Comments on EFSA opinions on Impossible Foods' application for EU market approval of its soy leghemoglobin product derived from GM yeast *K. phaffii*: Major gaps and errors identified

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Summary

The EFSA FAF (food additives) and GMO (genetically modified organism) Panels have issued favourable opinions on the safety of Impossible Foods' application for EU market approval of its soy leghemoglobin product (LegH Prep) derived from a genetically modified strain of the yeast *K. phaffii*. The product is intended to give meat substitute products a "bleeding" appearance and a meaty flavour. Neither panel saw any safety concerns with this GM yeast-derived ingredient.

However, there are serious concerns about the food safety of GM yeast-derived LegH Prep. Neither soy leghemoglobin nor *K. phaffii* (GM or not) have a history of safe use in food. Also, feeding studies with animals fed LegH Prep show adverse and potentially adverse health effects, including signs of anemia, decreased blood clotting ability, and kidney function problems. These findings were dismissed by the company and by the EFSA FAF and GMO Panels for what appear to be scientifically invalid reasons. Therefore EFSA should revisit its interpretation of these findings, objectively report them, and demand long-term animal feeding studies to clarify their implications to health.

A particular concern regarding the food safety of LegH Prep is that it is only 65% soy leghemoglobin. The other 35% is made up of contaminant proteins and (potentially) metabolites derived from the GM *K. phaffii* production strain. This high level of contaminants, an unknown number of which remain unidentified and unanalysed for safety, means that the safety of GM *K. phaffii*-derived LegH Prep is in question.

Moreover, the EFSA panels accepted safety data on LegH Prep produced with the wrong strains of yeast. Safety data should all be derived from the strain (MXY0541) that will be used for the commercialised product. Yet some of the data are derived from a previous less "optimised" strain, MXY0291, and the panels' opinions sometimes do not make clear which strain was used. This is unacceptable and poses a risk to public health.

As a precautionary reminder, it is instructive to recall the L-tryptophan catastrophe in the 1980s, when contaminants in the GM bacteria-produced food supplement manufactured by the company Showa-Denko caused a new disease, known as eosinophilia-myalgia syndrome (EOS), which severely and chronically sickened over 1500 people and caused 37 deaths. The contaminants resulted from the manufacturing company's progressive optimisation of the bacterial strains for higher L-tryptophan production. After the event, scientists emphasised the need for "close monitoring of the chemical purity of biotechnology-derived products, and for rigorous

testing of such products following any significant changes to the manufacturing process" through optimisation of the GM microorganism production strain.

In line with such a precautionary approach, the FAF Panel stated in its opinion that the EU authorisation of LegH Prep "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". Yet this strong statement is contradicted by the fact that the FAF and GMO Panels have accepted safety data derived from the earlier strain, MXY0291. EFSA should instruct the company to re-do its safety tests on the commercially relevant strain and re-submit its application.

Recommendations

The EFSA panels should withdraw and revise their assessments, based on requiring Impossible Foods to

- supply safety data on LegH Prep derived from the commercially relevant *K. phaffii* strain, MXY0541
- investigate the global biochemical and thus compositional consequences of the genetic modifications that made *K. phaffii* strain MXY0291 into the commercially relevant MXY0541, e.g. using untargeted proteomics and metabolomics
- conduct analysis of all the proteins in LegH Prep for toxicity as well as allergenicity
- conduct an untargeted metabolomics analysis to check for potential toxic metabolites
- make all the above data public.

Meanwhile EFSA should publish the full details of, and objectively evaluate, the animal feeding studies that revealed adverse and potentially adverse effects from LegH Prep consumption.

For all safety data provided to date, EFSA must clarify which GM *K. phaffii* strain was used in the production of the LegH Prep in each test applied.

The EFSA GMO Panel has published a favourable opinion (EFSA GMO Panel, 2024) on Impossible Foods' application (Impossible Foods, 2019) for EU market approval of its soy leghemoglobin product derived from the genetically modified (GM) yeast Komagataella phaffii (*K. phaffii*), sometimes referred to by its former name Pichia pastoris (P. pastoris). The GMO Panel's opinion follows and relies significantly upon an earlier favourable opinion by EFSA's Panel on Food Additive and Flavourings (FAF Panel) (EFSA FAF Panel, 2024). Neither panel saw any safety concerns with this GMO yeast-derived ingredient.

Impossible Foods' application to the EU is for approval of soy leghemoglobin derived from the GM yeast strain MXY0541, the strain that Impossible Foods currently uses for production (Impossible Foods, 2019). This strain has been genetically modified to

produce soy leghemoglobin in a fermentation process. Soy leghemoglobin is red in colour and has a meat-like flavour. Impossible Foods uses GM yeast-derived soy leghemoglobin in its meat substitute products, such as the Impossible Burger™, to give them a “bleeding” effect and a meaty flavour.

At the end of the fermentation process, the haem molecule is purified out from the soy leghemoglobin and is made into a liquid preparation called LegH Prep – a mixture containing soy leghemoglobin protein, *K. phaffii* yeast proteins, and stabilisers, such as sodium chloride and sodium ascorbate (Impossible Foods, 2019). The LegH Prep is then used as an additive in Impossible Foods’ meat substitute.

Soy leghemoglobin – no history of safe use in food

Soy leghemoglobin in its natural form is a protein present in the root nodules of soy plants, but not in the part of the plant that is eaten by humans – the beans. It has never formed part of the human food supply. Also, the soy leghemoglobin produced from genetically modified *K. phaffii* – which will be different from the natural form – has no history of safe use as a food ingredient.

