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SCIENTIFIC OPINION

Current practice, challenges and future opportunities in the safety assessment of newly expressed proteins in genetically modified plants

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

Current risk assessment strategies for protein safety of newly expressed proteins (NEPs) in genetically modified (GM) plants are based on chemical risk assessment principles and Codex Alimentarius guidelines for biotech-derived foods, initially published in 2003. These guidelines were designed for proteins with multiple testing options and for GMOs expressing a low number of NEPs. However, two decades of experience in assessing GMO and biotech products, along with recent advances in the field, underscore the need to update best practices for protein safety assessment. Furthermore, new types of products challenge the application of the current international guidelines, as assessments become more complex due to NEPs that are difficult to test using existing approaches or products with numerous NEPs. This document outlines a strategy that strengthens the stepwise, weight-of-evidence approach, incorporating new methodologies as complementary or alternative studies. An improved strategy for protein safety assessment could include: (1) considering history of safe use (HoSU), read-across and phylogeny defining the type of data required and remove the need for specific in vitro or in vivo studies; (2) applying advanced in silico tools, including predictive computational models and improved phylogenetic analysis to enable more accurate comparisons with known allergens, toxins or 'safe' proteins; (3) using standardised in vitro gastrointestinal models that replicate physiological conditions; (4) developing targeted in vivo studies; (5) evaluating the role of exposure in the safety assessment; and, where necessary, (6) considering post-market monitoring for risk characterisation. Consensus on the definition of HoSU and the effective integration of novel methodologies into the current NEP safety assessment will be essential to meet society's demand for safer, healthier and more sustainable food/feed in a growing world. This calls for revisiting and refining the goals of protein safety risk assessment to ensure that NEPs in biotech products are evaluated appropriately, consistently and proportionately.

KEYWORDS

allergenicity, GMO, newly expressed proteins, protein safety, risk assessment, toxicity

CONTENTS

1 | INTRODUCTION

Current risk assessment strategies for protein safety assessment of GMOs are based on principles from the chemical risk assessment area and guidelines of *Codex Alimentarius* for the safety assessment of foods derived from 'modern' biotechnology initially published in 2003. These guidelines were mainly aimed at the assessment of proteins with ample possibilities for testing and at GMOs containing only a few newly expressed proteins (NEPs). Experience gained in the assessment of GMO or biotech products and new developments in the field of protein safety over the last 20 years call for a revision of the best practices for protein safety assessment. Moreover, new products challenge the practical implementation of the current international guidelines, with protein assessments becoming increasingly challenging due to the presence of NEPs, such as membrane-bound proteins or transcription factors, which, by their nature and function, are difficult to evaluate using current approaches (Bushey et al., 2014; Hurley et al., 2016; Wang et al., 2024).

This work aims to utilise key lessons learned from previous assessments of NEPs in GM plants and to critically evaluate how emerging methodologies can serve as alternative or complementary strategies to improve future GMO assessments. Preparedness for even more complex future risk assessments is a priority for EFSA.[1]

1.1 | Background and Terms of Reference

Commission implementing Regulation EU No 503/2013[2] (hereafter referred to as 'IR503/2013') is in force on applications for authorisation of genetically modified (GM) food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. The specific scientific requirements for the risk assessment of NEPs in GM plant-derived products regarding their toxicity and allergenicity potential in relation to human and animal health can be found in Annex II of such Regulation.

Briefly, the safety assessment of NEPs in genetically modified organisms (GMOs) follows a case-by-case approach, depending on the knowledge available on the protein's source and the protein itself, including its function and history of human or animal consumption. In the case of the toxicity assessment, if history of safe consumption is duly documented, specific toxicity studies might not be required. Where specific additional testing is required, the applicant shall provide: (i) molecular and biochemical characterisation of the NEP; (ii) bioinformatics-derived searching for homology to proteins known to cause adverse effects; (iii) stability of the protein, e.g., influences of temperature, pH; (iv) potential degradation of the NEPs to proteolytic enzymes (pepsin test); and (v) a 28-day toxicity study.

In the case of the allergenicity assessment, the information shall include: (i) bioinformatics-derived searching for sequence similarity with known allergens; (ii) specific serum screening, on a case-by-case where there is a sequence homology or structure similarity and/or where the source of the gene is considered allergenic; (iii) pepsin resistance and in vitro digestibility tests; and (iv) additional studies in vitro or in vivo, if needed.

The Panel on Genetically Modified Organisms (GMO Panel) has assessed the safety of NEPs following such requirements set 15–20 years ago and mainly targeted for the assessment of one or few NEPs in each GMO. Two decades later, this assessment is becoming increasingly challenging because of the nature and function(s) of the NEPs and the possible interactions of various NEPs expressed in GM plant-derived products, that in some cases, are difficult to characterise and test, e.g., membrane-bound proteins, transcription factors. Experience gained with the current approaches, as well as new developments in protein safety assessment methodologies lead to the need to revisit and improve current practices and provide alternative methods for GM-derived products, in order to offer the highest level of protection possible for the consumers and the environment.

To move forward the field of protein safety and to advance and 'future proof' the safety assessment of complex GM derived products, the GMO Panel identified the need to publish a scientific opinion reflecting on current practice, challenges and future opportunities of protein safety in GMOs.

The European Food Safety Authority (EFSA) asked its GMO Panel to issue a scientific opinion on protein safety assessment. This scientific opinion should encompass the following four terms of reference (ToR):

1. Lessons learnt from experiences in the assessment of NEPs in the last 20 years, including more recent complex cases.
2. Building on experience gained in the past years and issues identified, a critical appraisal of new methodologies available with the potential to be used as complementary/alternative testing strategies to current methodologies described in legal frameworks. This point could also contribute to the principles of the 3Rs[3] (replacement, reduction and refinement of animal testing).
3. Road map for future implementation of such complementary/alternative methods in risk assessment strategies.
4. Recommendations for further research or for addressing method development needs.

[1] https://www.efsa.europa.eu/sites/default/files/2021-07/efsa-strategy-2027.pdf.

[2] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. OJ L157, 8.6.2013, p. 1–48.

[3] https://single-market-economy.ec.europa.eu/events/commission-roadmap-phasing-out-animal-testing-chemical-safety-assessments-2023-12-11_en.

2 | DATA AND METHODOLOGIES

2.1 | Expert working group and its methodology

The standing expert Working Group (WG) Food/Feed (FFWG) of the EFSA GMO Panel led the development of this scientific opinion and received contributions from the Molecular Characterisation (MC) and Comparative Analysis and Environmental Risk Assessment (CompERA) WGs. The methodology adopted by the WG to address the ToRs, including the use of the relevant case studies to answer the specific questions of the ToRs, was published on EFSA's website as a supporting document of this scientific opinion (see Annex A – Protocol).

In delivering its scientific opinion, the GMO Panel also considered:

- The current legislation and relevant EFSA guidance documents for the safety assessment of toxicity and allergenicity of proteins.
- The results of a survey on protein safety launched by EFSA (see supplementary information in Annex B – Survey).
- A public consultation on the draft scientific opinion (Annex C and see Section 2.2) and a stakeholder meeting with the participants of the public consultation.
- EFSA procurement reports (Bebi et al., 2024; Cobigo et al., 2024; Martinez et al., 2024; Mills et al., 2024; Palazzolo et al., 2024; Urbani et al., 2024) and up to date information published in the literature (see Section 3 and the reference list).

2.2 | Consultations

In line with EFSA's policy on openness and transparency, an online public consultation was launched after the endorsement of the opinion by the GMO Panel in December 2024. Stakeholders were invited to submit their comments on the draft scientific opinion between mid-January 2025 and mid-March 2025. Following this consultation process, the document was revised by the members of the FFWG and the EFSA GMO Panel. The comments received were considered and, when appropriate, incorporated into the current scientific opinion. The outcome of the public consultation is reported in detail and published on EFSA's website as a supporting document together with this scientific opinion, as adopted by the EFSA GMO Panel as Annex C – Public Consultation.

3 | ASSESSMENT

To facilitate the assessment of the ToRs, each of them was broken down in assessment questions (see Annex A – Protocol) and addressed in the subsequent Sections.

3.1 | ToR1: Lessons learned from EFSA experience in the assessment of NEPs in the last 20 years, including more recent complex cases

3.1.1 | What is the current common strategy for the assessment of NEPs in GM food and feed?

In the European Union (EU), Commission Implementing Regulation EU No 503/2013 provides specific scientific requirements for assessing the potential toxic and allergenic risks of NEPs in GM plant products to humans, and farmed and companion animals' health.

The EFSA safety assessment is based on principles defined by *Codex Alimentarius* (2003–2009) framed for the safety assessment of individual NEPs. To date, the *Codex Alimentarius* and EFSA guidance documents (EFSA GMO Panel, 2011a, 2017b) have been effective in assessing protein safety of single and stacked event GM plant applications. The GMO Panel has assessed more than 40 NEPs (see supplementary information Annex D). It is also worth noting that most of these proteins are not particularly difficult to work with. They have simple enzymatic activity, are soluble in water and offer ample possibilities for purification, structural and functional characterisation and testing to conduct safety studies.

The elements considered in the NEP assessment are selected on a case-by-case basis and follow a weight-of-evidence approach (see Section 1.1 for a brief description and the illustration in Figure 1).

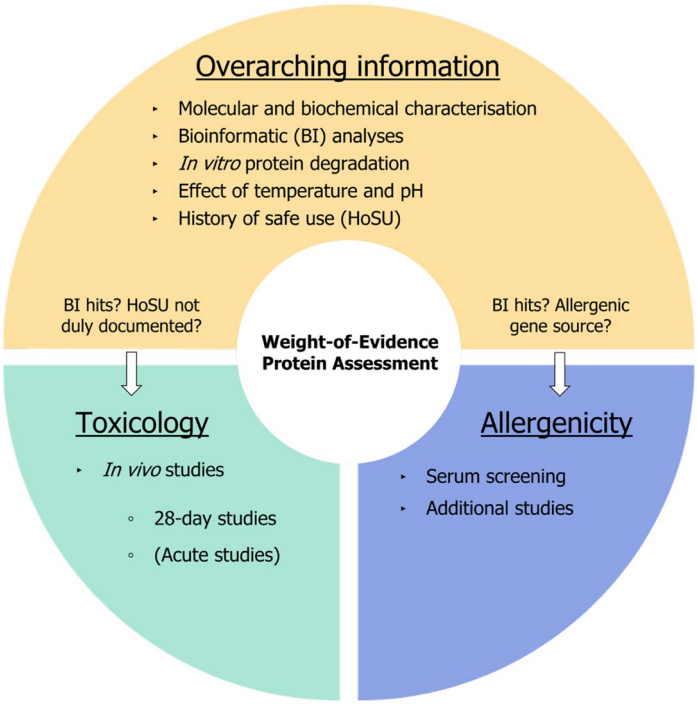

FIGURE 1 Weight-of-evidence approach in the protein safety assessment of GMOs.

In the protein safety assessment, the value of the weight-of-evidence approach is the evaluation of the results combined from several distinct studies rather than relying on a single piece of information. This approach includes methodologies that stakeholders and EFSA consider valuable. The experience gained by the GMO Panel in the assessment of NEPs is further detailed below.

Molecular and biochemical protein characterisation data

This refers to the information provided in applications to demonstrate the inherent molecular and biochemical characteristics of the NEPs as produced in the plant, forming a fundamental component of the core data requirements for the safety assessment. EFSA has not established specific requirements for the type of characterisation data to be submitted. Thus, assessments are conducted on a case-by-case basis, which are tailored to the specific characteristics of the NEP under evaluation. Typically, using well-established methods and approaches that demonstrate the NEP's biochemical, physicochemical, structural and functional properties are sufficient to adequately meet the requirements of the Commission Implementing Regulation (EU) No. 503/2013 (Annex II, section 1.4.1).

According to these requirements[4] the following information is needed:

- Determination of the primary structure.
- Molecular weight.
- Studies on post-translational modifications.
- Description of its function.

Additionally, if the NEP is an enzyme, further information is needed to characterise its activity by providing:

- The temperature and pH range for optimum activity.
- Substrate specificity.
- Possible reaction products.
- The potential interactions with other plant constituents (e.g. if any endogenous compounds could also be substrates for the enzyme).

[4]The text for the below listed requirements is derived from Commission Implementing Regulation (EU) No. 503/2013.

The data provided to EFSA are typically generated using the following methods:

- Amino acid sequence analysis, methods such as mass spectrometry and N- or C-terminal sequencing analyses are used to confirm whether the plant-produced protein aligns with the predicted sequence specified by the inserted gene, thereby fulfilling the requirement for information on the primary structure of the NEP.
- Molecular weight determination, the protein characterisation data includes information from methods such as SDS-PAGE and Western blot analyses to experimentally determine the molecular weight.
- Immunoreactivity, methods such as Western blot analyses are used to further characterise the NEP identity by assessing its immunoreactivity to specific antibodies.
- Post-translational modifications (PTMs), sequence analysis data (e.g. mass spectrometry) are used to identify PTMs such as N-terminal truncations or N-terminal methionine acetylation which are common modifications in eukaryotic proteins (Polevoda & Sherman, 2000). Additionally, in vitro glycosylation detection assays are employed to determine whether the NEP is glycosylated. Glycosylation is a PTM known to influence protein folding, stability and resistance to proteolytic degradation. Early studies suggested a link between glycosylation and allergenicity (Barre et al., 2008; Fötisch & Vieths, 2001). However, more recent evidence indicates that glycosylation alone is not a reliable predictor of IgE reactivity and immunogenicity (Mari et al., 2008; Zhang et al., 2019). These findings highlight the need for a case-by-case, weight-of-evidence approach when assessing the relevance of glycosylation in protein safety evaluation.
- Description of the function, data from activity assays are typically provided to determine if the NEP functions as intended, e.g. inactivation of or tolerance to a certain herbicide. For NEPs with insecticidal activity, data from feeding assays involving target organisms are commonly provided. In the case of enzymes, which currently represent the majority of biological functions of the NEPs in GM plant applications assessed by EFSA, additional characterisation data is provided for the assessment of the enzyme's activity in a range of pHs and temperatures, substrate specificity, potential interactions with other plant constituents and any possible reaction products or any other unintended metabolic impacts.

In those cases where technical constraints make it infeasible to extract enough protein from the GM plant for the needed studies, the NEP is produced in a heterologous expression system (e.g. *Escherichia coli*). The plant and heterologous produced (surrogate) NEPs are then directly analysed and compared side-by-side using the above-mentioned methods to demonstrate that they are physicochemically, structurally and functionally equivalent.

Based on the experience gathered from the NEPs assessed in GM plant applications submitted to EFSA, it is often difficult to provide all types of the analyses described above, in particular for a plant-produced NEP. This may be due to the NEP, for example, not being expressed at sufficient levels, not being adequately (bio)-chemically stable during processing to purify it at least partially (e.g. achieve the limit of detection required to perform Western-blotting), or not having a function that can be readily assessed (e.g. potential yield increase or R-genes). Wherever this is not possible, alternative data are used to conclude whether the protein is expressed and functions as intended. Some examples illustrating these challenges are briefly discussed below:

- IPD072Aa protein (insecticidal protein) in maize DP-023211-2 (EFSA GMO Panel, 2024a_AP163); the applicant was unable to purify a sufficient amount of the plant-derived protein for a feeding bioassay to demonstrate functional equivalence. Instead, the insecticidal activity of IPD072Aa was demonstrated by feeding target insects with plant tissues.
- GAT4621 protein (glyphosate acetyl transferase) in oilseed rape 73496 (EFSA GMO Panel, 2021b_AP109); although sufficient amounts were obtained from the plant, the purified protein was inactive. Plant-derived GAT4621 protein activity was indirectly demonstrated by the tolerance of the GM plants to glyphosate and by compositional analyses.
- GOXv247 protein (glyphosate oxidoreductase) in oilseed rape GT73 (EFSA GMO Panel, 2020b_RX002). In this case, harsh purification conditions applied to obtain the membrane-bound GOXv247 protein from *E. coli* led to the inactivation of the protein used in the 28-day toxicity study. To conclude on the safety of GOXv247, the GMO Panel considered additional data such as functional data from the starting material of the test substance (i.e. bacterial cell paste) and relevance of the GOXv247 function in humans and animals (EFSA GMO Panel, 2020b_RX002).
- AtHB17Δ113 (transcription factor) in maize MON 87403 (EFSA GMO Panel, 2018_AP125); no functional data could be obtained for the plant-derived protein due to the low yield and purity of the obtained samples. Functional equivalence was indirectly demonstrated by considering the activity in the *E. coli*-produced AtHB17Δ113 and evaluating the results based on available information of the activity of other *Arabidopsis* HD-Zip II proteins.

