Adviesraad voor Bioveiligheid
Conseil consultatif de Biosécurité

Advice of the Belgian Biosafety Advisory Council on application EFSA-GMO-NL-2019-162 (soy leghemoglobin produced from GM *K. phaffii*) from Impossible Foods under Regulation (EC) No. 1829/2003

18 December 2024
Ref. SC/1510/BAC/2024_1572

Context

Application EFSA-GMO-NL-2019-162 was submitted by Impossible Foods for the authorisation for the marketing of soy leghemoglobin produced from GM *Komagataella phaffii* (previously *Pichia pastoris*) for use in food within the European Union, within the framework of Regulation (EC) No. 1829/2003[1].

K. phaffii strain MXY0541 contains the *LGB2* coding sequence for leghemoglobin from soybean (*Glycine max*). The final liquid preparation containing the soy leghemoglobin (called LegH Prep) additionally contains residual proteins and (recombinant) DNA from the *K. phaffii* production strain.

The application was validated by EFSA on 15 December 2021 and a formal three-month consultation period of the Member States was started on 22 December 2021 until 28 June 2022 (including an interruption), in accordance with Articles 6.4 and 18.4 of Regulation (EC) No. 1829/2003 (consultation of national Competent Authorities within the meaning of Directive 2001/18/EC designated by each Member State in the case of genetically modified organisms being part of the products).

Within the framework of this consultation, the Belgian Biosafety Advisory Council (BAC), under the supervision of a coordinator and with the assistance of its Secretariat, contacted experts to evaluate the dossier, chosen from the common list of experts drawn up by the BAC and the Service Biosafety and Biotechnology (SBB). Three experts answered positively to this request, and formulated a number of comments to the dossier. See Annex I for an overview of all the comments and the comment sent to EFSA.

Upon a request from the Council, the Competent Authority provided in May 2022 a clarification on the scope of the advice it expects to receive for this dossier, namely the molecular characterisation of the GMM, the evaluation of the efficacy of the inactivation of the GMM, and the environmental risk assessment of the presence of recombinant DNA.

EFSA's FAF Panel (Food Additive and Flavourings) evaluated the safety of LegH Prep as a food additive in accordance with Regulation (EC) No 1331/2008[2], and published its scientific opinion on 28 June 2024 (EFSA Journal. 2024;22:e8822[3]).

EFSA's GMO Panel assessed the impact of the genetic modification on the safety of LegH Prep for food use, and the environmental risk linked to the presence of recombinant DNA in the product. Its scientific opinion, including the responses from the Panel to comments submitted by the Member States during

[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).
[2] 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)
[3] See https://doi.org/10.2903/j.efsa.2024.8822

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the three-month consultation period, was published on 15 November 2024 (EFSA Journal. 2024;22:e9060[4]).

In delivering the present advice, the BAC considered in particular the request by the Competent Authority, the comments formulated by the experts on application EFSA-GMO-NL-2019-162 and the opinion of EFSA's GMO Panel.

Scientific evaluation

1. Molecular characterisation

With regard to the genetic modification, the Biosafety Advisory Council is of the opinion that the information provided is sufficient and does not raise safety concerns. In particular, the Biosafety Advisory Council wants to highlight that:

(1) the absence of viable cells in the product was shown, demonstrating the efficacy of the inactivation of the GMM;
(2) the commercial strain does not contain any antimicrobial resistance genes;
(3) it agrees with the GMO Panel of EFSA that the available bioinformatics data on the soy leghemoglobin protein expressed by *K. phaffii*, does not raise safety concerns regarding toxicity and allergenicity.

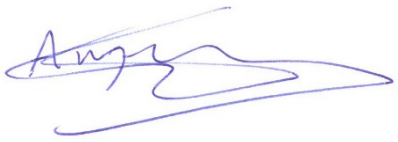
2. Environmental risk assessment

The Biosafety Advisory Council wants to note that, as the product considered in this application does not contain viable material, an assessment of the potential environmental impact, e.g. on ecosystem functions, is in principle legally not necessary. Nevertheless, the Biosafety Advisory Council agrees with the GMO Panel that it is unlikely that the presence of recombinant DNA in the product will lead to environmental harm.

Conclusion

Based on the whole set of data on soy leghemoglobin produced from GM *K. phaffii* provided by the applicant, the scientific assessment of the dossier done by the Belgian experts, the opinion of EFSA's GMO Panel, and the answers of the GMO panel to the question of the Biosafety Advisory Council, the Council:

1) Agrees with the GMO panel of EFSA that the LegH Prep derived from genetically modified *K. phaffii* is safe for human consumption with regard to the effects of the genetic modification;
2) Agrees with the GMO panel of EFSA that the LegH Prep derived from genetically modified *K. phaffii* is unlikely to pose any threat to the European environment.