***K. phaffii* – no history of safe use in food**

K. phaffii (genetically engineered or not) has no history of safe use in human food.

Genetically engineered versions of *K. phaffii* have long been used to produce pharmaceutical proteins and industrial enzymes. Enzyme production in GM *K. phaffii* includes enzymes for use in food and animal feed production or as animal feed supplements (Ahmad M et al, 2014; Karbalei M et al, 2020); Kuruti K et al, 2020). However, this is not the same as production of substances intended for direct introduction into the human food supply, such as Impossible Foods’ GM *K. phaffii*-derived soy leghemoglobin. Pharmaceutical proteins are highly purified (often over 99% pure (Puetz J, Wurm FM, 2019) and cannot be taken as evidence of food safety for GM *K. phaffii*-produced soy leghemoglobin. Enzymes used for food or feed production are often removed or inactivated in the final product (US FDA, 2010) and food-grade enzymes must be of a high level of purity (Ramos OS, Malcata FX, 2011).

In contrast, Impossible Foods’ LegH Prep is only 65% soy leghemoglobin (EFSA FAF Panel, 2024, Table 1). No viable *K. phaffii* cells remain in the final LegH Prep, but 35% of LegH Prep is made up of contaminant proteins and (potentially) metabolites, all derived from the GM *K. phaffii* production strain, which have likely never been a component of a human food product. This high level of contaminants, an unknown number of which remain unidentified, uncharacterised, and unanalysed for safety, means that the safety of GM *K. phaffii*-derived LegH Preps cannot be inferred from the safety of other more highly purified substances produced from GM *K. phaffii*, such as pharmaceutical proteins and enzymes.

As evidence for the food safety of GM *K. phaffii*-derived substances, the EFSA FAF Panel only repeated Impossible Foods’ statement that other food ingredients (a phospholipase C enzyme preparation, a soluble egg-white protein and a myoglobin

preparation) using *K. phaffii* as a production system are currently available in the US marketplace and no reports of adverse reactions have been reported (EFSA FAF Panel, 2024, para 3.4.7). But the limited use of *K. phaffii* to produce these substances is irrelevant to the risk profile of *K. phaffii*-derived LegH Preps. The *K. phaffii* used to produce them will have different genetic modifications from the strains used by Impossible Foods to produce soy leghemoglobin and the final products will have different levels and types of contaminants, as well as different end-use and consumption patterns.

Given the absence of a history of safe use for soy leghemoglobin (both natural and GM yeast-derived) in food and a similar absence for GM *K. phaffii*-produced proteins in human food, a high standard of proof should be provided to establish the safety of LegH Prep for human consumption. However, neither Impossible Foods' application, nor the two EFSA panels' opinions, supply such proof. The application and the opinions are full of data gaps, misleading statements, and misrepresentations of scientific findings, which together give a false impression of safety that is not supported by evidence.

Confusion around different strains used in safety tests

Impossible Foods' application for authorisation is for soy leghemoglobin made from the production strain MXY0541, but in the company's studies submitted to various regulatory authorities and in its peer-reviewed publications, it uses safety data from an earlier, less "optimised" strain, MXY0291 (for MXY0291's less "optimised" status, see EFSA FAF Panel, 2024, para 3.4.5), from a "parent" strain of MXY0291 called NRRL Y-11430, and from MXY0541, interchangeably.

For example:

- For the company's application to the US FDA, an initial 28-day toxicity study in rats was done with LegH Prep from GM *K. phaffii* strain MXY0291 (US FDA, 2017: para 6.3.2.1 following. It seems likely that this is the same study referred to by the EFSA FAF Panel (para 3.4.4) and GMO Panel (para 3.8.1.1) opinions).
- A second 28-day toxicity study in rats was done LegH Prep derived from MXY0291, as reported in a peer-reviewed journal alongside the results of an initial 28-day toxicity study (Fraser RZ et al, 2018). It is unclear whether the initial 28-day study is the same one as was cited in the company's application to the US FDA – but the available details published in the journal and in the FDA's report match and strongly suggest that the two studies are the same (US FDA, 2017: para 6.3.2.1 following).
- In a peer-reviewed publication separate from the application, *in vitro* and *in vivo* safety studies (the latter including a 90-day toxicity study in rats) were done with a LegH Prep from strains MXY0291 and NRRL Y-11430 (Reyes TF et al, 2023).
- It is often unclear in the EFSA FAF Panel's opinion which strain was used in the company's various tests cited in the application. For example, the FAF Panel describes subchronic in vivo toxicity studies commissioned by Impossible Foods (one 14-day, two 28-day, and one 90-day), without mentioning the strains used (EFSA FAF Panel, 2024, para 3.4.4). The GMO Panel, on the other hand, does mention the *K. phaffii* strains used – MXY0291

> for the 14- and 28-day studies and MXY0541 for the 90-day study (EFSA GMO Panel, 2024, para 3.8.1.1). This indicates that the GMO Panel evaluated data from toxicity studies using LegH Prep derived from an irrelevant *K. phaffii* strain (MXY0291) for a significant part of the safety assessment.

This conflation of different strains is unacceptable. The risk assessment should be based solely on the *K. phaffii* production strain that will be used commercially.

It is also unclear whether the studies that Impossible Foods published in peer-reviewed journals are the same studies as were reported in the company's application to the EU and evaluated by the EFSA FAF and GMO Panels. Relevant to this question is the discrepancy between the GMO Panel's statement that the 90-day study described in Impossible Foods' application used the MXY0541 strain (EFSA GMO Panel, 2024, para 3.8.1.1) and the company's statement in its 90-day peer-reviewed study that the strains used were MXY0291 and NRRL Y-11430 (Reyes TF et al, 2023).