The GOXv247 (membrane-bound) and AtHB17Δ113 (transcription factor) examples represent two protein classes within the category of intractable proteins, which are generally difficult to characterise experimentally due to their molecular and/or functional properties (Bushey et al., 2014).

Enzyme substrate specificity

When a NEP is an enzyme, its catalytic properties should be considered during the assessment, including the determination of the substrate specificity as required in Commission Implementing Regulation (EU) No 503/2013. This requirement is part of the hazard identification, which is the first step in the risk assessment of GM plants.

A newly expressed enzyme safety assessment requires the investigation of the catalytic properties of the enzyme, which are typically kinetic parameters in relation to its substrate(s). In addition to the intended substrate(s), other endogenous compounds can interact with the newly expressed enzymes serving as substrate analogues or enzyme inhibitors.

Currently, there is no detailed guidance addressing the specific methodologies for substrate specificity testing in the GMO risk assessment. Enzymes newly introduced into a GM plant may exhibit differential substrate specificity, which might result in the unintended formation of metabolites that are potentially hazardous to humans and animals. Another possible scenario is that enzyme analogues exhibit a different performance (e.g. in terms of kinetic parameters) within the metabolic network compared to their endogenous enzyme analogues. Examples for this scenario are 5-enolpyruvylshikimate-3-phosphate (EPSPS) enzyme analogues that can replace endogenous EPSPS enzymes in the plant's shikimic acid metabolic pathway (EFSA GMO Panel, 2023b_AP149).

The relevance of substrate specificity studies for hazard identification, as well as for structuring the risk assessment, was demonstrated in previous EFSA Opinions. For example, it was shown that some endogenous proteinogenic amino acids such as aspartic and glutamic acids (in addition to serine, threonine and glycine but with very low affinity) can serve as substrates for the newly expressed GAT4621 protein resulting in the accumulation of acetylated amino acids in the GM plant and in some other derived products (EFSA GMO Panel, 2011b_AP043; 2013b_AP045; 2021b_AP109) (see case study number 7 for further details).

Determining enzyme substrate specificity typically involves evaluating the intended substrate and substrate analogues in relevant in vitro assays. While systematic and standardised methodologies for discovering and pre-selecting substrate analogues for subsequent in vitro testing are advanced in drug discovery, they are still not available in food safety risk assessments.

Examples of enzymes expressed in GM plants assessed by EFSA are listed in the supplementary information Annex E.

Functional properties or traits conferred to the crops include:

(i) Herbicide inactivation/tolerance, achieved by either metabolising the herbicide toxic molecule into compounds not toxic to the plant such as performed by dicamba monooxygenase (DMO) or by expressing an enzyme, similar to the target endogenous enzyme, but with lower herbicide-sensitivity, e.g. the newly expressed CP4 EPSPS versus the endogenous EPSPS (EFSA, 2008_AP036).
(ii) Newly expressed enzyme(s) (e.g. fatty acid desaturases) conferring a metabolic pathway that is not present in the conventional counterpart (EFSA GMO Panel, 2014_AP076).
(iii) Selectable marker proteins, like aminoglycoside phosphotransferase (APH4) or phosphomannose isomerase (PMI), which facilitate the selection of genetically transformed cells/plants, in the presence of a selective agent such as an antibiotic or an auxotrophic nutrient, respectively (EFSA GMO Panel, 2023a_AP141; EFSA GMO Panel, 2015_AP095).

Substrate specificity testing was waived in cases where structural similarities to previously assessed enzymes could be demonstrated. For example, DMO enzymes with minor amino acid changes in the N-terminal region were considered functionally comparable, as these changes occurred far from the active site and did not impact the enzyme's catalytic activity or the initially reported substrate specificity (EFSA GMO Panel, 2022a_AP169; D'Ordine et al., 2009; Wang et al., 2024).

Bioinformatic analyses

Current assessments of NEPs for allergenicity and toxicity include a similarity search of the new protein with known allergens or toxins and open-ended searches for similarly annotated proteins. Sequence alignment-based methods like the FASTA local alignment algorithm (Pearson & Lipman, 1988) or the basic local alignment search algorithm (BLAST) (Altschul et al., 1990) are widely used for sequence comparison and searches, respectively.

In terms of allergenicity, the current practice for the in silico assessment of a protein consists of an amino acid sequence similarity search against an allergen database, and a default threshold value of 35% identity over an (at least 80 amino acids) sliding window (WHO/FAO, 2001). This approach, embedded in Codex Alimentarius guidelines (2003–2009) and EFSA guidance documents (EFSA GMO Panel, 2010, 2011a), has been considered highly conservative (EFSA GMO Panel, 2022c) and inadequate when broadly applied to a large number of protein sequences (EFSA FAF Panel, 2024; EFSA GMO Panel, 2024b_AP162) or for the assessment of putative open reading frames (Harper et al., 2012; Young et al., 2012), because all these examples result in too many false positives. Moreover, the current approach heavily relies on expert judgement to interpret a posteriori the outcome of the bioinformatic analyses of the assessment, which can lead to a lack of harmonisation, reproducibility, and transparency of the risk assessments (Fernandez et al., 2021). The EFSA GMO Panel (2022c) has previously discussed the need for advanced relevant in silico approaches (i) to improve sensitivity, specificity and accuracy compared with the classical sequence alignment algorithms for assessing the allergenic potential of food proteins for immunoglobulin E (IgE) cross-reactivity; (ii) to develop in silico methods to assess the potential of new proteins for de novo sensitisation; (iii) to refine and harmonise the existing allergen databases emphasising the importance of clinical relevance to create more targeted/fit-for-purpose databases for the allergenicity risk assessment.

In terms of toxicity, one of the primary steps in predicting potentially toxic proteins is to compare the protein sequence to known toxic proteins. In this case, BLAST or FASTA are used to compare the query protein against protein or translated nucleic acid databases. The search for similarities to proteins exhibiting adverse effects uses very large resources. For example, the NCBI (National Centre for Biotechnology Information) non-redundant databases containing hundreds of millions of determined or predicted protein sequences, or more targeted databases can be used, generated by using a smaller subset

of proteins which are identified by filtering larger databases (e.g. https://www.uniprot.org/) to specifically select proteins that are predicted or known to be toxins. The need to define more specific criteria for bioinformatic searches for similarity to known protein toxins has been previously highlighted (Cattaneo et al., 2023).

In vitro protein degradation, effect of temperature and pH

In vitro testing is currently used for risk assessment mainly to inform on protein degradation and stability. If a protein is completely digested in the gastrointestinal (GI) tract, the potential for its absorption from the digestive tract as an intact and functionally active macromolecule would be negligible in most cases. This minimises the likelihood that the protein could exert adverse effects such as allergenicity and/or toxicity (EFSA GMO Panel, 2021c, 2022c). Therefore, data concerning the resistance of NEPs to proteolytic enzymes (i.e. in vitro digestion studies) are used in protein safety risk assessment. Likewise, proteins are usually denatured and inactivated by high temperatures and/or extreme pH values, as would often occur in processing and cooking, thus minimising the dietary exposure in their active form (Martinez et al., 2024).

In practice, the classical pepsin-resistance test is used in the risk assessment following international guidelines, such as Codex Alimentarius (2003–2009) and Commission implementing regulation (EU) No. 503/2013. However, the usefulness of this test is questionable because it does not reflect the physiological conditions of human digestion or livestock (EFSA GMO Panel, 2011a, 2017b, 2021c; IR503/2013), and might not always fully demonstrate that a protein is effectively degraded during the digestion process.

Therefore, there is a need for more reliable systems to predict digestion, to better understand the fate of the protein or fragments in the GI tract and how they interact with relevant human/animal cells (EFSA GMO Panel, 2021c). Studies on NEPs subjected to a sequential digestion (e.g. pepsin followed by pancreatin) to investigate the possible persistence of peptide fragments throughout the pepsin-resistance test are being increasingly incorporated into dossiers assessed by the EFSA GMO Panel (EFSA GMO Panel, 2022b_AP161; 2024d_AP174). This is in line with Codex Alimentarius, which indicates that alternative in vitro digestion protocols may be used where adequate justification is provided (Codex Alimentarius, 2009). However, there are still gaps in these types of protocols that will need further considerations (e.g. the lack of validation for risk assessment purposes) (EFSA GMO Panel, 2021c, 2022c). Furthermore, extrapolations from in vitro degradation studies between humans and animals should also be considered (see Section 3.2.1.2).

The outcomes of in vitro protein stability and digestibility studies are considered difficult to interpret, and further guidance is needed. Furthermore, this type of in vitro studies can be difficult to perform for certain proteins (Bushey et al., 2014; EFSA GMO Panel, 2022c, 2022d; Roper et al., 2021). Traditionally, protein digestibility has been measured using SDS-PAGE and Western-blotting on single purified proteins. However, when employing gel analysis solely, to judge the completeness of digestion, a high level of purity might be required (Colgrave et al., 2019). As mentioned above, a high degree of purification can be unachievable for membrane-bound proteins or other intractable proteins. Additional practical challenges can be also present for GMOs expressing a high number of new proteins (EFSA GMO Panel, 2022c). Solutions to overcome these challenges could be based on: (i) the selection of alternative starting material of the test substance (e.g. enriched fractions of active proteins like purified microsomes prepared from eukaryotic expression hosts (Madduri et al., 2012) or whole seed/grain extracts (Schafer et al., 2016); and (ii) the use of complementary and more powerful analytical tools like liquid chromatography–mass spectrometry (LC–MSn) to carry out a comprehensive peptide mapping of digesta and identify stable digestion fragments (Colgrave et al., 2019; EFSA GMO Panel, 2017b).

History of safe use (HoSU)

In the GMO assessment, Regulation (EC) No 1829/2003 established the term HoSU that was mainly linked to the selection of a conventional counterpart for the comparative assessment. Furthermore, the Commission Implementing Regulation (EU) No 503/2013 also considered the concept of HoSU for the safety assessment of NEPs in GM plants. The underlying assumption of this approach is that traditionally cultivated crops have a HoSU for human and animals. However well-established the use of the term, HoSU is not formally defined. Thus, it is indicated that in the case where the HoSU for consumption as food and/or feed of both the plant and the NEPs is duly documented, specific toxicity testing shall not be required. However, it is not specified what could be the necessary information to duly document the HoSU of proteins. The demonstration of a HoSU is based on evidence that some or all parts of a plant have been consumed in the diet (food and/or feed and derived products) for a considerable time with no evidence of adverse effects for the consumer, and that exposure from a new use will be within the range of that of the 'historic' use. An operational definition of HoSU should be developed in the near future to support the risk assessment. Over the years, similar terms to HoSU have been used, such as history of use, history of consumption, history of safe food use and familiarity (Capalbo et al., 2020; Constable et al., 2007; Engel et al., 2011). Furthermore, different regulatory agencies have made use of these concepts. As examples, Health Canada (2006) defines HoSU as: significant human consumption of food over several generations and in a large genetically diverse population, for which there exists adequate toxicological and allergenicity data to provide reasonable certainty that no harm will result from consumption of the food. In the USA, any substance that is intentionally added to food is subject to pre-market review by the FDA, unless the substance is generally recognised, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use (GRAS, generally recognised as safe). The updated EU Regulation on novel foods (Regulation (EU)

2015/2283, 2015) defines 'novel foods' and 'history of safe food use in a third country'. A 'novel food' means any food that was not used for human consumption to a significant degree within the EU before 15 May 1997, irrespective of the dates of accession of Member States to the EU. However, a definition of 'human consumption to a significant degree' was not given. A 'history of safe food use in a third country' means that the safety of the food in question has been confirmed with compositional data and from experience of continued use for at least 25 years in the customary diet of a significant number of people in at least one third country, prior to a notification. There is a need for international agreement and consensus on a precise fit-for-purpose definition for HoSU that is acceptable and implementable worldwide (Constable et al., 2007; Fernandez & Paoletti, 2018).

A broader consideration on this topic is that the safety assessment of the NEP does not necessarily require that the exact protein has previously been consumed in food/feed. Thus, protein safety could be established based on structural or functional similarity to proteins with a HoSU derived from bioinformatic analyses on a case-by-case basis (Bushey et al., 2014; Delaney et al., 2008). The EFSA GMO Panel has previously used the HoSU concept to waive the need for specific in vitro and/or in vivo studies (EFSA GMO Panel, 2024e_AP159; 2020a_AP111). However, the use of such a concept is not always straightforward. We have included case studies below to illustrate the concept (see Section 3.3.2). Determining what constitutes a sufficient degree of information to consider a protein safe and establishing when two proteins can be considered similar for safety assessment purposes are challenging and relevant questions. Experience gained with the assessment of difficult cases (e.g. membrane-bound proteins) has proven that there is an urgent need to address these questions.

Animal studies

Although Codex Alimentarius (2003–2009) provides a global framework for GMO risk assessment, EFSA, unlike other authorities, requests a 28-day toxicity study instead of an acute toxicity testing of proteins, following Commission Implementing Regulation (EU) No 503/2013. Studies in which the NEP is fed to animals for 28 days are typically required on every NEP, unless a HoSU or a strong similarity to a previously assessed protein has been demonstrated. So far, studies seen by the GMO panel have identified only trivial findings readily considered as not adverse and/or not treatment related. For some studies, additional information was requested which sufficiently confirmed that there were no adverse effects associated with dosing of the NEP. The 28-day study requires the use of 64–160 animals, typically 16 animals (mice or rats) per sex per group; one to three test groups plus one or two control groups. For several of the NEPs there have been technical issues associated with producing enough protein to comply with the upper dose required by the study test guideline (1000 mg/kg bw per day), being highly resource-consuming (EFSA GMO Panel, 2015_AP095) and sometimes very difficult to perform, e.g. testing of a membrane-bound protein in a 28-day toxicity study (EFSA GMO Panel, 2013c_AP087).

Serum screening

To fulfil the regulatory requirements, testing using human sera from well-characterised allergic individuals should be performed on a case-by-case basis (IR503/2013; EFSA GMO Panel, 2010, 2011a). Although no criteria are provided for the specific assessment of this testing, the WHO/FAO expert consultation (Codex Alimentarius, 2009; WHO/FAO, 2001) provided the minimum number of sera necessary for determining major and minor allergens. EFSA recommends that individual serum and not pooled sera should be used (EFSA GMO Panel, 2010, 2011a) and acknowledged the difficulty in obtaining sufficient number, quantity and quality for screening purposes (EFSA GMO Panel, 2020a_AP111; 2022c).

Interactions between NEPs

According to Commission Implementing Regulation (EU) No. 503/2013, the applicant should perform studies with combined administration of proteins when the genetic modification results in the expression of two or more proteins in the genetically modified plant and when, based on scientific knowledge, a possibility of synergistic or antagonistic interactions raising safety concern was identified. The potential for such interactions among NEPs is assessed systematically by the GMO Panel. The assessment includes current scientific knowledge on the biological function of the NEPs involved and whether synergistic/antagonistic interactions are expected between NEPs that could raise safety concerns for food and feed. This is an evolving area of knowledge where further research is needed to develop robust databases and experimental models to assess synergistic/antagonistic effects. The novel stepwise approach described below in this scientific opinion is intended to serve as a pragmatic starting point for incorporating emerging data on combinatorial exposures in the future.

3.1.2 | Which are the complex cases requiring a different approach for their assessment?

In the EFSA GMO Panel experience, examples of complex cases challenging current approaches requiring alternative/complementary methods are NEPs that are difficult to produce and/or isolate in sufficient quantities (e.g. membrane-bound proteins), those with potential bioinformatic hits to toxins and/or allergens, and potential future proteins 'new-to-nature'.

Production and isolation of NEPs: Sufficient amounts of NEPs from plants are often difficult to acquire to conduct protein safety studies, such as animal toxicity and/or in vitro studies (Figure 1), and in those cases alternative approaches such as producing the NEP in microbial-based expression systems are used instead. However, expression of the NEP in the microbial system in an active form can be challenging and technically difficult, or even impossible for some proteins. Some examples assessed by the GMO Panel in the last few years are membrane-bound proteins and transcription factors (see Section 3.1.1). Together with integral membrane-bound proteins and transcription factors, there are other types of proteins that could also be considered intractable like signalling, glycosylated and resistance proteins (Bushey et al., 2014).

