH Prep produced with the strain MXY0541 for minerals introduced in the production process as part of the fermentation medium. The highest concentrations reported for Cu, Mn, Mg, B and I were 7.49, 2.44, 130, 0.30 and < 0.02 mg/kg (< LOQ), respectively.

Dietary intakes for the different minerals under assessment (Cu, Mn, Mg, B and I) were estimated across different European population groups, combining the highest concentrations of the minerals in the three batches with the amounts of LegH Prep derived from the exposure estimations to leghemoglobin protein as described in EFSA FAF Panel Opinion (EFSA FAF Panel, 2024) (95th exposure estimations, whole population). To derive the amounts of LegH Prep at the maximum proposed use level of the leghemoglobin protein (0.8%), it was assumed that the leghemoglobin protein was present in the LegH Prep at the lowest concentration (4%) as described in Section 3.1.2 'Proposed specifications' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024). Dietary intake estimations for B, Cu, I and Mg were compared to the corresponding tolerable upper intake levels[9] and no safety concerns were identified.

For Mn, whilst no Tolerable Upper Intake Level has been set, a safe level of intake of 8 mg/day was established for adults ≥ 18 years (including pregnant and lactating women), and between 2 and 7 mg/day for other population groups (EFSA NDA Panel, 2023). Dietary intake estimations for Mn would range between 0.006 mg/day in toddlers and 0.014 mg/day in adolescents, representing less than 0.5% of the corresponding safe level of intakes in all cases. Therefore, no safety concerns were identified.

Haem iron

The nutritional assessment of iron was focused on haem iron which is absorbed with better efficiency by the body than non-haem iron. The applicant stated that the amount of haem iron provided by the soy leghemoglobin protein from its proposed uses in meat analogue products will be comparable to those provided by an equivalent amount of a corresponding meat product. As also described in the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024), the GMO Panel estimated that 100 grams of the meat analogue will contain ~ [redacted] mg of haem iron, considering that one protein molecule of soy leghemoglobin contains one molecule of haem iron and the maximum proposed use level of the soy leghemoglobin protein in meat analogue products (0.8%). This amount of haem iron is, in fact, comparable to those provided by similar amounts of different types of raw meat (Lombardi-Boccia et al., 2002).[10] Therefore, no safety concern was identified in the general population[11] for the intake of haem iron based on the consumption of meat analogues containing soy leghemoglobin protein.

3.8.4 | Exposure assessment/characterisation related to food

Anticipated dietary exposure to soy leghemoglobin protein was estimated considering different exposure scenarios using the FAIM and DietEx tools. Details on the scenarios used and the exposure estimates across different European population

[9]Overview on Tolerable Upper Intake Levels as derived by the Scientific Committee on Food (SCF) and the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Version 9 (December 2023). https://www.efsa.europa.eu/sites/default/files/2023-11/ul_summary_tables-version-8.pdf.

[10]Levels of haem iron in different types of raw meat as described by Lombardi-Boccia et al. (2002): beef sirloin (1.72 mg/100 g); beef fillet (2.11 mg/100 g); roast beef (1.77 mg/100 g), horse fillet (1.75 mg/100 g); ostrich fillet (1.76 mg/100 g).

[11]EFSA FAF Panel Opinion. (2024): *'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'.*

groups are described in Section 3.3.2 'Exposure to soy leghemoglobin from genetically modified *Komagataella phaffii*' of the EFSA FAF Panel Opinion (EFSA FAF Panel, 2024).

3.9 | Post-market monitoring of the GMM and/or its product for food or feed

The GMO Panel concludes that based on the information considered during the safety assessment of the LegH Prep in this opinion, a post-market monitoring plan is not necessary.

3.10 | Potential environmental impact

As stated by the applicant, finished food products containing soy leghemoglobin will be imported. Thus, the genetically modified *K. phaffii* production strain will not enter the environment. Furthermore, the applicant states that, should production of soy leghemoglobin be carried out within the EU, it would be done by facilities which meet the requirements for level 1 contained use as described in Directive 2009/41/EC and will follow any other requirements as prescribed by applicable national regulations.

The product preparation process (Section 3.6) and the additional information provided by the applicant, demonstrated that the soy LegH Prep does not contain viable cells of the production strain *K. phaffii* MXY0541.

However, the preparation process does not prevent the presence of residual DNA in the product including the possibility to identify genes in full-length. The production strain does not contain antibiotic resistance gene sequences and the only exogenous sequences present in the construct is the *LGB2* from soybean. A potential transfer of DNA of plant origin from the yeast to environmental bacteria would not provide any selective advantage and thus pose no safety concern. Therefore, no environmental impact from the use of this product is expected regarding the recombinant DNA sequences possibly remaining in the product. The GMO Panel considers that the use of soy LegH Prep as food additive will not adversely affect the environment.