If the GMO Panel's and company's statements are both accurate, it would mean that Impossible Foods carried out two separate 90-day studies, one with LegH Prep from GM *K. phaffii* strains MXY0291 and NRRL Y-11430 and the other from MXY0541. EFSA should clarify if this is the case.

Why the strain matters

In a key statement, the EFSA FAF Panel explicitly stated that any safety assessment should be linked to a specific *K. phaffii* strain. The Panel noted that Impossible Foods had originally "proposed that the authorisation should not be linked to any specific production strain, to accommodate future improvements to the strain" (EFSA FAF Panel, 2024, para 3.1.2; and 4. Discussion).

However, the FAF Panel disagreed with the company: "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." Accordingly, the Panel recommended the introduction of the production strain MXY0541 in the Definition of the specifications for the proposed food additive (EFSA FAF Panel, 2024, para 3.1.2; and 4. Discussion).

It is the more baffling, then, that in clear contradiction of these strong and scientifically based statements, the FAF and GMO Panels have accepted safety data obtained from the MXY0291 and probably from the NRRL Y-11430 strain and are using it to support the authorisation of soy hemoglobin derived from the MXY0541 strain.

Are the MXY0291 and MXY0541strains significantly different? It appears that they are. Food Standards Australia New Zealand (FSANZ) stated in its opinion on Impossible Foods' application: "Some of the data provided to FSANZ for the risk assessment analyses was obtained from a predecessor of MXY0541, designated

MXY0291. The major differences between these two strains is [sic] the copy number of the leghemoglobin gene (MXY0291 contains fewer copies) and MXY0541 contains extra DNA sequences associated with one of the haem-synthesis enzyme genes" (FSANZ, 2020, para 2.1.1).

The differences in the genetic sequences of the two strains mean that the contaminating proteins that co-purify with the soy leghemoglobin protein will be different, too. The differences could appear in either the protein profile or in the amounts of each protein present.

Due to these differences, MXY0541 will have a different risk profile (including potential differences in the toxicity or allergenicity of the soy leghemoglobin derived from it) from MXY0291. All safety data provided in support of market authorisation should be based on MXY0541-derived soy leghemoglobin only.

Extensive genetic modifications made to the yeast

Impossible Foods states, "The only heterologous [from another species] donor gene introduced to the production strain is the LGB2 gene encoding leghemoglobin from the soybean plant (Glycine max), which was synthesised and optimised for expression in P. pastoris... The other changes introduced into the production strain involve genes that are native to P. pastoris" (Impossible Foods, 2019). This statement is echoed by the EFSA GMO Panel (EFSA GMO Panel, 2024, para 3.2.10).

However, this statement is misleading and even disingenuous. It is true that only one heterologous gene has been inserted into the yeast. But multiple other extensive genetic modifications have also been made to the native yeast genes to maximise the production of haem. These modifications are listed in:

i. A peer-reviewed study by Impossible Foods' scientists using strain MXY0291 and used to support the EU regulatory authorisations. The scientists write that compared with the parent strain, "MXY0291 was modified to overexpress the gene encoding the soy LegH as well as all 8 enzymes in the native Pichia heme biosynthesis pathway (aminolevulinic acid (ALA) synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, uroporphyrinogen (UPG) III decarboxylase, coproporphyrinogen oxidase, protoporphyrinogen oxidase, and ferrochelatase) using the Pichia alcohol oxidase 1 promoter (pAOX1). MXY0291 was also modified to overexpress the Mxr1 transcriptional activator using the pAOX1 promoter. The Mxr1 protein activates the pAOX1 promoter leading to increased expression of pAOX1-driven LegH, heme biosynthesis genes, and Mxr1 itself" (Fraser RZ et al, 2018).

   "Transgene" means "a gene which is introduced into another organism" (Oxford English Dictionary, 2024), not necessarily from another species. "Transgenesis" means "the process of introducing an exogenous or modified gene (transgene) into a recipient organism of the same or different species from which the gene is derived" (Blanco A, Blanco G, 2017). Therefore the artificial genetic constructs in Impossible Foods' GM yeast are transgenes,

introduced in a transgenic procedure. According to Impossible Foods' entries in the EUginius database, 16 copies of the soy leghemoglobin gene have been incorporated in the GM yeast in order to maximise production levels (EUginius, 2024). And according to the company's description in a peer-reviewed paper, there appear to be at least 9 transgenes in the GM yeast strain MXY0291 to code for an enhanced haem biosynthesis pathway (Fraser RZ et al, 2018). This makes a total of at least 25 transgenes in the GM yeast MXY0291. As the optimised strain MXY0541 is derived from MXY0291, it too minimally contains 25 transgenes, as described above.

ii. The entries for the MXY0291 and MXY0541 strains in the EUginius database (EUginius, 2024).

    The entries for both strains state that the transgene encoding the heme biosynthesis enzyme coproporphyrinogen-III (CPG) oxidase is truncated. It is unclear whether the truncated transgene produces a protein. Also the Mxrl protein produced by the transgene encoding the methanol expression regulator 1 (Mxr1) contains 6 extra amino acids on its N-terminus compared to the native yeast Mxrl. The toxicological and/or allergenic consequences of these genetic errors are not addressed by EFSA.

iii. The EFSA FAF Panel's opinion, the relevant section of which is not informative due to its heavily redacted nature (EFSA FAF Panel, 2024, para 3.3.1).