Potential bioinformatic hits to toxins and allergens: The safety assessment of NEPs with hits brings additional challenges. It is not always clear when a hit should be followed up with more specific/extensive in silico analyses and experimental data, or instead with theoretical considerations only. The criteria to use in these cases are not available and differences in the assessments between Authorities might occur (e.g. EFSA GMO Panel, 2021a_AP153; 2024b_AP162; 2024c_AP173). For example, bioinformatic analyses might reveal significant similarities with allergens using the criterion of 35% identity in a sliding window of 80 amino acids, requiring an in-depth analysis of the quality of the pairwise sequence alignments and the specific similarity regions between the novel protein and the allergen, as well as an assessment of the known epitopes and the clinical relevance of the allergen (EFSA GMO Panel, 2021a_AP153; EFSA FAF Panel, 2024). Even when experimental data are considered necessary, it is difficult to identify the appropriate study to address potential concerns derived from the bioinformatic analyses (Cobigo et al., 2024).

'New-to-nature' proteins: This concept has recently emerged, coinciding with advances in protein engineering, synthetic biology, and computational biology. These proteins have no equivalent in nature and are synthetic or engineered proteins designed to perform tasks beyond what natural proteins can achieve (Chen & Arnold, 2020). They can be produced by modifying existing proteins or creating entirely novel sequences, through directed evolution, iterating mutations and selections, rational design, predicting mutations computationally or de novo design, including proteins whose primary sequence can be designed directly from the 3D sequences (Anishchenko et al., 2021), resulting in unique structures and functions. These proteins have diverse uses. In medicine, they enable enzyme-based therapies and targeted drug delivery, in industrial biotechnology they can be used as efficient catalysts or agents for biodegrading plastics. They can also serve in agriculture by improving plant resilience. One example of potential application of these 'new-to-nature' proteins in agriculture is nanobodies (single-domain antibody fragments isolated from camelids) that can be selected to interact specifically with other proteins. These nanobodies can be designed to directly block a pathogen protein (Hemmer et al., 2018) or fused with a resistance receptor, allowing it to be triggered by new effectors (Kourelis et al., 2023). It remains to be determined if current assessment tools will be suitable for the assessment of new-to-nature proteins in GMOs.

Large number of NEPs: The current assessment of NEPs in GM plants is usually performed protein by protein, individually. In case of GM plants with a large number of NEPs this is challenging due to the costs incurred by the high number of in vivo and in vitro studies needed, and the need to reduce the use of animal studies (3Rs)[2] (Hubrecht & Carter, 2019). Importantly, these novel products may contain one or more NEPs which might not be purifiable in a functional manner adding an additional level of complexity in the safety assessment, this challenge being further amplified when multiple NEPs are present.

Other examples: Complex cases may also include the evaluation of allergen-free plant variants and bioactive peptides, for which additional future risk assessment strategies might be necessary.

3.2 | ToR2: Building on experience above and issues identified, a critical appraisal of new methodologies available with the potential to be used as complementary/alternative testing strategies to current methodologies described in legal frameworks

3.2.1 | Which complementary/alternative methodologies can be used for protein safety assessment, considering hazard identification and hazard characterisation?

New approach methodologies (NAMs) covering in silico and in vitro methods are becoming available and fully applied as research tools[5,6,7] (e.g. Escher et al., 2022), but their use is still limited in risk assessment (Cattaneo et al., 2023) for the following reasons.

- New targeted in vitro assays for investigating the potential hazards of NEPs are not available for risk assessment.
- Limitations of the current protein safety assessments, which rely on the chemical risk assessment paradigm and tools that are not entirely fit-for-purpose for GM plants with numerous proteins (Brune et al., 2021; Cattaneo et al., 2023; Fernandez et al., 2018).
- There is a lack of comprehensive guidance or criteria defined for in silico approaches such as bioinformatic searches for structural/functional similarity to known toxins and allergens and known safe proteins.
- Specific protocols and criteria for interpreting readouts and outcomes of in vitro testing are required (EFSA GMO Panel, 2017b, 2021c, 2022c). For in vitro testing, the behaviour and the fate of proteins is important when considering

[5]https://www.eu-parc.eu/.
[6]https://single-market-economy.ec.europa.eu/sectors/chemicals/reach/roadmap-towards-phasing-out-animal-testing_en.
[7]https://www.ineris.fr/en/ineris/news/launch-european-namwise-project-new-methodological-approaches-eco-toxicology.

conditions mimicking environments relevant for the risk assessment, e.g. digestive tract (Akkerdaas et al., 2018; Fernandez et al., 2019; Hammond et al., 2013; Markell et al., 2017; Pali-Schöll et al., 2018; Shan et al., 2002).

There is a need for discussion and consensus on how new methodologies could be used in the safety assessment of NEPs and their value when integrated with current approaches, including in vivo testing. A potential way forward in the use of alternative in silico, in vitro and/or in vivo studies, as well as the role of exposure in the current safety assessment is shown below.

3.2.1.1 | *In silico studies*

Over the last two decades, in silico prediction tools have largely evolved and include tools for determining similarities to known allergens, toxins and safe proteins, phylogeny analysis, digestion and protein aggregation. Moreover, recent advances have been made in computational prediction of 3D models of protein structures and new phylogenetic analysis software tools, but they are not routinely used in protein safety assessments. Despite great strides in the development of tools, there remain limitations. For example, the allergenicity assessment still relies on the 35% sequence identity over 80 amino acids sliding window approach framed in late 90's (Codex Alimentarius, 2003–2009; WHO/FAO, 2001), considered highly conservative and of low predictive value (Abdelmoteleb et al., 2021; EFSA GMO Panel, 2022c; Herman et al., 2021; Ladics et al., 2007). Considering the relevance for risk assessment, the following Section focuses on advanced in silico tools searching for comparisons such as similarities to known allergens, toxins and safe proteins, phylogeny analysis, digestion and protein aggregation.

Allergenicity assessment: EFSA has previously stressed bioinformatic approaches for predicting allergenicity in addition to those defined by Codex (EFSA GMO Panel, 2022c), which includes alternative or complementary methodologies as follows:

(i) increasing the match criteria above 35% identity and decreasing the E-score below 1e-7 or smaller (Abdelmoteleb et al., 2021);
(ii) utilising numerical descriptors representing the physicochemical properties of the amino acid protein sequence and machine learning approach for classification of allergens (Dimitrov et al., 2013; Dimitrov et al., 2014a);
(iii) using similarity of the 3D protein structure and the amino acid sequence (Maurer-Stroh et al., 2019);
(iv) searching similarity to a data set of allergenic and non-allergenic proteins represented as binary fingerprints (Dimitrov et al., 2014b);
(v) using machine learning approaches based on mapping of IgE epitope, motif search and/or other selected variables (Sharma et al., 2021; Westerhout et al., 2019); and
(vi) considering novel approaches with human leucocyte antigen (HLA) binders from known allergens (Dimitrov & Atanasova, 2020).

Furthermore, EFSA has recently published a report (Mills et al., 2024) addressing the following points:

(i) the development of a ranking method for proteins with different allergenic potential according to their clinical relevance and screen existing tools to assess allergenicity risk;
(ii) investigation of potential in silico tools and follow-up actions needed for an improved allergenicity assessment; and
(iii) proposal of a novel approach for allergenicity assessment integrating in silico, in vitro and in vivo methods considering the ranking strategy of known allergens. In this report, several novel bioinformatic tools described above were also assessed, including AllergenFP, AlgPred and AllerCatPro.

The authors found that the novel tools provided a good range of outputs that sought to address issues such as 3D structure assessment and IgE-epitope analysis. However, none of the tools were able to provide an output that could be linked to a clinical relevance score, and many suffered from identification of both false positive and false negative allergens. The systematic review and the ranking for clinical relevance undertaken in this report will help the development of more fit-for-purpose databases and the designers of in silico methods to refine their outputs and improve its usability in risk assessment. Specific refinements to the current approach were identified to further improve the outcomes of the allergenicity risk assessment (see Figure 7, Mills et al., 2024).

Toxicity assessment: EFSA recently published an external report on in silico methodologies to predict the toxicity of novel proteins (Palazzolo et al., 2024). As a result, a curated dataset has been released to assess protein toxicity, which is based on primary sequences of annotated toxins and non-toxins downloaded from UniProt. Additionally, the authors developed an artificial intelligence (AI)-based consensus model that accounts the results of the best ranked tools (i.e. ToxinPred2 and Toxify) and an in-house developed machine learning model built on BLAST alignment. Subsequently, the most up to date tools for protein toxicity prediction were tested and evaluated using this dataset as benchmark and the results were ranked in terms of their predictive accuracy. Ideally, future in silico models for protein prediction would prioritise the use of open-source and freely available software to ensure transparency, long-term maintenance, and result reproducibility and reliability. This type of platform should be accessible to non-bioinformatician risk assessors, enabling them to conduct preliminary evaluations of new proteins intended for introduction into the food and feed industry. Although the classical sequence alignment with BLAST is effective (Palazzolo et al., 2024), it is strongly dependent on the dataset used. Despite

the proposed machine learning models that incorporate tertiary structure data into the pipeline (e.g. motifs), it was not yet possible to add true comparisons of tertiary structure. However, this could potentially be addressed with AI, as some tools have solved this problem with a level of accuracy unmatched by any prediction method to date (Jumper et al., 2021). In the future, such platforms should embed different methodologies spanning from AI and machine learning to classical primary sequence alignment accounting for structural information (e.g. motif annotations) and structure alignment.

Functional and structural similarity: Classical sequence alignment is insufficient when an assessment of the structural and/or functional similarity is needed. For example, functional similarity to commonly consumed proteins might be used in the assessment of HoSU or protein familiarity, or when the toxicity of some proteins might be determined by their function (e.g. pore-forming toxins). Because structural alignment serves as a method to identify similarities in shape and three-dimensional arrangement between two or more protein structures, it might also become particularly valuable when comparing proteins that exhibit limited sequence similarity. To this end, the search for functional similarity might be assessed by combining information on protein structure, sequence and biochemical activity through tools like Gene Ontology (GO) annotations and/or pathway analysis software. Furthermore, functional implications of structural differences need to be considered as structural variations may affect protein function, binding interactions or stability (Notin et al., 2024).

Comparing the 3D structures of two proteins involves superimposing or aligning their atomic coordinates in 3D space to assess structural similarities and differences. The type of methods for protein structure comparison are: (i) Sequence-dependent methods of protein structure comparison that assume strict one-to-one correspondence between target and model residues; and (ii) Sequence-independent methods that perform structural superimposition independently, followed by the evaluation of residue correspondence obtained from such superimposition.

When applying structural alignment to predicted structures, the first step is to assess their quality by comparing the model to the true known structure through structural alignment. For example, some AI tools consist of a trained multiple sequence alignment (MSA), paired residues and PDB (Protein Data Bank) templates of thousands known protein structures (validated experimentally by NMR, X-ray crystallography, Cryo-electron microscopy) from metagenomic databases (Berman et al., 2003). These AI tools can predict the structures of proteins for which a primary sequence is known (Abramson et al., 2024; Varadi et al., 2023). Despite the advancements, it is posited that the AI models in question have been trained on inherently biased datasets, which include protein structures of varying resolutions and proteins in crystallised forms (Abbas et al., 2023). Consequently, the structural predictions generated by these models are highly valuable but require a careful assessment (Abbas et al., 2023; Terwilliger et al., 2024) as they are likely to inherit these biases, necessitating in some cases the application of molecular dynamics simulations for relaxation of the predicted structures.

When the superimposed structures are analysed to identify regions of similarity and dissimilarity, attention could be paid to the overall structure and structural features such as active sites, binding pockets, N- or C-terminal modifications and secondary structure elements. There are different metrics in the assessment regarding the quantification of structural differences, e.g. RMSD, GDT, TM, SSM. A combination of these metrics is often used to gain a comprehensive understanding of structural relationships and to draw meaningful biological conclusions. The metric requirements could differ depending on the purpose of the structural comparison and the characteristics of the proteins being analysed.

The choice of alignment method and the interpretation of results should be related to the specific goals and objectives of the structural assessment. The sequence alignment and the 35% identity over 80 amino acid sliding window approach are the default procedures for assessing allergenicity. Briefly, the concept of the 35% sequence identity over 80 amino acids long windows after the sequence alignment of a query protein and known allergens was developed based on the potential cross-reactivity of Bet v 1 homologues. As mentioned above, this concept is criticised mainly for its conservative approach and the generation of too many false positive hits. It is principally applied as a consensus position of experts rather than having a specific scientific rationale. Although potential thresholds depend strongly on the alignment length, structural homology can be inferred from the level of sequence similarity. Thus, some studies quantified the relationship between sequence similarity, structure similarity and alignment length by exhaustively surveying alignments between proteins of known structure. The authors produced a homology threshold curve as a function of alignment length (Rost, 1999; Sander & Schneider, 1991). These relationships were derived in 1991 and updated in 1999, when there were a limited number of known crystallographic structures. Therefore, improvements to the thresholds set considering new development of tools and new knowledge are needed. For example, the number of crystallographic structures in the databases has drastically increased and following the derived equations for calculating the percentage of sequence similarity, the determined threshold could be applied for the in silico assessment of protein allergenicity and toxicity.

For the toxicity assessment based on structural similarity, novel strategies will be required, particularly because protein toxicity is more often associated with biological function, which may not be evident from the primary sequence alone. The potential toxicity mechanism is of major importance in choosing the alignment method for assessment. For example, in cases where toxicity is due to the primary structure of a protein/peptide (e.g. alpha-amanitin), the sequence alignment between a NEP and sequences of known toxins with similar mechanisms of action is the appropriate approach. However, in cases where specific potential function-driven toxicity exists, the secondary and tertiary structure similarity is of utmost importance (enzymes, pore-forming toxins, lectins, etc.). In the latter case, the assessment would require structural alignment and assessment of the possible similarities with toxins having comparable mechanisms (e.g. potential bioinformatic hits with toxins, see EFSA GMO Panel, 2024c_AP173).

This process can be further strengthened by applying the concept of read-across, which considers the use of known information from data-rich source entities to predict the same property for a data-poor target entity. This concept is

well-established in the risk assessment of chemical compounds, and its principles and methodological frameworks are already outlined by different bodies (ECHA, 2017; EFSA Scientific Committee, 2025; OECD, 2017). Due to the inherent complexity of proteins, established guidelines and frameworks might not always be directly applicable. For example, proteins often lack linear structure–function relationships, and their behaviour can be influenced by post-translational modifications, conformational flexibility or the surrounding biological context. Nevertheless, the concept remains valid also for proteins and can be applied through a more refined and precise case-by-case approach to contextualise the assessment in a biologically relevant manner. For instance, shared functional characteristics, structural features and mechanistic pathways can be employed between the NEP and well-characterised proteins.

When the structural similarity is used in the context of HoSU or protein read-across, an appropriate approach could involve the searching for structure similarity to safe proteins based on a close relationship between function and structure. However, it is necessary to determine 'how similar is similar' and what is/are an acceptable comparator(s) allowing for the extrapolation and final acceptance of HoSU/read-across in the overall safety assessment.

Phylogeny and evolutionary conserved clusters of proteins

The concept of phylogeny considers multiple protein alignments to construct a phylogenetic tree. As mentioned previously, alignments such as BLAST rely on the assumption that proteins aligning at close sequence distances may be homologous, and vice versa. These alignments are often used by protein engineers to trace back to ancient, or presumed ancient, proteins and to endow them with specific properties (Spence et al., 2021). What is intriguing is that 'ancient proteins' may have had more than one function (Siddiq et al., 2017), unlike modern proteins, which are more specific. Utilising such techniques could lead back to scaffolds for which toxicity might have been a function and highlights the risks of ingestion of those proteins. Likewise, this concept can also be applied to understand the extent to which a NEP differs from or is similar to proteins in a family of ubiquitous proteins that are already present in the diet. Here, phylogenetic trees based on primary sequence alignments can be used. This concept might be particularly useful when the NEP falls into a class of proteins that is ubiquitous and serves a similar function across several order of species. Fatty acid desaturases constitute an example. Thus, a broad-scale family phylogeny of fatty acid desaturase genes in *Streptophyta* covering different order of species (i.e. green algae, mosses, gymnosperms, magnoliids, monocots and dicots) has been described (Zhao et al., 2022). The number of desaturases in the phylogenetic tree is very large, but the different groups are not restricted to a particular order of species; rather, they are found across all studied orders. This likely implies that humans and animals could have already been exposed to many different desaturases in the diet, and that the variability/diversity between those desaturases, at least at the primary amino acid sequence level, is known. This knowledge might be used to predict the likelihood of safety of a fatty acid desaturase as a NEP. If it falls within the already known variability/diversity of NEPs in the diet, it might be considered safe. Conversely, if it is completely different from proteins to which humans and animal have been exposed, additional risk assessment consideration and testing might be required.