Dr. ir. Geert Angenon
President of the Belgian Biosafety Advisory Council

Annex : Outcome of the assessment of the application and comments sent to EFSA

[4] See https://doi.org/10.2903/j.efsa.2024.9060

Annex : Outcome of the assessment of application EFSA-GMO-NL-2019-162 by the Biosafety Advisory Council during the formal consultation of the Member States (3-month commenting period in accordance with Articles 6.4 and 18.4 of Regulation (EC) No 1829/2003) and feedback from the EFSA GMO Panel

Coordinator: Dr. Lieve Gheysen
Experts: Henri Batoko (UCL), Marc De Loose (ILVO), André Huyghebaert (UGent)
SBB: Fanny Coppens

Application: EFSA-GMO-NL-2019-162
Applicant: Impossible Foods
Product: Soy leghemoglobin from genetically modified *Pichia pastoris*
Validation of dossier by EFSA: 22 December 2021

Categorisation of the GMM:
☐ Category 1: Chemically defined purified compounds and their mixtures in which both GMMs and newly introduced genes have been removed (e.g. amino acids, vitamins);
☐ Category 2: Complex products in which both GMMs and newly introduced genes are no longer present (e.g. cell extracts, most enzyme preparations);
☒ Category 3: Products derived from GMMs in which GMMs capable of multiplication or of transferring genes are not present, but in which newly introduced genes are still present (e.g. heat-inactivated starter cultures);
☐ Category 4: Products consisting of or containing GMMs capable of multiplication or of transferring genes (e.g. live starter cultures for fermented foods and feed).

Scope: food produced from or containing ingredients produced from GMOs

Given the characteristics of the GMO and its intended uses, experts were consulted to cover the following areas of expertise:
☒ Molecular characterization
☒ Environmental aspects
☒ Allergenicity
☒ Toxicology
☒ Food and Feed aspects

The experts were asked to evaluate whether the information provided in the application is sufficient in order to state that the marketing of the genetically modified plant for its intended uses, will not raise any problems for the environment or human or animal health. If information is lacking, the expert was asked to indicate which information should be provided and what the scientifically reasoning is behind this demand.

None of the comments formulated by the experts were selected to be sent to EFSA. It should be noted that all the comments received from the experts are considered in the evaluation of this dossier and in formulating the final advice of the Biosafety Advisory Council.

The following comment was sent to EFSA:
In the context of the consultation of the MS under Regulation 1829/2003, the Belgian Biosafety Advisory Council evaluated the molecular characterisation of the GMM (*P. Pastoris*) and the efficacy of the inactivation of the GMM. The Council neither has comments, nor requests for additional information on these two items.

Further, we want to note that according to the GMO legislation, an ERA only needs to be conducted in case the application concerns products containing or consisting of a genetically modified organism, i.e. viable material. As the product considered in AP162 does not contain viable material, an assessment of the potential environmental impact, e.g. on ecosystem functions (Chapter E of dossier) is not necessary. We would appreciate EFSA's feedback on this issue.

Feedback from the EFSA GMO Panel: The GMO Panel takes note of the comment. Following the GMM guidance 2011 Section III. B.4.2., the potential for HGT has been checked.

List of comments/questions received from the experts

PART I - GENERAL COMMENTS

Comment 1
This application concerns the marketing of a preparation of a soy haemoprotein produced by genetic modification of the yeast *Pichia pastoris*. The final product protein composition is made of more than 65% soy haemoprotein, protein contaminants and nucleic acid from the *P. pastoris* production strain, and stabilizers. The preparation called LegH Prep is intended to be used as an ingredient for simulated meat products as a nutritional, aroma and flavour component. The finished food product to be sold within the EU will be imported.

PART II - SCIENTIFIC INFORMATION

1. HAZARD IDENTIFICATION, HAZARD CHARACTERISATION AND EXPOSURE ASSESSMENT

1.1. INFORMATION RELATING TO THE GMM

Comment 1
I've evaluated this section and I consider the information adequate.
The genetically modified strain characteristics is considered CBI by the applicant. The *P. pastoris* production organism was developed from a parental strain with an established history of safe use in the food industry.

Comment 2
What are the experimental conditions for the PCR tests? Is the analysis carried out on a purified strain? What are the amounts of DNA extracted and how much was used in the PCR reaction? What are the positive controls for the PCR reaction on this DNA (e.g. reference gene?, internal gene?)