The effects of these multiple modifications to the native genome sequence of the yeast are not properly addressed by the FAF or GMO Panels. Yet as a consequence of these modifications, various yeast proteins and (potentially) metabolites will be present in the final LegH Preps, including potentially toxic or allergenic substances that could have harmful consequences to consumers.

Furthermore, as noted above, the current production strain MXY0541 is different from MXY0291, as the former has been "optimised" over the latter for higher haem production (EFSA FAF Panel, 2024, para 3.4.5). So the final composition of the LegH Preps will vary, depending on the production strain used. This consideration is not addressed by the FAF or GMO Panels, despite the FAF Panel's acknowledgement of its importance.

In connection with this issue, it is instructive to recall the L-tryptophan catastrophe in the 1980s, when contaminants in this GM bacteria-produced food supplement manufactured by the company Showa-Denko caused a new disease, known as eosinophilia-myalgia syndrome (EMS). The disease severely and chronically sickened over 1500 people and caused 37 deaths. The contaminants resulted from the manufacturing company's progressive optimisation of the GM bacterial strains used for production, which involved insertion of an increasing number of transgenes. It is noteworthy that the final marketed L-tryptophan from the optimised GM bacterial strain was greater than 99.6% pure and the suspected toxins that caused the EMS was present only at around 0.01% of the product. As these toxins was unexpected and even unknown as a possibility, the purification procedure, which by normal standards was good, nevertheless turned out to be inadequate.

This tragic incident highlights how a large number of transgene insertions can inadvertently and unpredictably alter core GMO biochemistry, leading to unexpected toxin production. The authors of a peer-reviewed paper on this L-tryptophan-EMS event concluded: “This outbreak [of disease] highlights the need for close monitoring of the chemical purity of biotechnology-derived products, and for rigorous testing of such products *following any significant changes to the manufacturing process*” (Mayeno AN, Gleich GJ, 1994) (our emphasis).

This cautionary tale offers a lesson for the case of LegH Prep. The global biochemical and thus compositional consequences of the genetic modifications that made *K. phaffii* strain MXY0291 into MXY0541 must be investigated, e.g. using untargeted proteomics and metabolomics; and all safety data must be based on MXY0541, the strain that is to be used for commercial LegH Prep production.

Toxicity assessment inadequate

In assessing the toxicological impact of any changes related to the genetic modification of *K. phaffii*, the EFSA GMO Panel makes the error of narrowing its focus to “the assessment of the newly expressed soy leghemoglobin protein in strain MXY0541” (EFSA GMO Panel, 2024, para 3.8.1). This is inadequate because it neglects to take into account the importance of the proteins and/or metabolites that make up the 35% contaminants present in LegH Prep.

Again, the L-tryptophan incident is instructive. People were sickened and in some cases died even though the contaminating suspected toxin responsible for causing EMS made up a tiny proportion (around 0.01%) of the final consumed product. The original bacterial strain was optimised for L-tryptophan production via four successive genetic modification procedures, generating a total of five increasingly productive strains. The epidemic of EMS disease coincided with the introduction of the more optimised strains of bacteria into the manufacturing process (Mayeno AN, Gleich GJ, 1994).

Regarding only the soy leghemoglobin protein expressed in *K. phaffii* (strain MXY0541), the GMO Panel says bioinformatics analysis (which, however, is only predictive and not comprehensive or conclusive) “revealed no significant similarities to known toxins” (EFSA GMO Panel, 2024), para 3.8.1.1). The qualifiers “significant” and “known” are not reassuring, since the similarities may not be deemed “significant” but may still be sufficient to result in toxicity; and not all toxins are “known”. More importantly, the soy leghemoglobin protein is only 65% of the LegH Preps. The GMO Panel should have broadened its focus to include the 35% non-soy leghemoglobin proteins (and potentially metabolites), as they could include toxins and/or allergens. EFSA fails to properly address this possibility.

Regarding metabolites, the EFSA FAF Panel says, “The recipient strain [*K. phaffii* strain MXY0541] qualifies for QPS [qualified presumption of safety] status; therefore, no concern is expected from yeast metabolites produced during the fermentation process” (EFSA FAF Panel, 2024, 4. Discussion).

However, the EFSA BIOHAZ Panel stated, “The species Komagataella phaffii... can be recommended for the QPS list *only when the species is used for enzyme production*” (EFSA Panel on Biological Hazards, 2018) (our emphasis), a process that demands a high level of purity (Ramos OS, Malcata FX, 2011). Unlike enzymes produced by yeast, soy leghemoglobin is not produced by secretion, so the product is not as pure. The result is a product containing 35% contaminants that are not purified out, but consumed by people in the final product. Therefore QPS status cannot be applied to the production of *K. phaffii*-driven soy leghemoglobin. The FAF Panel appears to be mis-applying the QPS concept to imply a level of safety that has not been established for LegH Prep.

Impossible Foods provided information on the similarity of the soy leghemoglobin protein to haemoglobin proteins found in plants and animals. The GMO Panel states, “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%)” (EFSA GMO Panel, 2024, para 3.8.1.1).

Understandably, given the inadequacy of the data provided, the GMO Panel concluded that it “considers the information above as not sufficient to duly document the history of safe use for consumption of the newly expressed soy leghemoglobin protein” (EFSA GMO Panel, 2024, para 3.8.1.1)

It is left, then, to the *in vitro* and *in vivo* toxicity studies to establish the safety of LegH Prep to the Panel and general public. These are addressed below.