Such a 'phylogenetic approach' could be complemented by a 'structure prediction approach' based on novel AI-based models to predict 3D protein structures based solely on linear amino acid sequences. A database is now available that contains over 200 million predicted protein structures and Barrio-Hernandez et al. (2023) developed an algorithm that groups predicted structures into 2.3 million clusters. They observed that a large proportion of such clusters are represented in almost all life forms, i.e. the algorithm categorises protein structures in evolutionary conserved clusters that are widespread. This idea brings together the evolutionary and structural characteristics of proteins. Strikingly, only 4% of the analysed protein sequences did not match known structures or domains, so relatively straightforward predictions can be made for most proteins. Combining different AI approaches, it is likely to increase confidence in the predictions.

In the future, algorithms like these may enable the classification of NEPs, based on their known or predicted structures, into evolutionary clusters with no safety concerns, as such clusters include numerous proteins already present in the diet (an approximation of HoSU/read-across). Conversely, a NEP could fall into an evolutionary cluster of safety concern as this cluster contains proteins known to be harmful (requiring further risk assessment consideration or discouraging their use in food/feed products). Animal studies could then be limited to those proteins that, based on their structure, do not belong to any established evolutionary conserved cluster. Importantly, this concept would apply to NEPs that are expressed in their native form, so unmodified. When NEPs have been altered to enhance or change their properties, additional steps might be required to ensure safety. Specific modifications to enhance bioactivity, for example, will require an analysis of potential side effects of such a modified protein.

To determine the feasibility of such approaches, further research would be required.

Similarly, evolutionary conservation of protein structure and function suggests that mechanisms of toxicity associated with toxic proteins common among species are conserved within phylogenetic groups. In addition, the mode of action of toxic proteins within a given phylogenetic group may differ from toxic activities across phylogenetic groups. Since the clustering/grouping of toxic effects generated by toxic proteins is tightly associated to their phylogeny, knowledge on the phylogenetic origin of a (novel) protein (e.g. in the assessment of NEPs) in combination with an AI-driven structural analysis, could help guide a more focused testing strategy.

Gastrointestinal digestion

In silico tools aiming at predicting the behaviour of a protein in relation to GI digestion can complement but not substitute in vitro digestibility experiments. The outcome of these tools is more limited when dealing with full-length proteins

because important factors determining proteolysis like protein structure and folding or post-translational modifications (e.g. glycosylation, disulfide bridge formation) are not regularly considered by these tools.

Protein aggregation

Proteins aggregate to fulfil a physiological role, e.g. some globular proteins; but aggregated proteins can also lead to potential adverse effects (Louros et al., 2023; Stefani & Dobson, 2003). Therefore, the introduction of tertiary structure into a pipeline for protein safety prediction is critical. This information requires more information than the primary sequence because proteins that aggregate have aggregating protein regions (APR) that are not signatures within the amino acidic sequence but depend on factors such as pH and temperature (Yagi-Utsumi et al., 2020). To improve reliability, tools and computational models to predict protein aggregation require a protein structure to run molecular dynamics simulation. AI programmes including several tools and databases that are freely available on the web are promising for predicting protein aggregation (Pinheiro et al., 2021). Examples of these can be found in Prabakaran et al. (2021), Navarro & Ventura (2022) and in the supplementary information in Annex F. Although still in the early stages, such information might be incorporated into the protein safety assessment in the future, as reliable tools for predicting aggregation at the tertiary structure level may soon become available (Sun et al., 2024).

3.2.1.2 | *In vitro studies*

The in vitro studies used in the current safety assessment inform on protein characterisation, protein stability and protein toxicity/allergenicity potential. Considering new developments and experience gained, below there are considerations for the potential use of alternative/complementary methods for protein safety assessments.

Protein characterisation

Characterising proteins is becoming more challenging because the events assessed by EFSA are more complex as are the GM products that will be designed to express more proteins at low levels. These could be more difficult or impossible to purify in sufficient amounts (e.g. if they are membrane-bound) and their function could be difficult to demonstrate in vitro, for example in case of plants developed by synthetic biology (see EFSA GMO Panel, 2021d for hypothetical case studies). For such events, certain analyses typically provided by the applicants might not be possible (e.g. antibody-based assays). Thus, the risk assessment requirements should allow for the use of any robust and standardised techniques that generate the data required by the Commission Implementing Regulation (EU) No 503/2013. For example, the fast progress in the resolution of mass spectroscopy methods and other proteomic techniques could soon allow for the detection and characterisation of proteins present in amounts below the detection levels of current methods (Bennett et al., 2023).

Enzyme substrate specificity

The substrate specificity assessment generally starts from a database of potential candidate substrates followed by in silico modelling to select compounds with a chemical structure that is comparable to the intended substrate (Simoben et al., 2023). Examples of databases, which may not all be open access, include the Dictionary of Natural Products (Sorokina & Steinbeck, 2020), the NAPRALERT database (Bisson et al., 2016) and Food Explorer.[8]

Examples of approaches to select substrates for phosphinothricin acetyltransferase (PAT) were based on structural similarity to the natural substrate (L-phosphinothricin). The structurally close analogues glutamate, methionine sulfoximine and hydroxylysine were selected for testing (Hérouet et al., 2005). In another example, D'Ordine et al. (2009) tested the specificity of DMO for O-anisic acid and vanillic acid, as both are structurally similar to dicamba. Additionally, in the assessment of aryloxyalkanoate dioxygenase-12 (AAD-12), other criteria to select potential substrates were similar physiological function or their presence in major metabolic pathways of plants (EFSA GMO Panel, 2017a_AP106; 2017c_AP097; Griffin et al., 2013; Mendonça & Marana, 2011).

Once potential substrates in plants are identified, the next step utilises an in silico approach to narrow them down leading to in vitro assay testing. Although there are no validated or officially recognised methods, in silico methods can include molecular docking to a known crystallographic structure of the enzyme using fit-for-purpose software and rank the potential substrates based on the binding affinity or energy score (Agu et al., 2023; Asiamah et al., 2023).

On a case-by-case basis, compounds present in animals and humans could also be considered when selecting the substrates of an enzyme newly expressed in a plant. As an example, the aminoglycoside phosphotransferase (APH4) enzyme catalyses the phosphorylation of the 4-hydroxyl group of hygromycin B 2-deoxystreptamine ring, inactivating its antibiotic activity. Due to this property, APH4 was used as a selectable marker for the transformation of a GM cotton (EFSA GMO Panel, 2023a_AP141). The substrate specificity of APH4 was tested for 14 aminoglycosides and among those only hygromycin B was recognised by the enzyme (Stogios et al., 2011).

[8] https://www.eurofir.org/.

Additional evidence on substrate specificity and kinetics of newly or differentially expressed enzymes can be obtained from the results of a targeted comparative compositional analysis which is required in accordance with applicable guidance on the risk assessment of genetically modified plants. The compositional endpoints generally included in the comparative analysis following the recommendations set out in applicable OECD consensus documents cover key components representing nutrients, anti-nutrients, allergens and toxicants stemming from various metabolic pathways. The comparative analysis of those endpoints is therefore considered to provide a comprehensive picture of the total composition. Such steps were followed when evaluating the activity of 2mEPSPS, a variant of the endogenous EPSPS enzyme (EFSA GMO Panel, 2009_AP051). This protein acts in the synthesis pathway of aromatic amino acids, and therefore, no change of the amino acid composition of the GM crop was considered part of the weight-of-evidence to conclude on the safety of the NEP.

The key components analysed are used as indicators of whether effects of the genetic modification influencing plant metabolism have occurred or not. This may help as well collect evidence on the mode of action of newly expressed enzymes involved in key metabolic pathways. In the case of GM soybean 305423, changes in the level of odd chain fatty acids were further investigated and considered unintended effects probably caused by the introduction of the ALS enzyme (EFSA GMO Panel, 2013b_AP045).

The characteristics of the introduced trait may trigger further analysis of specific compounds including metabolites of potentially modified metabolic pathways. In the case of newly expressed enzymes, additional compounds may be selected based on prior knowledge on their functional characteristics and catalytic properties. The targeted analysis of intended substrates, known enzyme inhibitors and compounds selected from in vitro substrate specificity screening may help obtain a more complete picture on the effect of enzyme expression in different tissues in vivo.

The results obtained from the targeted comparative compositional analysis may inform the identification of hazards resulting from the expression of a newly expressed enzyme and help build a hypothesis on the effects of the genetic modification which may further structure the risk assessment.

Established compositional analysis methods remain the primary tools for risk assessment. However, a targeted and/or hypothesis-driven analysis of substrate specificity may be biased depending on available knowledge regarding compound selection and potentially affected metabolic pathways. To gain a more unbiased understanding of the effects of enzyme expression in metabolic networks, non-targeted approaches may be additionally explored. Analytical methods may span from those covering specific classes of compounds until those capturing a wide range of constituents. On a case-by-case basis following a specific hypothesis, omics-based methodologies may be explored in the future. Nevertheless, although omics-based analyses can be used in some specific cases to support GM plant assessments, their wide application in regulatory science still face challenges such as the lack of validated analytical methods, insufficiently harmonised data interpretation and integration approaches (Benevenuto et al., 2023; Harrill et al., 2021; Radio et al., 2024; Sauer et al., 2017).

Protein degradation

From the large numbers of existing proteins, very few are known to be toxic via the oral route in animals (e.g. mammals, birds and fish), with a few exceptions (e.g. the octapeptide of alpha-amanitin, prions, lectins), which often have unusual structures (e.g. cyclic, misfolding), which resist digestion. One of the reasons for this is that consumed proteins are extensively digested by proteases. In humans and monogastric animals, initial digestion is in the stomach (e.g. by pepsin), subsequently in the small intestine (e.g. by trypsin and chymotrypsin) and by enzymes secreted by the intestinal lumen. Protein digestion in polygastric animals (e.g. ruminants) occurs in the rumen where microorganisms degrade proteins into peptides and amino acids locally. These are further transformed into ammonia, used by bacteria to synthesise microbial proteins, essential for the animal's nutrition when they reach the intestine. Absorption of the components of proteins is usually as oligopeptides (typically di- or tri-peptides). These small fragments will not maintain the core structure of the protein required for receptor binding, enzymatic or additional biological activities.

Proteins having a specific insecticidal action (e.g. Cry proteins) retain this activity in the insect due to the insect gut having no equivalent of the acid conditions of the mammalian (vertebrate) stomach. Many of the proteins, which are toxic following ingestion, do not act systemically but locally on the GI tract. Among the proteins known to be toxic upon ingestion, α-amanitin, a cyclic octopeptide, exhibits systemic toxicity through the bloodstream, affecting liver, kidney, central nervous system and GI tract; prions which spread systemically through the lymphatic and nervous systems, accumulate primarily in the central nervous system; lectins, which are prevalent in food and feed sources due to their abundant presence in plants, primarily exhibit local toxicity in the GI tract.

Data obtained from bioinformatics and in vitro studies (e.g. GI *digestion*) can be used to identify those NEPs which are related to known toxins or are resistant to digestion and, therefore, more likely to be toxic and merit further investigation.

Significant advances in standardised and harmonised alternative in in vitro GI protocols using physiological digestion conditions (e.g. addition of surfactants, phospholipids and bile salts) and pancreatic proteases (trypsin and chymotrypsin in physiological amounts) have been developed during the last decade (Brodkorb et al., 2019; Minekus et al., 2014). Some of these protocols have been further adapted to infants (Ménard et al., 2018), older adult populations (Menard et al., 2023), as well as to estimate the in vitro digestible indispensable amino acid score (DIAAS) to determine food protein quality (Sousa et al., 2022) in the context of the nutritional assessment of proteins. In addition, in the small intestine, enteropeptidases embedded in the brush border membrane (BBM) (e.g. aminopeptidases, carboxypeptidases, endopeptidases and dipeptidases) are responsible for the final stage of peptide digestion (prior to their absorption into the enterocytes) by reducing

most poly- and oligopeptides to their monomer constituents (Hooton et al., 2015). Before being released in the bloodstream, peptides may undergo further intracellular digestion by cytosolic, lysosomal and microsomal enzymes (Ozorio et al., 2020). Indeed, antigen-presenting cells (APC) digest proteins internally with endolysosomal enzymes (e.g. cathepsins, prolylcarboxypeptidases) before presenting to antigen-specific T cells, suggesting that in vitro assays determining endolysosomal degradation may be an efficient tool providing information on the immunogenicity or allergenicity of proteins and T cell reactivity (Öztemiz et al., 2024; Verhoeckx et al., 2019).

Therefore, in the eventual case that stable fragments, such as those having a relatively high molecular weight, persistence and abundance, could be identified, the in vitro GI digestion process could be complemented by further in silico (if available) or in vitro steps. These steps could be attained using enteropeptidases embedded in the small intestine epithelial brush border membrane, as well as the consideration of the contribution of the endolysosomal degradation to protein digestion during antigen uptake. However, there are still some shortcomings with BBM and lysosomal enzymes preventing its straightforward use in risk assessment protocols, such as (i) a lack of harmonisation of this type of assays, (ii) the regular supply of commercial peptidases from mammalian species is non-existent or very restricted or (iii) their expression under in vivo conditions is not well-established. A solution to these limitations could be the further development of in silico digestion approaches using a selection of representative peptidases involved in BBM and endolysosomal digestion processes. It is important to consider that slow or limited protein digestibility does not indicate, on its own, that a protein is necessarily allergenic or toxic. However, proteins that are at least partially resistant to digestive proteolysis may need additional risk assessment considerations as they might be more effective at stimulating the immune system (Fernandez et al., 2019). Immunogenic structures that persist longer in the gut might be absorbed by epithelial cells of the small intestine in an active form (Delaney et al., 2008; EFSA GMO Panel, 2021c; Pilolli et al., 2019; Shan et al., 2002). The Commission Implementing Regulation (EU) No 503/2013 requires that stable breakdown products should be characterised and evaluated regarding their potential to cause adverse health effects linked to their biological activity. However, one of the main drawbacks is that the in vitro protein digestion data lacks the necessary guidance about how to interpret the outcome for risk assessment (Fernandez et al., 2024). Therefore, it is important to reach a consensus and/or definition, among other aspects, about:

- the criteria to identify digestion fragments as relevant for risk assessment of toxicity and allergenicity considering both sensitisation and/or elicitation (i.e. abundance, persistence, molecular size and/or others) (EFSA GMO Panel, 2017b). In relation to allergenicity, a WHO/FAO Joint Expert Consultation Group indicated that collecting evidence of protein fragments less than 3.5 kDa would not necessarily raise issues of protein allergenicity and the data could be taken into consideration with other decision tree criteria (WHO/FAO, 2001). In agreement with this, Huby et al. (2000a, 2000b) stated that an allergen must contain at least two IgE binding sites or epitopes, each of which with a minimum of 15 amino acid residues long, to make possible the antibody binding. This IgE binding places theoretical limits on the peptide size of at least 30 amino acids or ~3kDa (Colgrave et al., 2019); and.
- the criteria for classifying a protein as resistant or labile to digestion, and the risk implications of such data. This lack of knowledge impairs the setting of appropriate limits for digestibility in assessing the safety of a protein by using an appropriate set of reference control proteins ('allergenic' and 'non-allergenic' or 'readily degradable' and 'non-degradable'). Krutz et al. (2019) reported a set of non/low-allergenic plant proteins based on their significant exposure to humans (such as abundant proteins from spinach, corn, potato, rice, tomato or wheat) and being not (or only rarely) associated with allergy.

The extrapolation between humans and animals indicated that the fate of protein digestion is similar between terrestrial farmed and companion animals and humans, when monogastric animals are considered. Ingested proteins are broken down initially by pepsin and hydrochloric acid in the stomach, followed by intestinal digestion by pancreatic proteases. The mechanisms of pre-caecal utilisation of dietary protein in equids are like that of other monogastric terrestrial animals. Although protein digestion in stomach and foregut in terrestrial farmed and companion animals is like humans, differences exist considering anatomical and physiological conditions of different species, e.g. capacity of the stomach, length and capacity of small intestine and hindgut, gastric emptying time, small intestinal transit. This involves a species-specific difference in the environmental conditions of the digestive tract depending on dietary habits (e.g. carnivore, herbivore, omnivore).