Comment 3
What is the history of safe use concerning the soy leghemoglobin?

Note Coordinator/SBB: The applicant notes on p. 51 that soy leghemoglobin is not widely consumed by humans, and the applicant's assessment of toxicity and allergenicity of the protein are part of the dossier.

1.2. INFORMATION RELATING TO THE PRODUCT (INCLUDING CASES WHEN THE GMM ITSELF IS THE PRODUCT)

1.2.1. Information relating to the production process

Have evaluated this section and consider the information adequate: 1 expert

1.2.2. Information relating to the product preparation process

Comment 1
The production of LegH Prep consists of the expression of the soybean LGB2 gene in *P. pastoris* during fermentation. The yeast cells are then harvested and lysed and the soy hemoprotein (about 16 kDa) concentrated by filtration. The preparation is then heated to inactivate any possible contaminating

microorganisms during the preparation process. The final product is described as containing at most 17 yeast proteins (probably small proteins as well). It is not specified whether this protein composition in yeast peptides is constant quantitatively and qualitatively from one batch to another. Furthermore, to ensure production of soy holoprotein, the biosynthetic pathway of tetrapyrroles was boosted in the producing yeast strain. It is unclear whether all of the soy proteins present in LegH Prep are heme-complexed or not. The holoprotein can lose its prosthetic group (heme) during heat treatment (Leghemoglobin is denatured if heated >65°C) and free heme is toxic in particular for biological membranes.

Comment 2
C.2.1. Although no viable *P. pastoris* cells remain in the final LegH Prep (see Section C.2.2), some residual amounts of the *P. pastoris* proteins (representing up to 35% of the total protein) and DNA (averaging 21 µg/g) remains. Are these proteins characterised and have these proteins been checked for potential allergenicity and/or toxicity?

Note Coordinator/SBB: The toxicity and allergenicity studies reported in the dossier were conducted on this final LegH Prep.

Comment 3
C.2.2. *P. pastoris* cells are lysed using suitable processes such as bead mill mechanical shearing or high-pressure homogenization. The lysate is then heat treated to remove potential for growth of any remaining *P. pastoris* cells. Are the conditions described in the document? How will it be checked in the context of enforcement that real productions will make use of these conditions?

Note Coordinator/SBB: This application only concerns the import of LegH Prep for food use; the production process will take place outside of the EU.

Comment 4
C2.3. The average concentration of *Pichia* DNA in 3 production lots of LegH Prep was determined using quantitative PCR. Why quantitative PCR, which is a relative method for detection/quantification (what is the reference material) and not digital PCR which is an absolute quantification method?

1.2.3. Description of the product

Have evaluated this section and consider the information adequate: 1 expert

Comment 1
In the specifications, table C.3.3.1-1 it is mentioned that the soy leghemoglobin protein content is < 9% with a protein purity of > 65 %. Is there any information about the 35 % of the protein constituents present in the preparation? It is mentioned that the other proteins are residual *Pichia* proteins. Is there information about these proteins and their properties; This question has been answered in further sections of this document. So this observation is no longer relevant.

Comment 2
About the stability of LegH at -20°C: upon storage as a liquid preparation there seems to be a decrease in legH content. What are the degradation products? Is the underlying mechanism of the degradation process known? Is there any toxicity of the degradation products due to oxidation processes?

Comment 3

From the results, shown in table C.3.5.2-1 of the stability van LegH in a meat analogue at 4 °C, it is clear that a significant decrease in the residual content is observed. There is in some cases a decrease of up to 15 to 20 % in the target recovery. My question is similar to the previous case of storage at -20 °C: what is known about the degradation products and their potential toxicological effects? Have there been tests about human health with meat analogues that have been stored for 9 days at 4 °C?

Comment 4
Flavouring properties:
The mechanism of flavour development is well documented in the application.

Safety Assessment:
The applicant considers the potential risks of the yeast *Pichia pastoris* and of LegH according to the EFSA guidelines.

1.2.4. Considerations of the GMM and/or its product for human health

Comment 1
I have evaluated this section and I consider the information adequate (no comment/question)

P. pastoris has a good history of safe use in the food industry and the only foreign gene in the production strain is the soy leghemoglobin gene.

Comment 2
“Contains Soy” as these products contain ingredients obtained from soybeans, and Impossible Foods will comply with all applicable requirements set forth in Regulation (EU) No 1169/2011 with regards to allergen declaration. Is this interpretation of the legislation 1169/2011 correct?