In vitro digestibility studies not representative of real conditions

To assess the digestibility of the soy leghemoglobin protein expressed in strain MXY0541, Impossible Foods provided in vitro degradation studies on the resistance to pepsin (an enzyme made in the stomach that breaks down proteins in food during digestion) of the LegH Prep at a pH 2 acidity level. The FAF Panel concluded, “The in vitro studies showed rapid digestion (within 2 min) of soy leghemoglobin and of *K. phaffii* proteins present in LegH Prep” and concluded that there were no safety concerns (EFSA FAF Panel, 2024, 3.4.1).

It is assumed that leghaemoglobin will denature and release the haem co-factor on exposure to the low pH (acidic) environment of gastric fluid, at pH 2. However, between 4 and 5% of people suffer from low stomach acid (hypochlorhydria) or an inability to produce it (Fatima R, Aziz M, 2024). The incidence of hypochlorhydria in the population has been estimated to be about 20-50% (on average, 30%) of people aged above 65 years (Wolters M et al, 2004). In countries where data were available, around one-quarter of adults take acid blockers that reduce acid secretion in the stomach (Shankia LGT et al, 2023). Therefore tests at pH 2 do not reflect the reality for many people.

In addition, digestion of proteins into smaller peptides will not necessarily prevent immunologic and potential allergic reactions. Indeed, digestion, starting in the mouth and extending through the length of the gastrointestinal tract, can exacerbate allergic

reactions as it may expose allergens in the peptides that were previously hidden within the complex three-dimensional structure of the protein from which they came (Sun N et al, 2022).

Finally, the validity of the pepsin digestibility test in the allergenicity assessment for GM and novel proteins is in question. A comprehensive review concluded, “Protein digestion is relevant for allergenicity of some proteins, but not for all”, “There is no rationale for a clear readout that is predictive for allergenicity”, and “We suggest to omit the digestion test from the allergenicity assessment for now and put effort into filling the knowledge gaps” regarding the mechanisms of food allergy (Verhoeckx K et al, 2019).

Allergenicity assessment – data gaps, risks downplayed

The FAF Panel describes and comments on Impossible Foods’ allergenicity assessment. Impossible Foods conducted a proteomics (protein profile) analysis to identify and quantify the contaminating (non-soy leghemoglobin) *K. phaffii* proteins that constitute 35% of the commercial LegH Prep. However, of all the protein sequences they obtained, they only identified some: “The applicant identified 17 of the most abundant proteins in strain MXY0291, each of the proteins representing ≥ [greater than or equal to] 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 with known allergens over 80 amino acids windows. Some of these matches were against contact or inhalation allergens. Other matches were identified against fish, crustaceans and carrot” (EFSA FAF Panel, 2024, para 3.4.7).

This assessment raises three major concerns:

i. The EFSA FAF Panel – and the GMO Panel – should not have accepted data on strain MXY0291 when MXY0541 is the strain under consideration for regulatory authorisation and commercial production.

ii. The analysis was partial and incomplete. For strain MXY0291, they identified 17 of the most “abundant” proteins for analysis. For strain MXY0541 they identified 11 of the most abundant proteins (EFSA FAF Panel, 2024, 3.4.7). The analysis begs the following questions, which remain unanswered:
    - Identifying “17 of the most abundant proteins in strain MXY0291” and “11 of the most abundant proteins in strain MXY0541” leaves an unknown quantity of proteins unidentified and unexamined for allergenicity. How many proteins, including those that were “abundant”, remained unidentified? What is their allergenic potential?
    - What percentage of total proteins were identified and what percentage were unidentified?
    - Why did Impossible Foods not subject the unidentified proteins to the same allergenicity investigations as the identified ones? As the amino acid sequences of these unidentified proteins was known, they could easily have been compared against known allergens in the Allergenonline database.

- Why did Impossible Foods fail to identify and examine for allergenicity all of the proteins present at less than 1% of the total protein fraction, given that allergens can elicit reactions at very low levels of exposures?
- What evidence is there that Impossible Foods has not “cherry-picked” the data, analysing only those proteins that (in its view) are less likely to be problematic?
- Why did Impossible Foods not look at toxicity as well as allergenicity in the proteomics analysis?
- Why did EFSA allow Impossible Foods to present partial and potentially selective and biased data?

There is no excuse for leaving such large gaps in the data submitted.

iii. In spite of the extremely partial and selective nature of the analysis, suspected allergens were still identified among the *K. phaffii* contaminant proteins. Impossible Foods analysis revealed that the majority of the “most abundant” proteins it identified – 10 out of the 17 – from *K. phaffii* strain MXY0291 as suspected allergens (sharing more than 35% identity over 80 amino acids windows with known allergens), as flagged up in the Allergenonline database. For MXY0541, four of the 11 identified proteins were suspected allergens (EFSA FAF Panel, 2024, 3.4.7). These similarities make the risk of even low level exposure a gamble with public health.

However, the implications were brushed aside by Impossible Foods and the EFSA panels. Impossible Foods argued 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 (EFSA FAF Panel, 2024, para 3.4.7). No data or arguments are presented to support the implication that this should reassure the public about the potential allergenic properties of LegH Prep. Yet it is known that skin allergies can lead to food allergies, since inflamed skin is “leaky” and can let in small amounts of foods through the skin. These food particles are then seen by immune system cells, which react to the food as if it was attacking the body. This can lead to developing a food allergy (NHS Oxford University Hospitals, 2024). A review of the in vitro digestibility assay used by EFSA for allergenic evaluation of GMO and novel proteins noted, “Other routes (skin, lung) of exposure might play a role in food allergy” (Verhoeckx K et al, 2019). Therefore Impossible Foods’ attempt to dissociate dermal allergens from food allergens is scientifically invalid.