Protein digestion in ruminants is different because feedstuffs are partially digested in the rumen by microorganisms capable of producing enzymes to degrade plant polysaccharides and transform nitrogen into bacterial protein. Two sources of protein are available for absorption in the intestine of ruminants, one is the plant protein that escapes rumen fermentation, and the other is the bacterial protein produced by rumen bacteria.

The fate of protein digestion in fish is to some extent comparable to terrestrial monogastric animals. However, some anatomical and physiological differences exist, mainly based on if the feeding strategy is carnivorous, omnivorous or herbivorous. Most fish species have a stomach where pepsin and hydrochloric acid digestion of protein occurs. Unlike terrestrial vertebrates, many fish have a collection of blind gut sacs called pyloric ceca after the stomach where the carnivorous fish species seem to have more pyloric ceca. In most fishes, the pancreas is not a distinct structure but exist as diffuse tissue interspersed in mesenteric tissue along the gastrointestinal tract. The fish 'pancreas' produces most of the same proteases as mammals and birds; trypsins, chymotrypsins, elastases and carboxypeptidases. Following the pyloric ceca is the midgut and distally the hindgut where, as opposed to mammals, proteins may be further hydrolysed and absorbed.

The protocols used to study protein digestion in vitro for monogastric animals are mainly based on protocols used for humans. The INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014) is used to simulate the human GI tract (mouth, stomach and small intestine) during the digestion of foods. Briefly, the procedure is composed of three phases: preparation, digestion procedure and sample treatment with subsequent analysis. It is a static digestion method that uses constant ratios of meal to digestive fluids and a constant pH for each step of digestion. The digestive physiology of pigs is like that of humans, and pigs were identified as a model to compare INFOGEST in vitro protein digestibility to in vivo digestibility (Egger et al., 2017). Zaefarian et al. (2021) report that, even though differences between digestion in pigs and poultry exist, in vitro pig protocols are often used to study digestion in poultry. As recently reviewed by Deschamps et al. (2022) up to now, only a restricted number of in vitro models has been developed to simulate the canine upper or lower digestive tract.

The principal in vitro methods available for measuring intestinal digestibility of protein in ruminants are the three-step procedure (Calsamiglia & Stern, 1995), the modified three-step (Gargallo et al., 2006) and a revised three-step method (Ross et al., 2013). All three methods effort to simulate ruminal, abomasa and intestinal processes with different enzyme types and concentrations, incubation times, methods of termination and equipment. In ruminants it is important to consider that the protein available in the intestine comprises bacterial protein produced in the rumen, and the feed protein non-fermented in the rumen. Recently, results indicated the possibility of using ANKOM gas production system to estimate the protein fractions and in vitro degradability of protein-rich feeds (Demirtas et al., 2019; Tunkala et al., 2023).

The in vitro digestibility studies carried out for regulatory purposes are mostly done on purified proteins produced by heterologous expression systems. However, the production of some types of proteins (e.g. membrane-bound proteins) in active form can be technically non-feasible. To overcome this limitation, some methods based on enriched preparations of the active proteins like microsomes prepared from eukaryotic expression hosts (Madduri et al., 2012) or whole seed/grain extracts (Schafer et al., 2016) have been successfully used to produce test material for in vitro protein stability studies.

The classical pepsin-resistance test is not intended to mimic the physiologic conditions of gastric digestion (WHO/FAO, 2001), and the use of currently available standardised and harmonised in vitro protocols (e.g. INFOGEST) to provide information on the GI fate of proteins in humans and monogastric animals seems feasible. If stable fragments of a relatively high molecular weight, persistence and abundance are identified, further in silico (if available) or other in vitro steps using enteropeptidases could be considered.

Protein toxicity

Currently, there is no existing in vitro testing strategy available for the evaluation of protein toxicity, and construction of such a strategy must take into account several considerations unique to protein toxicity testing. Current in vitro approaches for toxicity assessment, including NAMs, are largely based on strategies developed for chemical toxicity. The applicability and predictive power of in vitro approaches for the assessment of protein toxicity requires further investigation, and must take into consideration protein-specific criteria. Considerable discussion and consensus concerning the applicability of these approaches are required in order to assess how in vitro methodologies could be incorporated in the context of risk assessment of NEPs.

Toxic proteins are produced by a diverse array of species over a wide range of phylogenetic groups, and the mechanisms of toxicity associated with these proteins vary considerably among these groups. In light of this diversity, a generalised in vitro testing strategy for the evaluation of protein toxicity is not currently possible, and a hypothesis-driven stepwise approach, generated from the core lines of evidence including HoSU, mode of action and bioinformatic analyses, could be considered. A testing framework could be developed such that in vitro testing is only required when a hazard has been identified or unacceptable uncertainties remain such as in case of proteins with unusual structures and/or part of a protein family with members for which toxicity has been documented. A proposal for an in vitro-based testing strategy for the evaluation of protein toxicity was recently proposed by Cobigo et al. (2024). Key points raised in this strategy are presented below. If, following a preliminary step considering all available lines of evidence on the protein and the donor organisms, a hypothesis of potential protein toxicity could be identified, in vitro testing strategies could be applied. In the case of multiple cloning, it is relevant to assess the safety of the proteins individually but also in combination, in order to look for any synergistic or antagonistic effects.

In cases where a hypothesis of potential toxicity could be generated, a thorough protein characterisation is necessary before any in vitro testing, including biochemical characterisation of the protein and protein solutions, considering the concentration, purity and when possible, protein activity. Testing of the stability of the protein under storage conditions (freeze/thaw cycles) and identification of a suitable storage buffer is also necessary. Protein stability could be assessed to verify whether the protein retains activity throughout the duration of the in vitro test. Digestion of the protein of interest in relevant in vitro digestion methodologies representative of passage through the digestive tract, including mouth, stomach and intestine could also be required. A harmonised protocol such as INFOGEST could be applied (see above). The in vitro toxicity of proteins could be evaluated before and after in vitro digestion.

The primary aim of preliminary hypothesis-driven in vitro toxicity testing would be to evaluate principal endpoints of toxicity using 'simple' cellular models and rapid, high throughput/content assays. Two assays per type of endpoint could be evaluated to take into account mechanism-based differences in protein toxicity. In the case of basal cytotoxicity, well-established assays for membrane permeability (e.g. LDH, Propidium Iodide) together with an assay evaluating changes in cellular metabolism (e.g. ATP, MTT), or the neutral red uptake (NRU) assay would be recommended. The evaluation of these effects could be carried out using appropriate model systems expressing relevant target proteins.

Mechanism-based endpoints, including oxidative stress and (pro)-inflammatory responses, could be addressed using high throughput or high content-based approaches, including gene reporter or cellular image-based methods. The choice of mechanism-based tests to include in the battery of tests could be justified and appropriate for the protein under investigation. Genotoxicity testing could also be performed on a case-by-case basis, if indicated by the hypothesis generated by the core lines of evidence.

If toxic effects are observed in the preliminary toxicity testing in simple models, additional testing on relevant in vitro models would be warranted. Preliminary considerations could include local toxicity, barrier function, inflammatory effects as well as absorption and passage across the biological barrier. Methods assessing the absorption and crossing of biological barriers could follow test guidelines when available (for intestinal absorption DB-ALM Protocol no 142: Permeability Assay on Caco-2 Cells or the tri-culture model currently being considered at the OECD level including Caco-2 cells, HT-29/MTX cells and M-cells) (Castiaux et al., 2016; Cobigo et al., 2024).

If barrier integrity is compromised and/or absorption of the protein is observed in in vitro models of primary target organs, systemic availability of the protein can be assumed and further testing of toxicity on in vitro models of relevant secondary systemic target organs could be performed.

Assays to determine the effects of compounds on epithelial integrity have long been dependent on epithelial cell lines, mostly of colon and cancerous origin that form polarised monolayers in tissue culture, like Caco-2 cells. A drawback of such monolayers is that they do not accurately mimic the intestinal epithelium as various epithelial cell types – like stem cells, the secretory Paneth cells and mucus producing goblet cells – are not found in such monolayers. The most widely accepted 'complex' model of the intestinal epithelium is the tri-culture Caco-2/HT-29 MTX/Raji model which comprises enterocytes, mucus producing goblet cells and M-cells. Such a model is currently under evaluation at OECD within the Project 4.158: New Guidance Document on IATA for intestinal fate of orally ingested nanomaterials. An additional relatively recent development is the generation of intestinal organoids from LGR5+ stem cells present in intestinal crypts. Alternatively, they can be generated from pluripotent stem cells derived by reprogramming of skin fibroblasts. The 3D organoid structures that are generated are a more accurate approximation of the intestinal epithelium as they contain all cell types found in the epithelium in vivo (Pang et al., 2022; Pleguezuelos-Manzano et al., 2020). Moreover, they can be maintained in culture for up to years, providing a novel platform to perform functional studies in a 3D cellular structure that more closely represents the physiology of the intestine (Gómez & Boudreau, 2021).

Extensive literature documents that organoids can be generated from both human- and animal-derived samples and can be grown for many organs, including stomach, liver, intestine, kidney, skin, lung and brain. As such organoids hold great promise as tools for functional testing in vitro. It can be imagined, for example, that they can be used for determining the impact of novel food components on epithelial barrier function and the integrity of the various differentiated epithelial cell types present in intestinal organoids.

It should be noted that they are still relatively simple models that do not incorporate all cellular constituents of the intestine, notably stromal and immune cells are lacking. However, novel approaches may tackle this problem as air-liquid interface (ALI) organoids have been shown to preserve the intestinal stromal and immune compartment, at least for a limited number of days, potentially allowing an evaluation of the impact of novel food ingredients on these cellular constituents as well.

Finally, while organoid technology provides a powerful tool for research it is far from being a standardised and fully validated technology that can be applied today in a routine setting for screening for potential harmful effects of food components (Lee et al., 2024; Wang et al., 2023). Nevertheless, given the recent advances in the use of organoid technology for screening of the usefulness of drug compounds, it is reasonable to assume that soon this technology will become available for screening of food components as well. In future, intestinal organoids might represent a very interesting model in in vitro toxicology. However, also in this case, there are no standardised protocols for production and they are very labour-intensive. Testing throughput, protocol standardisation, reproducibility and Inter-laboratory transferability are also problems. There is also the problem of reversed apical-basolateral polarity with intestinal organoids – however, plating dissociated organoids on trans well inserts (2D) can also be an option. While interesting, considerable work is required before organoid models can be used in a regulatory context.

In addition to organoids, organ-on-a-chip (OoC) technology has developed rapidly in the past decade and is now widely applied in a research setting. OoCs are tissues that are grown in microfluidic devices. Within these devices, several physiological parameters can be applied and controlled, like liquid flow, sheer stress and mechanical stress. As such, OoCs have advantages over conventional 2D culture systems as they more closely resemble the in vivo conditions. In addition, they allow the testing of drugs and other compounds of interest in well controlled conditions. In fact, even multiorgan OoC platforms have been described. It can be envisaged that OoC may also become a useful tool for the screening of food components in the future.

Currently, only few in vitro methods designed for the evaluation of toxicological endpoints have been validated at the level of the OECD, and the process of integrating data generated from in vitro-based assays, including NAMs, in the context of risk assessment is at its beginnings. Considerable work, including case studies and proof of concept studies, are needed to evaluate the applicability of the integration of alternative in vitro approaches into a risk assessment workflow. In the short-term, data from these approaches could be used as support in a weight-of-evidence evaluation in combination with in vivo experimentation. Discussion and consensus concerning a possible roadmap towards the integration of in vitro approaches in risk assessment of NEPs is necessary.

Protein allergenicity

Currently, there is no validated in vitro testing strategy available for the evaluation of protein allergenicity. The EFSA GMO Panel has previously reviewed several in vitro models for allergenicity assessment, including the use of human sera testing (EFSA GMO Panel, 2022c). Furthermore, a range of in vitro models were considered to assess key immunological events such as antigen uptake through the intestinal mucosal barrier, epithelial and dendritic cell activation and migration, as well as T- and B-cell differentiation (EFSA GMO Panel, 2022c; Lozano-Ojalvo et al., 2019; van Bilsen et al., 2017). The EFSA GMO Panel's scientific opinion also includes the main challenges and research needs related to in vitro tools for allergenicity risk assessment of foods derived from biotechnology (EFSA GMO Panel, 2022c).

3.2.1.3 | *In vivo studies*

Protein toxicity

Considering that there are at present no validated and commonly accepted in vitro studies which are suitable to use as a screen for general protein toxicity in GMOs, it is foreseen that some form of in vivo testing on NEPs may be required to cover potential gaps/uncertainties on a case-by-case basis.

Some other authorities with responsibilities for regulating GMOs require a single dose acute toxicity test based on OECD TG for acute oral toxicity studies. The study design is aimed only at hazard identification and typically uses 24 to 40 animals (6 or 10 test and 6 or 10 control animals of each sex) with dosing at 1000 to 5000 mg/kg bw (the reason for the variability across studies is unclear). The use of only a single administration reduces the degree of discomfort compared with 28 daily administrations. Although sufficient to identify any severe toxicity, the existing acute study protocol includes only limited investigations performed within 14 days from dosing (no haematology or clinical chemistry analyses; limited gross pathology examination, no microscopic examination of organs and tissues), compared with those performed for the OECD guideline-compliant 28-day study required in the EU.

Both the 28-day repeated dose toxicity study, and the acute toxicity study are based on protocols developed for low molecular weight chemicals and the applicability to proteins tested by oral route is uncertain.

In the near future, until the predictability of bioinformatics and in vitro tests in the evaluation of protein toxicity has been demonstrated/validated, one way forward to combine a perceived need to provide adequate reassurance regarding the potential toxicity of NEPs, whilst meeting some of the 3Rs requirements of Directive 2010/63/EC[9] (refinement, reduction and replacement), would be to design a protocol for a required in vivo test focusing on the likely effects of proteins. This approach would also align with the EC roadmap for phasing out animal testing.[10]

On a case-by-case basis, and when scientifically justified, either for toxicological assessment or to support the evaluation of allergenicity, a single in vivo study could be developed to address toxicity and allergenicity, in line with the 3Rs principles. For instance, additional data might be considered to clarify specific pathways to harm. This could include immunophenotyping, serum antibody measurements or immunohistochemistry to evaluate potential effects on the immune system and to identify immunotoxicity. A protocol for such a study needs to be developed which might include:

- Administration of three to five oral gavage doses – toxic proteins normally produce toxicity after a single dose but detection of allergenicity requires multiple doses.
- Only two groups of animals (normally mice), one negative control group (containing an equivalent dose of a 'control' protein such as BSA) and one test group, consisting of the NEP (in its active/native configuration), dosed orally at the limit dose of 1000 mg/kg bw per day. The test is intended to identify potential hazards and there is no reason to use additional doses below the limit dose. An additional group exposed to a positive control for allergenicity could be included or laboratory historic control data could be used (as is the case for skin sensitisation and some genotoxicity assays), where scientifically justifiable.
- A group size of 10 animals of a single sex (the minimum group size in the OECD 28-day guideline 407 is 5/sex; but 10 to 16 animals/sex/group are routinely used in the tests performed on NEPs); the principle of using just one sex is accepted in the OECD acute test guidelines 420, 423 and 425.
- Blood samples for haematology and clinical chemistry are taken and histopathology examinations are performed. Investigations do not need to include all those required in existing test guidelines but could include a suite of endpoints of relevance to proteins, taking into account knowledge of their function and potential pathways to harm (e.g. markers of immunological effects and enhanced examination of the GI tract for local effects such as using 'Swiss roll' preparations and specific staining techniques; microbiome).

Any treatment related adverse findings would need to be investigated further, on a case-by-case basis.

If concerns regarding a NEP are identified by in silico or in vitro tests, these might trigger additional investigations within the in vivo study. If they cannot be resolved in another way, further in vivo studies might be necessary.

[9] https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32010L0063.

[10] https://single-market-economy.ec.europa.eu/events/commission-roadmap-phasing-out-animal-testing-chemical-safety-assessments-2023-12-11_en.

Protein allergenicity

An effective in vivo model for the allergenicity risk assessment could provide reliable predictions about the allergenic potential of proteins. Mice are commonly used for this purpose due to the availability of diverse food allergy models. These models (reviewed by Kazemi et al., 2023) offer a range of approaches that can be adapted for risk assessment, including the evaluation of NEPs in GMOs, as previously detailed (Marsteller et al., 2015). Despite the advancements, the field still lacks models that can universally predict allergenic responses, highlighting the need for further research, continued development and validation of these experimental systems.