Note Coordinator/SBB: The application or interpretation of Regulation (EU) No 1169/2011 on the provision of food information to consumers is outside of the remit of the Council.

1.2.4.1. Toxicology

Comment 1
I agree with the conclusion from the applicant that LegH did not induce mutagenic and clastogenic effects.

Comment 2
The soy leghemoglobin protein is the major constituent of LegH Prep. This particular isoform of the protein is exclusively expressed in nodule (roots) with no history of human consumption. However, soy leghemoglobin is structurally similar to other globin proteins including myoglobin from animal meat consumed in diet suggesting the safety of such hemoproteins and the accompanying heme b. Bioinformatics data presented and discussed by the applicant suggest that proteins in LegH Prep have no sequence homologous to any putative toxin and are susceptible to in vivo digestive processes. LegH component also did not show any mutagenic activity in in vitro reverse mutation assays and were not shown to be clastogenic in chromosome aberration test. These data provide ground to suggest that LegH Prep can not be considered genotoxic.

1.2.4.2. Allergenicity

Have evaluated this section and consider the information adequate: 1 expert

Comment 1
I have evaluated this section and I consider the information adequate.
Known soy allergens endogenous to the crop are absent from LegH Prep. In addition, data presented by the applicant suggest that the major polypeptide in LegH Prep has no significant similarity (i.e. >35% sequence homology over a window of 80 amino acids, and sequence homology with 8 contiguous amino acids) with known allergens. The final product (simulated meat product) containing LegH Prep will be cooked before serving, and given that all the polypeptides in LegH are digested in vitro under conditions mimicking normal digestion, it can be anticipated that LegH components are not expected to elicit an immune response.

1.2.4.3. Nutritional assessment

Comment 1
It is mentioned that the addition of LegH to meat analogue is self-limiting due to unacceptable organoleptic properties. Any further information available about these unacceptable properties?

Comment 2
It is claimed that the meat analogue containing LegH has the same level of haem iron as in ground beef. What about the bio-availability of iron from the meat analogue? Resorption may be disturbed by other constituents of the meat analogue, such as phytates and other anti-nutrients.

As it is intended to use LegH in other meat analogues, is iron resorption by the global population, especially vulnerable groups, not of concern?

Comment 3
I have evaluated this section and I consider the information adequate.
LegH Prep will be used essentially as a flavour ingredient for simulated meat product. However, the product could also be a source of heme-derived nutritional iron. In this context, data presented by the applicant seem to suggest that LegH Prep in simulated meat product can be a reasonable substitute to iron intake from ground beef consumption.

1.3. EXPOSURE ASSESSMENT/CHARACTERISATION RELATED TO FOOD AND FEED CONSUMPTION

Have evaluated this section and consider the information adequate: 1 expert

1.4. POTENTIAL ENVIRONMENTAL IMPACT OF GMMS AND THEIR PRODUCTS

Comment 1
The discussed data pertain more to horizontal gene transfer and the applicant convincingly suggest that this is unlikely or of no detrimental consequence to the environment. However, potential accidental release of the production strain is not well discussed. In particular, no experiment was conducted to assess the relative fitness of this strain which, in addition to encoding a plant gene resulting in an additional haemoprotein in its repertoire, also has a boosted tetrapyrrole biosynthetic pathway. It can be argued that in the context of this application LegH Prep will not be produced in Europe and the growth of the modified strain is within a confined environment (fermentation).

Note Coordinator/SBB: This application concerns the import of LegH Prep for food use; the production process will take place outside of the EU.

2. RISK CHARACTERISATION

2.1. ISSUES TO BE CONSIDERED

Have evaluated this section and consider the information adequate: 1 expert

2.2. CONCLUSIONS FROM THE RISK CHARACTERISATION OF GMMS AND DERIVED FOOD/FEED

Have evaluated this section and consider the information adequate: 1 expert

3. POST-MARKET MONITORING REGARDING USE OF THE GMM AND/OR ITS PRODUCT FOR FOOD OR FEED

Have evaluated this section and consider the information adequate: 1 expert

4. POST-MARKET ENVIRONMENTAL MONITORING (PMEM)

4.1. GENERAL

Comment 1
It is not clear to me that because restricted to confined environment the production organism could never be involuntarily released, see also comments on 1.4.

4.2. CASE-SPECIFIC MONITORING

4.3. GENERAL SURVEILLANCE

4.4. MONITORING SYSTEM

4.5. REPORTING THE RESULTS OF MONITORING

5. SUMMARY OF THE RISK ASSESSMENT REQUIREMENTS

Have evaluated this section and consider the information adequate: 1 expert