Regarding the remaining known food allergens flagged up by the Allergenonline database as being similar to the *K. phaffii*-produced contaminant proteins, according to the FAF Panel, “the applicant considers that there is a low percentage of patients that showed immunoreactivity” to these 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" (EFSA FAF Panel, 2024, para 3.4.7).

This paragraph contains multiple ethically unacceptable or scientifically untenable statements. The "low percentage of patients" that had reactions to these matched allergens should not be dismissed on the grounds that they are few in number. Also, it cannot be assumed that allergenic proteins will be inactivated by heat, as in cooking. Some proteins do not reduce in allergenicity on heating and can even acquire new allergenic properties (Wal JM, 2003).

The statement that "each *K. phaffii* protein in that final product is in trace amounts" is not reassuring, because (as stated above) allergens can cause reactions in trace amounts.

It must also be remembered that this discussion of potential allergenicity relates to proteins that co-purify from the GM yeast production strain alongside the soy leghemoglobin protein. Therefore the FAF Panel's recommendation that Impossible Foods label its product with the allergy warning "Contains soy" (EFSA FAF Panel, 2024, para 3.4.7) is inadequate and irrelevant to the issue of the other potential allergens in LegH Prep and puts public health at risk.

Impossible Foods should have conducted a proteomics analysis of all the proteins for toxicity as well as allergenicity. It should also have carried out an untargeted metabolomics analysis to check for potential toxic metabolites, production of which could arise from disturbances in biochemistry arising from the genetic modification of the *K. phaffii* strain. Unexpected, unpredictable toxin production, such as occurred in the GM bacteria-manufactured L-tryptophan, which sickened and killed over a thousand consumers of even though the suspected toxic metabolites were only present at around 0.01% of the final consumed product, serves as warning in this regard (Mayeno AN, Gleich GJ, 1994).

Toxicity studies in animals show worrying results, ignored by EFSA

28-day toxicity study: The FAF Panel describes the results of a 28-day toxicity study in rats fed LegH Prep, commissioned by Impossible Foods: "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" (EFSA FAF Panel, 2024, para 3.4.4).

As no further details are given, it is not possible to evaluate from the FAF Panel's opinion whether this is an objective assessment of the data. However, the 28-day toxicity study evaulated by the US FDA (US FDA, 2017) and reported by Impossible Foods in its peer-reviewed journal publication (Fraser RZ et al, 2018) is highly likely to be the same one that the FAF Panel considered, judging by the published details, such as doses, endpoints examined, and the decreased uterine weights in females fed LegH Prep.

If the FDA-cited study, the peer-reviewed study, and the FAF Panel-cited study are indeed one and the same, as appears likely, then the EFSA FAF and GMO Panels have misrepresented the study findings. This is because both the FDA-cited study and the peer-reviewed study report statistically significant adverse and potentially adverse health outcomes in groups of animals fed LegH Prep. They include (our own comments are in brackets):

- unexplained transient decrease in body weight gain
- increase in food consumption without weight gain
- changes in blood chemistry
- decreased reticulocyte (immature red blood cell) count (can be a sign of anemia and/or damage to bone marrow where red blood cells are produced)
- decreased blood clotting ability
- decreased blood levels of alkaline phosphatase (can indicate malnutrition and/or celiac disease)
- increased blood albumin (can indicate acute infection or damage to tissues) and potassium values (can indicate kidney disease)
- decreased blood glucose (low blood sugar) and chloride (can indicate kidney problems)
- increased blood globulin values (common in inflammatory disease and cancer) (US FDA, 2017; Fraser RZ et al, 2018).

In its peer-reviewed study, Impossible Foods' scientists state that these outcomes were "nontoxicologically relevant and nontest substance dependent" and that they were "generally of small magnitude, lacked a response in a dose-dependent manner, and are interpreted to be within expected biological variation and considered to be of no toxicological relevance and non-test substance dependent" (Fraser RZ et al, 2018).

The FAF Panel has adopted some of these arguments, stating that the changes seen in the treatment groups of animals "were not dose-dependent and not accompanied with adverse histopathological findings; therefore, the Panel concluded that they were not adverse" (EFSA FAF Panel, 2024, para 3.4.4).

However, regarding dose-dependence, it is well known that biological effects do not always increase with dose in a linear fashion (Hill CE et al, 2018; Goldsmith JR, Kordysh E, 1993). And regarding adverse histopathological findings, the study duration is too short for these to reliably show up. Adverse effects may be seen at first on the functional and biochemical level, as is the case with this study, and not be accompanied with adverse histopathological findings until some time has passed. The FAF and GMO Panels must address the adverse and potentially adverse findings revealed in this study, rather than excluding them from their opinions and/or dismissing them. In the interest of public health and the precautionary principle, it must be assumed that they are adverse unless strong evidence is obtained that they are not. Such evidence could be provided by extending the study duration and increasing the number of animals studied.

In addition, some adverse and potentially adverse effects were seen in more than one group of animals fed LegH Prep: e.g. statistically significant increases in mean

daily food consumption in group 3 males on days 7 to 14 and in group 4 males on days 7 to 10; increase in APTT (decreased blood clotting time) in groups 3 and 4 males; decrease in alkaline phosphatase in group 2 and group 4 females; and decreased GLUC (fasting glucose) and chloride in groups 2 and 3 females (Fraser RZ et al, 2018). Because these changes were consistent between different groups of animals (even if they did not occur in a linear dose-dependent manner – see above), this is confirmation that they should be taken seriously and not dismissed.