To develop in vivo food allergy mouse models for the allergenicity risk assessment, several factors must be carefully considered. Mouse models like BALB/c and C3H/HeJ demonstrate Th2-skewed immune responses that mimic human allergies, producing IgE and other immune responses similar to those seen in humans. They also display clinical signs, such as anaphylaxis and diarrhoea, which offer valuable insights into potential allergenicity. Sensitisation through oral or epicutaneous routes simulates human exposure pathways and yields relevant data. However, there are challenges to using these models effectively. Fundamental differences between mouse and human immune systems can limit predictive accuracy, while inconsistencies across laboratories can impact reproducibility. Moreover, calibration against known human allergens is necessary to ensure results are meaningful.

To overcome existing challenges, an integrated approach could be developed that merges a standardised food allergy model with in vivo feeding studies commonly used in toxicology assessments. This model could encompass multiple groups: a control group on a non-GMO diet, a test group consuming a GMO diet infused with the NEP and a positive control group exposed to a known allergen, such as peanut protein. It is crucial for all diets to maintain nutritional equivalence, except for the GMO component. Throughout the feeding period, monitoring immune responses by measuring specific IgE and IgG antibodies, T cell subpopulation cytokines (e.g. Th2, Th17) along with conducting T- and B- cell proliferation assays, could provide insight into allergenic responses. A post-feeding oral challenge with a higher dose of the GMO protein could then be used to induce immediate hypersensitivity reactions, including potential anaphylaxis. Additionally, conducting cross-reactivity testing with structurally similar proteins might help identify cross-reactive immune responses. Histopathological examination of the gastrointestinal tract, lungs and skin could be included to assess markers of allergic inflammation, such as eosinophils and mast cells and mediators. Complementing this in vivo allergenicity and toxicology model with in vitro digestibility assays and bioinformatic analyses would create a comprehensive assessment. By integrating these methodologies, we can address the limitations of individual approaches, ensuring a robust and realistic allergenicity risk assessment. This strategy also aligns with ethical standards, reducing the need for further animal testing and adhering to the 3Rs principles: replacement, reduction and refinement.

Furthermore, when considering protein allergenicity, it might be necessary to broaden the scope by including protein immunogenicity in the future. Generally, immunogenicity refers to proteins that induce protective immune responses. In contrast, antigenicity refers to proteins that cause immune reactions, and allergenicity is defined as a protein that induces an allergic, non-protective immune response. Although the safety assessment includes assessing allergenicity, including an immunogenicity assessment might provide a more accurate and robust approach to GMO protein safety assessment. However, widening the evaluation may lead to immunogenic but not allergenic proteins. A comprehensive immunogenicity evaluation might identify proteins that trigger an allergic response in sensitive individuals, even if they do not share structural similarities with known allergens. This is especially important because the current GMO safety assessment primarily relies on the amino acid sequence similarity to known allergens as a critical determinant of potential allergenicity. Indeed, some allergens exhibit low sequence similarity to known allergenic proteins and evade detection by sequence-based methods.

Integrating an immunogenicity assessment into NEPs safety evaluations could significantly enhance the accuracy of allergenicity risk assessment by including a range of assays (see supplementary information Annex G). By incorporating immunogenicity assessment into the safety evaluations, we may improve our ability to predict and mitigate allergenicity risks by providing a more comprehensive understanding of how NEPs interact with the immune system, helping identify potential allergenic triggers that might not be identified through sequence-based methods alone.

Selected publications cover various aspects of immunogenicity testing and allergenicity risk assessment, including in vitro assays, animal models, epitope mapping and regulatory considerations (see Annex G). They provide a foundation for developing methodologies to assess the potential allergenicity of genetically modified proteins, contributing to the overall safety evaluation of GMOs.

3.2.1.4 | *Exposure*

Currently, estimated exposure is not systematically used for the safety assessment of NEPs of products derived from GMOs. Expanding further the role of exposure is recommended as a future development in NEP risk assessments. As examples, a regulatory threshold of 20 ppm of gluten, equivalent to 20 mg of gluten per kg of the finished product, was recommended by the Codex Alimentarius in 2008 based on scientific evidence, consumer safety and practical testing limits. In the context of IgE-mediated allergenicity, reference doses for a series of priority allergenic food sources have been established by a FAO/WHO Expert Committee on risk assessment of food allergens (FAO/WHO, 2021). A reference dose is the eliciting amount in milligrams of protein (total protein from an allergenic food), not as grams of the food itself, below which only a given percentage of the population with that particular allergy will react with objective symptoms (Smits et al., 2024). Other

considerations based on the knowledge of the dose–response relationship between exposure, characteristics of exposure and response can be also taken into account to establish reliable thresholds of allergenic concern (Crevel et al., 2024).

In the toxicological assessment of most chemicals, the exposure assessment is fundamental to further characterise an identified hazard, as part of the risk characterisation assessment. The risk characterisation can conclude on the absence of a significant risk because: (i) no hazard is identified up to the limit dose of the test guideline; (ii) a hazard is identified, but the predicted exposure is negligible/non-existent; (iii) no hazard is identified up to the highest dose tested (so called 'limit dose') or a hazard is identified, but the predicted exposures provide an adequate margin of safety based on a point of departure derived from the dose–response for toxicologically relevant, adverse effects (*dosis facit venenum* – Paracelso).

In previous sections, there are some examples of proteins known to be toxic in vertebrates (e.g. mammals, birds and fish) following oral exposure. The reported doses at which adverse effects occur include 0.1 mg/kg bw for the octapeptide of amanitin (Bang et al., 2022), 1–20 mg/kg bw for ricin (lectins) (Moshiri et al., 2016), while for prions, even a few nanograms can be lethal under certain conditions (Swire & Colchester, 2023). In future, it might be possible to define a level of exposure of a NEP representing a threshold below which no adverse effects would be expected, provided that the NEP is well degraded in vitro and that there are no identified concerns based on bioinformatics. If predicted exposures are below this threshold, further studies might not be required. For NEPs where there are concerns relating to degradation or they are identified by bioinformatics as having similarity to known toxins, some additional testing for toxicity might be required. This could be in vitro to investigate if the bioinformatic data indicating similarity to known toxins are confirmed experimentally and/or in vivo to investigate if there is an actual hazard and, if so, there is an adequate margin between predicted exposures and the dose producing no toxicologically relevant adverse effects.

3.2.2 | What stepwise weight-of-evidence approach should be used for the safety assessment of NEPs in GMOs?

Historically, the NEP safety assessment has been divided into two main fields usually performed in an independent manner, protein toxicity and protein allergenicity. However, more similarities than differences exist between these two fields of protein safety. For example, they both rely on a weight-of-evidence approach that can be used as a common umbrella in the overall protein safety assessment. Under such a frame, the knowledge on the genetic modification and on the novel proteins themselves might be used as starting point guiding for the necessary steps to be taken. In particular, the HoSU, protein read-across, documented knowledge on the protein and phylogeny might be leveraged to define the type of data required in the assessment.

It is expected that the weight-of-evidence approach will continue to be used in the short/medium term. Within the weight-of-evidence approach two main elements are highlighted: (i) the comparative approach as the basis in any assessment; and (ii) expert judgement as the decision-making step. Today, the only universal approach available for the establishment of safety is the comparative approach (Fernandez & Paoletti, 2021; OECD, 1993; WHO, 1991). Furthermore, the current approach heavily relies on expert judgement to interpret a posteriori the outcome of studies considered in the evaluation, e.g. the bioinformatic analyses, which can lead to a lack of harmonisation, reproducibility and transparency of the risk assessment (EFSA GMO Panel, 2021a_AP153; 2022c; 2024c_AP173).

The specific methodology that can be used in the weight-of-evidence include concepts[11] such as HoSU, protein read-across, gene ontology, phylogeny, protein characterisation, mode of action, stability, structural/functional similarity to known proteins, similarity to toxins/allergens, fate in the GI tract, interactions between proteins (See Figure 2, adapted from Fernandez et al., 2023).

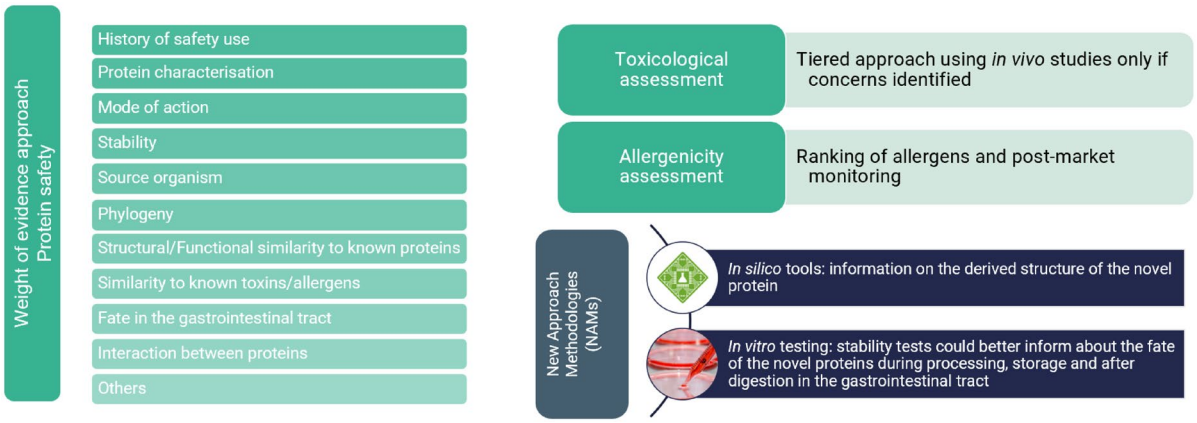

FIGURE 2 Methodologies that potentially add to the weight-of-evidence approach on a case-by-case basis.

[11] https://doi.org/10.1016/S0378-4274(23)00841-X.

A cornerstone in this approach is to consider all lines of evidence in a stepwise manner and use a tiered approach where in vivo studies are only requested if concerns are identified or if some specific information relevant to ensure safety is unavailable.

3.3 | ToR3: Road map for future implementation of such complementary/alternative methods in risk assessment strategies

3.3.1 | How can new methodologies be introduced as complementary/alternative testing strategies in the overall weight-of-evidence approach for protein safety?

The strategy proposed in this document is to reinforce the weight-of-evidence approach following it in a stepwise manner considering new methodologies as complementary and/or alternative studies and using animal studies only when there is a hypothesis or when key information to conclude on safety of a NEP is missing.

Figure 3 presents a schematic representation of a proposed tiered and proportionate weight-of-evidence approach for the safety assessment of NEPs. This approach provides flexibility and covers a range of scenarios which illustrate the level of requirements on a case-by-case basis, and demonstrates that there is a need for: (i) finding consensus on key terms; (ii) upgrading in silico/in vitro investigations; and (iii) using more targeted in vitro testing and/or animal studies as a second/third tier requirement and only if concerns about potential hazards still remain or if information from previous tiers is unavailable or inconclusive in proving safety.

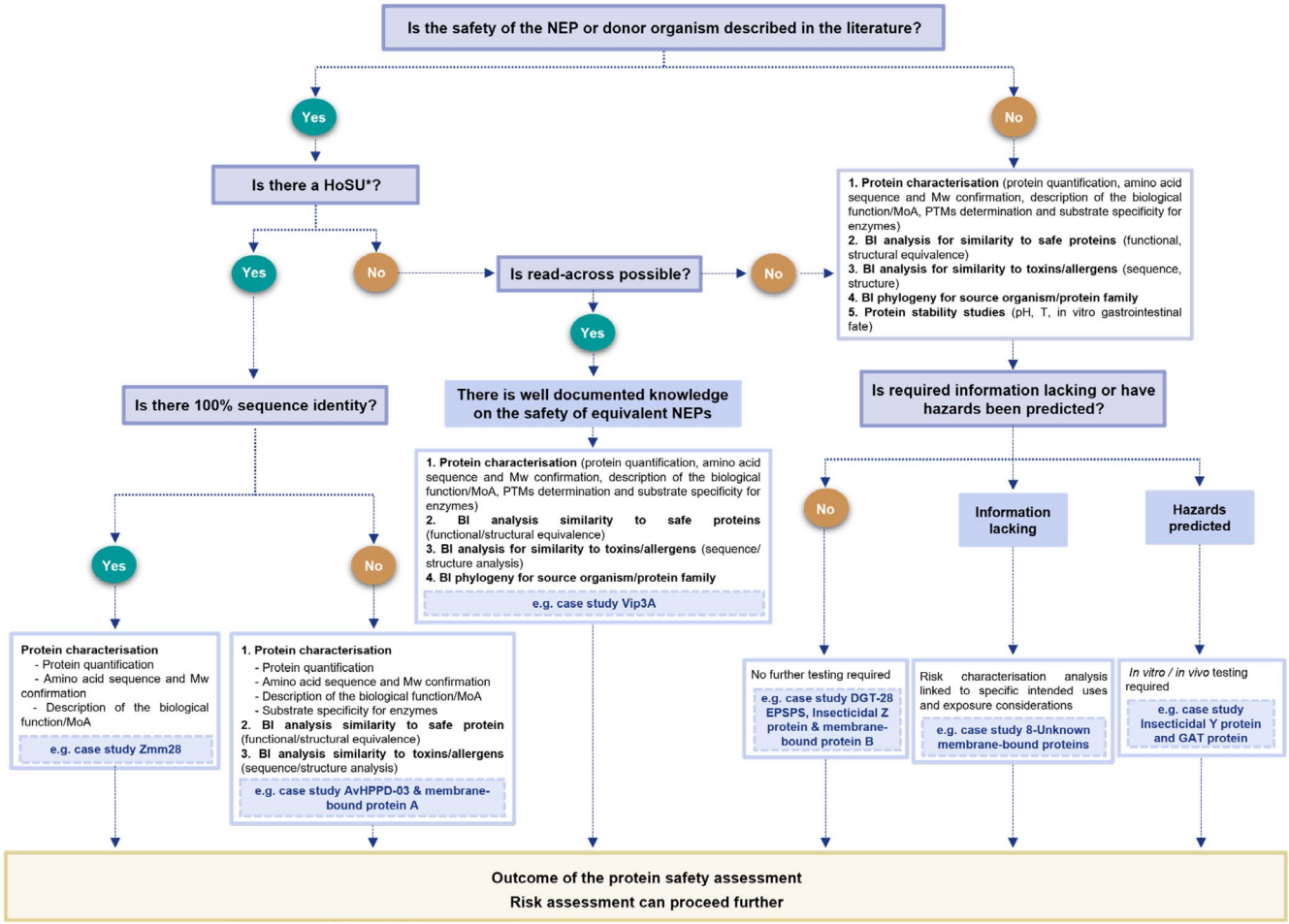

FIGURE 3 Stepwise weight-of-evidence approach. *The Working definition of HoSU in the context of this document is: Evidence of consumption of the NEP with well-documented quantitative levels, within a biological context and commonly considered as safe for humans/animals (e.g. consumed over several generations or for an appropriate number of years with no health-related issues reported or other terms).

This stepwise approach heavily relies on key terms for which better clarity is needed. In particular, what is meant with HoSU and when documented knowledge on the safety of a NEP is accepted. Some important factors in establishing a HoSU have been described, including: (i) knowledge and safety of the source from which the protein was obtained; (ii) evidence of dietary exposure to the protein (from that source); (iii) consumption levels of the protein in different populations (e.g. use of epidemiological studies, characterisation of the population exposed to the protein or source over generations, or number of consumers or specific consumer groups); and (iv) toxicology/allergenicity/nutritional information of the protein.

It is important to highlight:

- The lack of HoSU does not imply that a NEP is not safe, but it only indicates that further analysis to obtain evidence on safety is required (Figure 3).
- Protein safety could also be established based on structural and functional similarity to proteins with a HoSU or with proteins with a well-documented knowledge on safety. The safety of an introduced protein does not necessarily require that the exact protein has previously been contained in food/feed.
- A potential long-term solution could be the building of a database containing proteins with a well-established HoSU or considered safe for consumption.
- Experimental evidence of the levels of comparator proteins in consumed foods and feeds is important to verify their existence rather than relying on transcript levels, protein inferred homology or predicted from annotated coding regions.

3.3.2 | How should the outcome of these new methodologies be interpreted to inform the overall weight-of-evidence approach for protein safety?

To demonstrate the effectiveness of the stepwise approach outlined in Figure 3, we present eight selected examples of hypothetical and/or real case studies. These examples illustrate how a range of safety assessment scenarios can be conducted using this approach.