3.11 | Literature review

The GMO Panel assessed the applicant's literature searches on soy leghemoglobin protein produced in *K. phaffii*. The GMO Panel considered the overall quality of the performed literature searches acceptable. Based on the relevant publications, the GMO Panel does not identify any safety issues pertaining to the intended uses of the soy leghemoglobin product.

4 | CONCLUSIONS

The GMO Panel was asked to carry out a scientific assessment of the genetic modification for soy leghemoglobin produced from genetically modified *K. phaffii* in accordance with Regulation (EC) No 1829/2003. The molecular characterisation data establish that *K. phaffii* strain MXY0541 is the result of several genetic modifications aimed at the upregulation of the endogenous haem pathway and the expression of soy leghemoglobin protein. The quality of the sequencing methodology and datasets was assessed by the EFSA GMO Panel and is in compliance with the requirements listed in the EFSA Technical Note. Updated bioinformatics analyses of the sequence encoding the newly expressed protein do not raise any safety concerns. The stability of the inserted DNA and of the introduced trait is confirmed. The GMO Panel does not identify safety concerns regarding the toxicity and allergenicity of soy leghemoglobin protein as expressed in *K. phaffii* and finds no evidence that the genetic modification would change the overall allergenicity of *K. phaffii*. However, the GMO panel deems it in line with current practice to label the product with 'Contains Soy', as the gene responsible for expressing the leghemoglobin protein is derived from soy, a known allergenic source. The GMO Panel concludes that the LegH Prep derived from genetically modified *K. phaffii* strain MXY0541 is safe for human consumption with regard to the effects of the genetic modification. The analysis of a potential HGT from residual DNA corresponding to the LGB2 coding sequence present in the LegH Prep from *K. phaffii* strain MXY0541 to bacteria does not indicate a safety concern. Based on the relevant publications identified through the literature searches, the GMO Panel does not identify any safety issues pertaining to the uses of LegH Prep, as far as the genetic modification is concerned. The GMO Panel concludes that LegH Prep from genetically modified *K. phaffii MXY0541* is safe with respect to potential effects on human health and the environment at the proposed use and use level as far as the impact of the genetic modification is concerned. The overall conclusion is that the genetic modification does not lead to safety issues.

5 | DOCUMENTATION AS PROVIDED TO EFSA

- Letter from the Competent Authority of the Netherlands received on 15 October 2019 concerning a request submitted in accordance with Regulation (EC) No 1829/2003 by Impossible Foods Inc. (EFSA Ref. EFSA-GMO-NL-2019-162; EFSA-Q-2019-00651).

- The application was made valid on 15 December 2021.
- Additional Information (1) was requested on 21 December 2021.
- Additional Information (1) was received on 19 February 2022.
- Additional Information (2 JRC-EURL) was requested on 31 January 2022.
- Additional Information (2 JRC-EURL) was received on 20 June 2023.
- Additional Information (3) was requested on 20 June 2022.
- Additional Information (3) was received on 18 August 2022 partial, 30 September 2022 full.
- Additional Information (4 JRC-EURL) was requested on 15 July 2022.
- Additional Information (4 JRC-EURL) was received on 17 September 2024
- Additional Information (5) was requested on 21 December 2022.
- Additional Information (5) was received on 12 April 2023 partial, 23 May 2023 full.
- Additional Information (6) was requested on 13 July 2023.
- Additional Information (6) was received on 12 September 2023 partial, 17 November 2023 full.
- Additional Information (7 JRC-EURL) was requested on 3 November 2023.
- Additional Information (7 JRC-EURL) was received on 24 January 2024.
- Additional Information (8) was requested on 23 November 2023.
- Additional Information (8) was received on 23 February 2024 partial, 2 April 2024 partial, 12 April 2024 full.

ABBREVIATIONS

BIOHAZ Panel	Panel on Biological Hazards
Bp	base pair
Bw	body weight
CEP Panel	Panel on Food Contact Materials, Enzymes and Processing Aids
FAF Panel	Panel on Food Additives and Flavourings
FAIM	Food Additives Intake Model
GMM	genetically modified microorganism
GMO Panel	Panel on Genetically Modified Organisms
GRAS	generally recognised as safe
LegH Prep	liquid preparation containing soy leghemoglobin
LOQ	limit of quantification
OECD	Organization for Economic Co-operation and Development
PCR	polymerase chain reaction
QPS	Qualified Presumption of Safety
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
WGS	whole genome sequencing

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REQUESTOR

Competent Authority of The Netherlands

QUESTION NUMBER

EFSA-Q-2019-00651

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The relevant information or parts of this scientific output have been blackened in accordance with the confidentiality requests formulated by the applicant pending a decision thereon by the European Commission. The full output has been shared with the European Commission, EU Member States and the applicant. The blackening will be subject to review once the decision on the confidentiality requests is adopted by the European Commission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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