The FAF and GMO Panels do not address the changes in body weight in some treated groups of animals. However, these changes are especially striking in light of the wide range of starting body weights in the animals, as indicated in the company's peer-reviewed study (Table 4) (Fraser RZ et al, 2018) (assuming this is the same study as that evaluated by EFSA) – but omitted from the EFSA opinions. This wide range of starting body weights means that to show up as statistically significant, any changes in the animals' body weights would have to be of a magnitude of at least 5% ($p<0.05$) by the end of the study. This is highly unlikely after the short study period of 28 days, but such changes were observed, pointing to LegH Prep being a significant stressor to the animals' health and/or behaviour.

Did EFSA evaluate a study using the wrong *K. phaffii* strain – and make a false statement about it?

If the FDA-cited study, the peer-reviewed study, and the FAF Panel-cited study are the same investigation, it follows that the FAF Panel evaluated a study done with *K. phaffii* strain MXY0291-derived LegH Prep – an irrelevant strain to the risk assessment as this is not the strain that Impossible Foods uses for LegH Prep manufacture.

Moreover, this would also mean that the GMO Panel has falsely described the study as having been done with MXY0541-derived LegH Prep, the commercially relevant strain and the one that EFSA's risk assessments are supposed to address. The GMO Panel states: "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" (EFSA GMO Panel, 2024, para 3.8.1.1).

If the GMO Panel has misrepresented the study, it should correct its statement. Alternatively, if Impossible Foods repeated its original study with LegH Prep from the commercially relevant *K. phaffii* strain MXY0541, the GMO Panel should clarify this.

However, the latter scenario would leave the GMO Panel with the difficult task of explaining why not one study, but two, produced the same adverse and potentially adverse outcomes in animals fed LegH Prep. Two studies showing the same findings would make it highly unlikely that they were due to anything but the treatment substance (LegH Prep).

90-day toxicity study with 28-day recovery period (of no dosing with LegH Prep): This study is likely to be the same one that Impossible Foods published in a peer-reviewed journal (Reyes TF et al, 2023). EFSA should clarify if this is the case, since

if it is, the study that EFSA evaluated was done with LegH Prep derived from non-commercially-relevant strains of *K. phaffii*, MXY0291 and NRRL Y-11430.

The study as reported by the FAF Panel lists several adverse and potentially adverse findings from LegH Prep consumption, which the FAF Panel lists but dismisses without providing scientific reasons or evidence in justification (EFSA FAF Panel, 2024, para 3.4.4). The findings, the FAF Panel's dismissals, and our comments are below.

Mean red blood cell haemoglobin (MCH) and mean red blood cell volume (MCV) were slightly increased in high-dose males, but the FAF Panel said the increase was not "of toxicological relevance" (EFSA FAF Panel, 2024, para 3.4.4). However, this is not possible to establish without further studies of longer duration. These changes can be signs of macrocytic anemia onset.

Regarding clinical blood chemistry, LDL cholesterol was increased in mid-dose males – a change believed to raise the risk of cardiovascular disease. In urinalysis parameters, urine ketones were increased in mid-and high-dose males.

The FAF Panel said, "These changes were not dose-dependent, within the natural variability. Therefore, the Panel considered these changes not treatment-related or adverse" (FAF Panel, 2024, para 3.4.4).

However, cardiovascular disease also displays natural variability, but this does not mean it is acceptable to place on the market a food that is shown in an animal study to increase the risk of this disease, without informing the consumer of the risk. Increased urine ketones can be a sign that the blood is too acidic, a condition known as ketoacidosis, which is a complication of diabetes; its occurrence in two groups of animals fed LegH Prep shows that this finding should be taken seriously.

Dismissing significant changes on the grounds that they are "within expected biological variation" is unacceptable. The most relevant control for determining treatment-related effects in animal feeding studies is the concurrent control group, not the wide range of biological variation (Keenan C et al, 2009).

The wide range of biological variation is also known in carcinogenicity and toxicity studies as "historical control data" (Kluxen FM et al, 2021); the two concepts are used interchangeably by the pesticide and GMO industry group CropLife (CropLife, 2021).

The Organisation of Economic Cooperation and Development (OECD), which sets guidelines for industry toxicity studies, emphasises that if historical control data are used, they should be tightly controlled for variables: "Ideally, all historical control data submitted for consideration are obtained from the laboratory at which the study being assessed was carried out, and relate to animals of the same strain, age and sex, and obtained from the same supplier, as those used in the study. They should come only from studies conducted within 5 years, or two to three years on either side, of the study under review" (OECD, 2002).

The importance of tight controls to minimise variables is also emphasised by Haseman and colleagues (Haseman JK et al, 1984).

Using the wide range of “biological variation” drawn from unspecified data outside of the experiment under consideration in order to dismiss statistically significant changes in treatment group animals is not scientifically valid, as the data will be drawn from a wide range of different experiments done at different times and locations, under different conditions, with different strains or origin of animal, different feeds, and different housing materials, etc. There is no evidence that steps were taken by Impossible Foods or the EFSA FAF and GMO Panels to ensure the inclusion of only valid “biological variation” data in the toxicity studies of LegH Prep, nor is it clear where the data were obtained and whether they are publicly available. Therefore EFSA’s dismissal of these findings using the argument that they are within the range of natural variability is not scientifically valid.

Increases in thyroid-related hormones, i.e. TSH (in high-dose males +/− recovery) – can indicate hypothyroidism; and increase in T4 (in high-dose males + recovery) – can indicate hyperthyroidism; decrease in T4 (in high-dose females) can indicate hypothyroidism; and decrease in T3 (in all treated males) can indicate hypothyroidism. These obvious disruptions to thyroid functioning require further investigation via a study of longer duration.