Case studies:[12]

1. Zmm28 protein:

The ZMM28 is an endogenous protein in maize (*Zea mays*) with altered expression.[13] The ZMM28 protein is a MADS-box transcription factor (Castelán-Muñoz et al., 2019; Münster et al., 2002), involved in biochemical and physiological processes including photosynthesis, nitrogen assimilation and growth regulating hormone reception. The HoSU of the ZMM28 protein in humans and animals took into account the exposure to this protein through conventional food and feed products and was considered adequate (EFSA GMO Panel, 2024e_AP159). According to the approach proposed in Figure 3, cases like ZMM28 protein would require documented information supporting its HoSU complemented with a protein characterisation. For example, this characterisation could consist of protein quantification, amino acid sequence confirmation and a description of its biological function or mode of action. In this case and considering the mode of action, additional investigation might include the potential for unintended effects of the ZMM28 transcription factor on the composition of maize.

2. AvHPPD protein:

The AvHPPD-03 protein is a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme. The *avhppd-03* gene encoding for the AvHPPD-03 protein is originated from oat (*Avena sativa*). The native HPPD protein has a HoSU supported by dietary exposure estimations (EFSA GMO Panel, 2020a_AP111). The AvHPPD-03 protein differs by one amino acid from the native oat HPPD. Considering the amino acid sequence, the in silico structural analyses showed that the single amino acid change did not have an impact on the HPPD folding and function. The AvHPPD-03 protein expressed in the GM soybean was considered structurally and functionally similar to the native oat HPPD. According to the approach proposed in Figure 3, cases like AvHPPD-03 protein would require a documented information supporting its HoSU complemented with a protein characterisation, and a structural and functional equivalence between the NEP and the native protein. For example, the characterisation in this case could consist of protein quantification, amino acid sequence confirmation, a description of its biological function or mode of action and substrate specificity considerations. In this case, because the AvHPPD-03 protein differs by one amino acid only from the native oat HPPD it might not be considered necessary to carry out a bioinformatic analysis searching for similarities to toxins and allergens.

3. Vip3Aa19 protein

The Vip3Aa19 is a vegetative insecticidal protein derived from the native protein found in *Bacillus thuringiensis*. The Vip3Aa19 protein has insecticidal activity against several lepidopteran species, including cotton bollworm and fall armyworm. The Vip3Aa19 has documented knowledge on its safety as follows: (i) this protein is highly similar (99%) to the Vip3Aa20 protein, differing only by one amino acid; (ii) the Vip3Aa20 protein has been previously assessed and is supported by well-established safety assessment studies (EFSA GMO Panel, 2012_AP082); and (iii) structural and functional equivalence between the two Vip proteins is confirmed. According to the approach proposed in Figure 3, cases like the Vip3Aa19 protein would require documented information supporting its safety (HoSU or read-across), complemented by a

[12]Disclaimer: Please note that the information provided might not be real. These case studies have been selected for illustrative purposes only – this information should not be used to conclude on the safety assessment of ongoing or future applications.

[13]Referred by the EFSA GMO Panel as endogenous protein with altered expression and not as newly expressed protein (EFSA GMO Panel, 2024e).

protein characterisation, structural and functional similarity between the NEP and the protein holding a 'safe' status, and bioinformatic analyses searching for similarities to known toxins and allergens.

4. Membrane-bound proteins A and B

The membrane-bound protein A is an enzyme involved in fatty acid biosynthesis derived from *Komagataella phaffi*. This source organism is widely used as a host for expression of recombinant proteins and is included in the list of Qualified presumption of safety (QPS)-recommended biological agents intentionally added to food or feed (EFSA BIOHAZ Panel, 2018) and generally recognised as safe (GRAS). According to the approach proposed in Figure 3, cases like membrane-bound protein A would require documented information supporting its safety based, for example, on the QPS-status and exposure estimations, complemented with a protein characterisation as described in case study 1. If the NEP would be engineered and differ by few amino acids (e.g. two amino acid change), a structural and functional equivalence between the NEP and other appropriate comparator proteins known to be safe and bioinformatic analyses searching for similarities to known toxins and allergens would be needed, as described in case study 2.

The membrane-bound protein B is an enzyme involved in fatty acid biosynthesis derived from a marine alga that does not have a HoSU- or a QPS-status. In this case, the applicant could provide a bioinformatic analyses for: (i) similarity to safe proteins; (ii) similarity to toxins and allergens; and (ii) phylogeny comparison. Briefly, humans and animals could be exposed to many distinct enzymes of this type in the diet and the variability/diversity between those enzymes could be known. If the membrane-bound protein B falls within the already known variability/diversity of NEPs already consumed in the diet, it could be considered as strong argumentation for safety. Therefore, the main information for the overall assessment would rely on similarity to well-known proteins consumed in the diet. Furthermore, whenever possible, protein characterisation and protein stability studies might be required, as described in case study 5.

5. DGT-28 EPSPS protein

The DGT-28 EPSPS protein confers tolerance to glyphosate herbicide by converting shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate (Griffin et al., 2021). The HoSU use/documented knowledge on the safety of DGT-28 EPSPS protein cannot be duly documented because the amino acid sequence largely differs from previously assessed proteins. The DGT-28 EPSPS protein belongs to the newly discovered Class IV EPSPS (EFSA GMO Panel, 2025_AP175). According to the approach proposed in Figure 3, cases like DGT-28 EPSPS protein would require a stepwise full protein safety assessment. Briefly, the assessment of the DGT-28 EPSPS could consist of a protein characterisation, enzymatic activity based on kinetics, structural analysis based on crystallography studies, a phylogenetic analysis with known EPSPS proteins, a structural and functional comparative assessment with known EPSPS proteins, bioinformatic analyses searching for similarities with known toxins and allergens, and in vitro studies informing on protein stability and its fate in the GI tract. Considering that in this particular case no hazards were identified, no animal studies would be required.

6. Insecticidal proteins Y and Z

The insecticidal Y protein derives from a microorganism in soil and confers insect protection against certain species. The HoSU/documented knowledge on the safety of this insecticidal protein has not been duly documented. According to the approach proposed in Figure 3, cases like this insecticidal Y protein would require a stepwise full protein safety assessment. As mentioned above, the assessment of the insecticidal Y protein would consist of a protein characterisation, a phylogenetic analysis, a structural and functional assessment with known safe proteins, bioinformatic analyses searching for structural similarities with known toxins and allergens and in vitro studies informing on protein stability and its fate in the GI tract. In this case, the bioinformatic analysis revealed this protein as a potential toxin. According to the literature, the toxicity of this type of insecticidal proteins is targeted to insects. However, no scientific publications confirming the specificity to insects of protein Y were retrieved. Therefore, to support the safety of this insecticidal Y protein, it might require a hypothesis-driven and targeted in vitro and/or in vivo testing.

The protein Z is an insecticidal protein produced by combining the binding region of insecticidal protein A with the pore-forming region of insecticidal protein B. Both protein A and protein B are supported by extensive datasets in line with EFSA and regulatory guidelines. These data on proteins A and B have been previously evaluated by EFSA and no significant safety concerns were identified. A 28-day in vivo study on protein Z was not provided in the application. Data on bioinformatic analyses for similarity to toxins/allergens, protein stability and digestion of protein Z show consistency with data on proteins A and B and indicate that protein Z would be expected to present no additional safety concerns. A conclusion on the safety of protein Z can be made without requiring a 28-day in vivo study by reading across from the studies on proteins A and B. The assessment of the protein Z could consist of a battery of tests as described in case study 5.

7. GAT4621 protein

The GAT4621 protein is a *N*-acetyltransferase enzyme derived from *Bacillus licheniformis* which confers tolerance to the herbicidal active substance glyphosate. The HoSU or documented knowledge on the safety of this herbicidal protein and/or its source was not duly documented (EFSA GMO Panel, 2013a_AP053). According to the approach proposed in Figure 3,

cases like this enzyme would require a full protein safety assessment. Therefore, the assessment of the GAT4621 protein would consist of a protein characterisation (including substrate specificity studies), a phylogenetic analysis, a structural and functional assessment with known safe proteins, bioinformatic analyses searching for similarities with known toxins and allergens and in vitro studies informing on protein stability and its fate in the GI tract. Given the capacity of the GAT4621 protein to acetylate glyphosate, the substrate specificity of this protein was explored in vitro to obtain a more complete picture on the effect of enzyme expression in the alteration of levels of endogenous compounds (i.e. certain *N*-acetylated amino acids). The knowledge on the biological function of the GAT4621 protein and the outcome of the in vitro substrate specificity studies indicated the need to: (i) perform additional toxicological studies on the role of *N*-acetyl amino acids, (ii) consider other relevant information on the biological role and metabolism of *N*-acetylated amino acids and (iii) carry out human and animal dietary exposure assessments to *N*-acetyl amino acids, to complete the risk characterisation and conclude on the safety of the GM crop (EFSA GMO Panel, 2021b_AP109). Likewise, a post-market monitoring plan was recommended to confirm the predicted consumption data of the GM crop and/or its products and to verify that the conditions of use are those considered during the pre-market risk assessment. This case study shows how the characteristics of the introduced trait (e.g. a specific enzymatic activity) may trigger further analysis of specific endogenous compounds and the subsequent assessment of the biological role of possible reaction products, which are not directly linked to the safety assessment of the NEP itself.

8. GM plant containing multiple unknown membrane-bound NEPs

The newly expressed proteins in this GM plant are membrane-bound proteins from marine organisms with no information on their use as food or feed. Therefore, HoSU or documented knowledge on its safety could not be established. The fatty acid composition of the GM plant and/or derived products does not raise concern for human and animals. According to the approach proposed in Figure 3, this type of case would require a stepwise full protein safety assessment. However, in this hypothetical case, an additional issue encountered in the assessment would be the impossibility to obtain a protein characterisation, e.g. protein quantification and detection, enzymatic activity. Furthermore, there would be a lack of appropriate comparator proteins known to be safe to perform a structural and functional equivalence and a phylogeny analysis did not yield conclusive information. Considering key information on safety is missing, a proper full safety assessment would not be possible and a full-scope scenario of this GM plant for all food and feed uses could not be covered. Therefore, the risk assessment could be restricted to specific intended uses only following a product-driven safety assessment that could consider: (i) a minimum set of data such as in vitro/in vivo testing with a concentrated protein material from the final product relevant for safety that holds regulatory acceptance; and (ii) a selection of specific exposure scenarios/proposed used levels. This approach would require a risk characterisation analysis supported by an exposure assessment linked to the specific intended uses selected. In some cases, specific post-market monitoring might be requested to confirm over the years the protein safety assessment assumptions done during the pre-risk assessment process, similarly to the requirements for the nutritional assessment in nutritionally altered GM crops (EFSA GMO Panel, 2020c_AP126).

3.4 | ToR4: Recommendations for further research or for addressing methodological development needs

3.4.1 | What are the main gaps and/or uncertainties in the protein safety assessment that would need to be addressed in the future?

This document outlines a strategy that strengthens the stepwise, weight-of-evidence approach, incorporating new methodologies as complementary or alternative studies and reserving animal studies solely for cases where a specific hypothesis exists, or critical information is needed to assess the safety of a NEP (Figure 3). The main gaps for a full implementation of Figure 3 that should be addressed are the following:

- HoSU, or related terms such as read-across are key terms in the overall safety assessment of NEPs for which specific criteria for their use are still missing (see Section 3.1.1).
- In silico analysis, the bioinformatics assessment searching for known allergens and toxins are still framed following criteria defined 20 years ago. Limitations of these approaches and potential follow-up solutions are described in Sections 3.1 and 3.2 and elsewhere (EFSA GMO Panel, 2022c). The need for a strategy placing greater emphasis on the development of fit-for-purpose databases to better advance the predictability of in silico tools has been highlighted (Fernandez et al., 2021; Mills et al., 2024). A robust, reliable and verifiable database could better serve risk assessors as a benchmark to frame the risk assessment around the specific public health objectives. For example, it has been described for allergenicity assessment that current allergen sequence databases do not provide systematic data about their allergenic potential in terms of clinical relevance and often employ different inclusion criteria (EFSA GMO Panel, 2022c; Fernandez et al., 2024; Mazzucchelli et al., 2018; Mills et al., 2024; Radauer & Breiteneder, 2019). In addition, experience gained with the assessment of complex cases, such as the assessment of membrane-bound proteins, has also proven the urgent need to address key questions on the safety assessment of NEPs. Currently, there are no validated in silico tools (i.e. not confirmed by experimental data via in vitro/in vivo methods) and/or guidelines on how to assess the information derived from

bioinformatic approaches comparing structural/functional similarity between two distinct proteins. Furthermore, it is unlikely that in silico methods alone are sufficient to determine the safety of proteins without supporting experimental data (Roper et al., 2021). The selection of appropriate comparator proteins is a key step in the process and a potential long-term solution could be the building of a database containing proteins with a well-established HoSU.

- In vitro analysis, efficient GI digestion of proteins results in the loss of biological activity, thereby minimising the likelihood of proteins exerting adverse effects, such as allergenicity (Ma et al., 2024; Pali-Schöll et al., 2018) and/or toxicity (Hammond et al., 2013; Tafazoli et al., 2019). Given that the classical pepsin-resistance test is not intended to mimic the physiologic conditions of gastric digestion (WHO/FAO, 2001), the use of available standardised and harmonised in vitro protocols (i.e. INFOGEST) to provide information on the GI fate of proteins in human and monogastric animals could be feasible. However, specific and consensual criteria for interpreting the readouts and outcomes of the in vitro digestibility tests in risk assessment are required (see Section 3.2.1.2).

  A major challenge is to identify a testing protocol which focusses on the potential adverse effects of ingested proteins, rather than low molecular weight chemicals, while moving towards meeting the 3Rs requirement of Directive 2010/63/EC. Linking in vitro studies to toxicity and allergenicity is one possible strategy, but internationally accepted and validated in vitro studies are limited to specific effects such as genotoxicity, aspects of immune functioning, irritation/corrosion and sensitisation. Despite extensive literature on NAMs-based in vitro safety assessment, very few in vitro NAMs have reached full regulatory validation (see Section 3.2.1). This poses severe limitations on the usability of in vitro testing in the regulatory safety assessment. For example, many NAMs are still insufficiently characterised and standardised for use in routine evaluation, necessitating substantial investments in terms of equipment and personnel. Moreover, interpreting results require expert evaluation, which is not easy to draw definitive conclusions. This is particularly true when the safety evaluation is based on NAMs-derived -omics data (e.g. transcriptomics, proteomics, epigenomics, metabolomics), where the generated datasets require specialised expertise for bioinformatics on processing and analysis. Notably, international regulatory agencies (such as OECD, EFSA, ECHA) emphasised the necessity of additional validated models, and several research and validation projects are ongoing, e.g. OECD project on the development of a Guidance Document on 'Integrated *in vitro* approach for intestinal fate of orally ingested Nanomaterials', the EFSA research projects NAMS4NANO or ADME4NGRA.
- In vivo testing, there is a lack of targeted in vivo studies specifically designed for protein safety assessment that minimises the use of animals and performed in such a manner that the same animals can be used to address both general toxicity and potential allergenicity (see Section 3.2.1.3). There is a need to move away from a mandatory 28-day study based on a protocol for low molecular weight chemicals to a study more targeted at investigating the potential hazards of NEPs.
- Exposure, the role of exposure in the protein safety assessment is not described in detail in international or European guidelines (Codex Alimentarius, 2003–2009; EFSA GMO Panel, 2011a). EFSA published opinions on exposure assessment for humans and animals (EFSA, 2019; EFSA GMO Panel, 2023c). Currently, estimated exposure is not systematically used for the safety assessment of NEPs of products derived from GMOs to conclude on safety (see Section 3.2.1.4).
- Post-market monitoring (PMM), according to the EFSA GMO Panel (2011a) and Commission Implementing Regulation (EU) No 503/2013 guidelines, when there is a likelihood of allergenicity or side effects predicted, the food/feed derived from the GM plant should be further characterised and appropriate conditions for placing on the market may be applied, e.g. labelling (EFSA GMO Panel, 2011a). In addition, the outcomes of the protein safety risk assessment may give rise to various levels of uncertainty that could be better addressed through PMM. However, no criteria are provided on how PMM systems should be built and assessed. Furthermore, the assessment of complex foods will benefit from a developed PMM system that is linked with other ongoing initiatives on this topic in the healthcare sector (EFSA GMO Panel, 2022c).[14]
- Impact of processing, given the diversity of food matrices and food processing procedures, knowledge of their effects on susceptibility of proteins to digestion is still limited. Consequently, the effects of processing and of the food matrix on the susceptibility of a particular protein to digestion are difficult to predict (EFSA GMO Panel, 2017b, 2021c; Martinez et al., 2024).
- De novo sensitisation, it is not yet fully understood what makes a protein an allergen (EFSA GMO Panel, 2022c) as the mechanisms underlying the development of food allergy are not completely elucidated (Mills et al., 2024), making the allergy prediction of NEPs a challenge.
- Omics technologies, when specific traits may be introduced with the intention of generating complex changes in the composition of GM food and feed and a more comprehensive compositional analysis could be needed.
- Complex cases in risk assessment (e.g. membrane-bound proteins, computationally designed proteins or GM crops expressing many NEPs; see Section 3.2.1), challenging current approaches and requiring alternative/complementary methods.