The FAF Panel said these changes “were not correlated with microscopic changes in thyroid, not consistent between males and females, and the magnitude of the effects were not biological [sic] relevant and therefore not considered adverse” (EFSA FAF Panel, 2024, para 3.4.4).

However, microscopic changes may not appear within the short time frame of the feeding trial. In a subchronic study of only 90 days, functional changes may not be reflected by structural changes to an organ, as the latter can take time to manifest.

Also, it is not valid to dismiss changes that are not consistent between males and females as their different hormonal and metabolic makeup and functioning means they respond to stressors differently (Vahter M et al, 2007).

Regarding ‘small’ effects of limited “magnitude”, changes are either statistically significant or not – and these were. Dismissing such changes is especially unacceptable in relation to a study of short duration, as is the case here.

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. The FAF Panel said, “These decreases did not correlate with any histopathology findings in the thymus. No treatment-related histological findings were reported” (EFSA FAF Panel, 2024, para 3.4.4). But again, histopathology findings may require a longer study duration to show up, so this finding should not be dismissed. Decreased thymus weight can indicate accelerated ageing, response to infection or other stress, or malnourishment.

Conclusions and recommendations

Soy leghemoglobin has never previously been part of the human food supply. In addition, K. phaffii does not have a history of safe use in human food. Also, products derived from GM *K. phaffii* for human food use are extremely limited and no history of safe use can be claimed for them. Accordingly, a rigorous safety assessment of both soy leghemoglobin and GM *K. phaffii* is required. The need for regulatory rigour is highlighted by the fact that the product to be marketed, LegH Prep, is only 65% soy leghemoglobin, with the remainder being contaminating *K. phaffii* proteins and possibly metabolites. Thus the risk assessment needs to properly examine the potential toxicity and allergenicity of the LegH Prep as a whole.

However, the EFSA FAF and GMO Panels' assessments do not fulfil this requirement. They contain many data gaps and serious technical errors. The panels have inadequately addressed the potential toxicity and allergenicity of the LegH Prep, but have largely ignored the risks associated with the *K. phaffii* contaminants. This includes ignoring or dismissing multiple adverse and potentially adverse health effects in animals fed LegH Prep, including signs of anaemia, decreased blood clotting ability, and kidney function problems. These findings are serious enough to warrant further investigation.

Remarkably, the panels have (against the explicit advice of the FAF Panel) accepted safety data on LegH Prep derived from a GM *K. phaffii* strain, MXY0291, which is not intended for commercial manufacture for the EU. This has potentially serious consequences, as it could contain a markedly different spectrum of contaminating proteins compared to those that would be present in LegH Prep derived from the intended commercial strain, MXY0541. As a result, the toxicological and allergenic profile of the LegH Prep that was assessed by EFSA could be significantly different from the intended marketed product. Safety data must all be drawn from the commercially relevant strain of *K. phaffii* and this must be a prerequisite for market approval.

The potential production of toxic substances following complex genetic modification of microorganisms is not unprecedented. In the L-tryptophan tragedy of the 1980s, people were sickened and killed by toxic contaminants produced by the progressive optimisation of the GM bacteria production strains. The "optimised" strain of *K. phaffii* intended to be used for LegH Prep manufacture, based on the available information, contains a total of at least 25 transgenes (16 copies of the soy leghemoglobin gene and 9 genes for enhanced haem biosynthesis). With such a pronounced alteration in *K. phaffii* genetics, it is not beyond the realms of possibility that core biochemical pathways have been altered, raising the risk of novel toxins and/or allergens being produced.

Of the 35% contaminating proteins, an unknown number remain unidentified, uncharacterised, and unanalysed for safety. Among the relatively small number of contaminating proteins that were identified, some were found to possess amino acid sequences that matched known allergens. EFSA accepted Impossible Foods' argument that this finding did not pose serious risks to human consumers, but this was based on scrutinising only one allergen database (Allergenonline) and lax interpretation of the findings. They also neglected to assess the allergenic potential of the unidentified contaminating proteins. A focus on known proteins present at 1%

or more of the total LegH Prep ignores the fact that allergens can elicit reactions at trace levels of exposure.

It was also argued that since soy leghemoglobin was readily digestible, that any allergenic potential of this protein would be negated. However, this was based on inadequate *in vitro* digestion simulation testing that does not reflect real-life conditions and ignores the fact that digested food products can still pose an allergenic risk.

The EFSA panels should withdraw and revise their assessments, based on requiring Impossible Foods to

- supply safety data on LegH Prep derived from the commercially relevant *K. phaffii* strain, MXY0541
- investigate the global biochemical and thus compositional consequences of the genetic modifications that made *K. phaffii* strain MXY0291 into the commercially relevant MXY0541, e.g. using untargeted proteomics and metabolomics
- conduct analysis of all the proteins in LegH Prep for toxicity as well as allergenicity
- conduct an untargeted metabolomics analysis to check for potential toxic metabolites
- make all the above data public.

Meanwhile EFSA should publish the full details of, and objectively evaluate, the animal feeding studies that revealed adverse and potentially adverse effects from LegH Prep consumption.

For all safety data provided to date, EFSA must clarify which GM *K. phaffii* strain was used in the production of the LegH Prep in each test applied. EFSA should also explain why it has claimed QPS status for GM *K. phaffii* even though it appears that this status is only granted for enzyme production – a process that results in a purer product that is not intended for human consumption.

These are the minimum steps required to be in accord with the precautionary principle and protect public health.

** Prof Michael Antoniou's contributions to this document reflect his own views and do not represent the position of his university.*

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