It is important to recognise that requiring 'full' or 'official' validation (e.g. via OECD guidance documents) for every new method could hinder flexibility and delay the integration of novel, science-based approaches into risk assessment. While formal validation ensures reproducibility and regulatory acceptance, the process can be lengthy. In contrast, 'in-house' or functional validation can offer empirical evidence of a method's reliability and fit-for-purpose performance. For example, enzymes with unique catalytic properties often require tailored assays to confirm substrate specificity. Depending on the

[14] https://www.food.gov.uk/report-a-food-allergy-intolerance-reaction.

intended use, rapid functional validation may suffice for exploratory questions, whereas full accreditation is appropriate when replacing an established regulatory test. These considerations apply equally to any new methodology, whether in vitro, in silico or other NAMs, ensuring that novel tools can be applied flexibly and responsibly in safety assessments.

3.4.2 | What developmental and research activities are needed to address above gaps?

A case-by-case weight-of-evidence approach remains the most effective and flexible strategy for the safety assessment of NEPs. However, it would benefit from best-practice criteria and consensus on the applicability of new methodologies and their added value when integrated with current approaches.

This scientific opinion identifies new developments and challenges that question the usability of the classical strategy for the assessment of NEPs in GM food and feed. The GMO panel's experience serves to highlight developmental and research activities to address or implement in future risk assessments. Furthermore, the EFSA GMO Panel asked the public about the necessary developmental and research activities, and how these should be prioritised. To help guide the work program and the research activities, and to focus limited resources, the GMO Panel proposes the following ranked priorities for addressing methodological gaps and uncertainties in protein safety assessment of novel food/feed proteins (NEPs) (see Table 1 for a summary of gaps and recommended activities):

1. *HoSU/biological read-across (short-term)*. Currently, HoSU and read-across are key in the weight-of-evidence approach, but lack standardised definitions, best practice or quantitative thresholds in the context of protein safety assessment (e.g. minimum consumption levels, epidemiological benchmarks, minimum structural similarity). Therefore, we need to agree on meaningful criteria and/or international consensus on a fit-for purpose operational definition of HoSU- and familiarity-related terms and build open databases containing proteins known as safe for human/animal consumption (see Section 3.3.1). For example, consensus on the minimum consumption levels of the protein in different populations is needed (e.g. use of epidemiological studies, characterisation of the population exposed to the protein or source over generations, or number of consumers or specific consumer groups).

   Key activities:

   - Convene expert working groups (regulators, academia, industry) to draft an operational definition of HoSU and read-across in the context of protein safety.
   - Define measurable criteria (e.g. generations of exposure, consumer-group size, intake metrics) and minimum consumption thresholds.
   - Develop and launch an open database of comparator proteins, with metadata on consumption history, exposure levels and study quality.
   - Explore the use of advanced computational tools, such as protein structure prediction, phylogenetic analyses and AI-assisted similarity assessments, to strengthen read-across justifications and to feed into the updated in silico frameworks outlined in Priority 2.

2. In silico, *methodologies (short- to medium term)*. Current in silico allergenicity/toxicity screens rest on criteria that are now over 20 years old, limiting both predictivity and comparability. It is important to (i) modernise the methodologies considering current knowledge and experience gained from the safety assessment of NEPs in GM crops over the last 20 years, making the safety assessment of proteins more proportionate and effective (see Section 3.2.1.1); (ii) develop fit-for-purpose databases (e.g. toxins, allergens, plant endogenous compounds) to advance the predictability of in silico tools and their role in risk assessment; and (iii) implement accurate, robust and validated criteria and methods to establish protein structural and/or functional similarity with toxins/allergens or safe comparators (see Section 3.2.1.1).

   Key activities:

   - Review and update sequence- and structure-similarity thresholds against allergens, toxins and safe comparators.
   - Assemble fit-for-purpose, validated databases (toxins, allergens, plant endogenous compounds, proteins known to be safe) with standardised inclusion criteria and metadata.
   - Develop a standardised framework for interpreting in silico outputs, including 'red flags' (e.g. sequence motifs or structural features of concern), grey-zone hits needing expert review and no-concern zones to guide follow-up testing when needed.
   - Develop a framework for the implementation of phylogenetic and AI-driven structural analysis into the risk assessment to inform read-across and hazard grouping.

3. In vitro, *(medium- to long-term)*. Existing pepsin-resistance and other in vitro assays often fail to mirror physiological digestion or key toxicological pathways; few NAMs are harmonised and –omics data require expert interpretation. We need to (i) modernise in vitro methodologies considering current knowledge and experience gained; (ii) set criteria for interpreting the outcome of in vitro studies in the weight-of-evidence approach, for example understanding the fate of a protein in the GI

tract and how it informs the protein safety assessment as described in Section 3.2.1.2; and (iii) modernise and define which cases would require further additional information or studies (see Section 3.2.1.2). In the short-term, in vitro testing strategies can serve as complementary methods in the risk assessment strategies of NEPs. Furthermore, the integration of validated, well-established in vitro tests and models into current testing strategies will provide additional toxicological information that could be included in a weight-of-evidence based approach. In the future, we can anticipate an acceleration of the integration of in vitro methodologies, NAMs and -omics tools for the evaluation of protein safety. Proof of concept projects are needed to demonstrate the utility and effectiveness of in vitro toxicity testing strategies in risk assessment of (novel) proteins.

Key activities:

- Test whether harmonised in vitro digestion protocols are suitable for determining the gastrointestinal fate of proteins in the context of risk assessment.
- Develop and agree on interpretation criteria for digestibility readouts (e.g. peptide cut-off sizes, persistence, abundance) to ensure that outputs can be meaningfully used (e.g. integrated into broader assessment workflows or triggering follow-up investigations).
- Identify and qualify NAMs for key pathways to harm, such as gut barrier integrity and immune sensitisation.
- Conduct proof of concept studies to correlate in vitro endpoints with relevant biological effects.

Additional priorities described below could follow these core activities as resources permit, using a case-by-case weight-of-evidence approach. These main priorities are not closed: future stakeholder engagement may lead to elevating some 'additional' activities described below, such as better integration of exposure in protein safety assessment, post-market monitoring, to higher priority. It may also identify other areas of interest, e.g. adjuvanticity.

- In vivo, (i) studies specifically designed for protein safety assessment required on a case-by-case basis (hypothesis-driven) only; and (ii) how best to meet 3Rs commitments whilst performing investigations more targeted for the assessment of proteins (see Section 3.2.1.3).
- Evaluating the role of occurrence and resulting exposure, thereby defining a strategy in the overall toxicology and allergenicity assessment. For example, (i) expanding research into the dose–response relationships and improving testing methods; and (ii) the establishment of threshold levels for toxicology and allergenicity below which no safety concern is expected (see Section 3.2.1.4).
- PMM, developing post-market monitoring systems following a hypothesis-driven approach to confirm assumptions made during the initial risk assessment (e.g. exposure) or to reduce the level of uncertainties left in the pre-market assessment, for example to confirm exposure in the case of nutritionally enhanced crops or to confirm uncertainties of new tools used in the safety assessment of proteins.
- De novo allergen (protein) sensitisation, despite advancements, it is not possible to predict de novo allergenic potential (EFSA GMO Panel, 2022c). The research activities need to include basic research to understand the pathophysiology underlying sensitisation to food allergens and how this translates to clinical allergy.
- Impact of processing, determining the effect of processing on protein safety (e.g. cooking, dehydrating, enzyme treating, heating, freezing, drying) that potentially influences toxicity, immunotoxicity, allergenicity, nutrition and digestibility.
- Omics technologies, exploring the usefulness of -omics techniques in risk assessment for specific cases. For example, the interpretation of -omics data and how it can be incorporated in risk assessment needs to be determined.
- Complex cases in risk assessment (e.g. membrane-bound proteins, computationally designed proteins or GM crops expressing many NEPs), requires the evaluation of alternative risk assessment strategies to address the complexities and uncertainties, by implementing a strategy like the one defined in Figure 3 (see Section 3.3).

Advancing allergenicity and toxicity risk assessment will require active engagement and encouragement of experts across diverse disciplines, including immunology, molecular biology, bioinformatics, toxicology, protein chemistry, epidemiology and clinical research. Expertise in areas such as predictive modelling, in vitro and in vivo testing, basic research addressing the pathophysiology underlying sensitisation and -omics technologies would be essential for addressing existing gaps. Additionally, the recognition by the European Commission of the critical need for improved methods and dedicated funding for basic research is necessary to drive meaningful progress and foster innovation in this field.

TABLE 1 Summary table of the recommendations for further research or for addressing methodological advancements. The table summarises all identified gaps and uncertainties, along with the proposed developmental and research activities to address them. It is organised according to the priorities outlined in the text, balancing urgent needs with limited resources (i.e. short-term; short- to mid-term; mid- to long-term; and additional areas).

Gaps and/or uncertainties in the protein safety assessment	Developmental and research activities needed
1. SHORT-TERM core priorities: HoSU (History of Safe Use)/read-across	
HoSU/Read-across lacks criteria and definitions	Develop consensus definitions, meaningful criteria and databases of safe-use proteins
2. SHORT- to MID-TERM core priorities: in silico	
Outdated in silico methods & databases	Modernise tools, create fit-for-purpose databases, validate criteria for structural/functional similarity
In silico methods not validated/insufficient alone	Integrate in silico with experimental data; establish robust validation/regulatory acceptance pathways
3. MID- to LONG-TERM core priorities: in vitro	
Lack of standardised in vitro gastrointestinal digestibility tests	Update methodologies (e.g. INFOGEST), define interpretation criteria, demonstrate relevance in case studies
Limited validated in vitro/New Approach Methodologies (NAMs)	Validate more in vitro NAMs; integration into the weight-of-evidence
Additional priorities	
In vivo testing not protein-specific/overuse of 28-day test	Develop targeted, protein-focused in vivo protocols meeting 3Rs principles/Hypothesis driven
Exposure role not clearly defined in current frameworks	Define exposure assessment strategy; research dose–response; establish thresholds for toxicology/allergenicity
Post-Market Monitoring (PMM) lacks guidance on design/use	Design PMM systems to confirm exposure and reduce pre-market uncertainty; integrate with other systems
De novo allergen sensitisation, mechanisms unclear	Conduct basic research into immunological mechanisms underlying sensitisation
Unknown effects of food processing on proteins	Research processing effects on toxicity, allergenicity, digestibility, nutrition
Omics tools are hard to interpret in risk context	Explore how to incorporate and interpret -Omics data in compositional/protein safety assessment
Complex cases challenge current methods	Develop alternative risk assessment strategies tailored for membrane-bound, designed proteins, multi-Newly Expressed Proteins GMOs

3.4.3 | What factors could be considered for supporting scientific assessments with early and continuous engagement with key stakeholders contributing in this way to building trust and confidence in scientific outcomes?

Scientific complexity is constantly increasing, leading to more and new data and the incorporation of new methodologies for the scientific assessments and innovative product production. By bringing together the right expertise and knowledge from different organisations, we can better address scientifically complex or novel topics. EFSA operates in a complex ecosystem, made of multiple actors with different needs and interests. Engagement and collaboration with different actors throughout the risk assessment process helps to better navigate through this complexity. Intensifying engagement opportunities at various stages of the assessment process will bring stakeholders closer together, enabling fruitful collaboration driven by shared objectives fostering transparency, mutual understanding and trust between the scientific community and stakeholders. This approach has clear benefits in terms of defining problem formulation, developing assessment methodology, data needs, understanding of production processes, communication and information sharing.

Experience gained and new developments made in protein safety assessment methodologies calls for the need to complement and review current practices and explore alternative methods. Assessing the toxicity and allergenicity of alternative proteins encounters several challenges, which require continuous improvement and collaboration with various stakeholders. This involves clearly defining the purpose and the scope of the scientific assessments, co-developing methodologies and ensuring data sharing. In this frame, early involvement of key stakeholders and the establishment of effective engagement strategies facilitates an iterative review with all interested parties e.g. through dedicated calls for evidence, calls for data, public consultations and ad hoc feedback mechanisms.

For this scientific opinion on protein safety assessment of GMOs specifically, EFSA carried out several initiatives to seek input from various actors since the start of its assessment. Following the publication of the scientific mandate, EFSA initially outlined the areas of work for the development of its scientific opinion during an open webinar targeting all interested stakeholders with expertise in protein safety assessment. The aim was to better frame the complexity of the scientific context, identify challenges, reflect on current methodologies and look for future opportunities in the field. To ensure a broad collection of relevant input from knowledgeable stakeholders on the matter, EFSA simultaneously launched a survey to gather contributions on

issues relevant to frame this scientific complexity (see Annex B). The questions covered possible strategies to adopt for the assessment of NEPs, new or alternative methodologies to use for protein safety assessments or possible data gaps.

An open and ongoing dialogue continued throughout the assessment process via some regular engagement channels such as webinars, feedback surveys, public consultations and technical meetings. Industry representatives, national authorities, universities, (non-governmental) organisations and other stakeholders have actively participated to the various engagement windows. The engagement approach also included updating stakeholders at various dedicated forums with Member States (e.g. through EFSA's Advisory Forum meetings) and allowed for more stakeholders' feedback to refine the problem formulation in more technical meetings with the industry or Member State networks. Specific input was requested on current assessment practices to identify possible new protein safety assessment approaches to complex GMO cases, identify complementary or alternative testing strategies to current applicable methodologies and learn about any latest research or methodological development needs to fill the gaps in this field.

EFSA has organised an online event to introduce the main driving elements of the document and the key chapters subject to public consultation. After the public consultation, EFSA continued engaging with its stakeholders, allowing for an open discussion with public consultation respondents. Following adoption, EFSA will organise an informative session targeted at all interested parties to present the final adopted output while providing the rationale behind the scientific conclusions and recommendations.

4 | CONCLUSIONS

In conclusion, the principles and guidelines for protein safety assessment of GMOs, established two decades ago, were designed for simpler products with ample testing possibilities and relatively few NEPs. However, the evolving complexity of GMO products, including NEPs that are inherently challenging to test and evaluate (e.g. membrane-bound proteins, transcription factors), highlights the need to revise current practices. This changing product landscape triggered the importance of learning from past assessments and integrating emerging methods as alternative or complementary strategies to improve future risk assessment frameworks. Hence, this scientific opinion aims to illuminate the key challenges, gaps and uncertainties in this field and to explore potential avenues for addressing these issues in the near to medium term. It is not intended to provide prescriptive guidance on specific in silico or in vitro tools for future use.

The GMO Panel performs the safety assessment of GMOs following its guidance documents and Codex Alimentarius (2003–2009), which have effectively evaluated NEPs in single/stacked event GMO applications. However, the field is evolving, and more complex and challenging cases require the modernisation of the current strategy. To achieve this goal, there is a need to promote a dialogue with stakeholders and experts in the field and establish a consensus on the use of new methodologies in the safety assessment. While the GMO Panel considers that the weight-of-evidence approach is the best strategy for the assessment of NEPs, new tools and methodologies as outlined in this document could be incorporated to improve the risk assessment.

The first step in an improved strategy for the risk assessment should be the HoSU, read-across and phylogeny defining the type of data required and waive the need for specific in vitro or in vivo studies. Additionally, although it is unclear when two proteins can be considered similar in terms of their safety in humans and animals, the GMO panel's new and increasing experience with advanced methodologies for structural prediction and analysis (e.g. ongoing risk assessments[15]), will facilitate the assessment. To update the current risk assessment approach, a consensus is needed on:

(i) the criteria to define HoSU/read-across and related terms;
(ii) the selection of appropriate comparator proteins known to be safe; and
(iii) the best practice to establish sequence similarity/homology between proteins.

The second step is to modernise in silico, in vitro and in vivo methods based on scientific advances and accumulated experience making the safety assessment of proteins more proportionate and effective.

For in silico analysis, advanced bioinformatic tools based on sequence alignment principles can be used to make comparisons of NEPs with known allergens, toxins or 'safe' proteins. However, criteria for developing fit-for-purpose databases are still lacking. Furthermore, best practices for using in silico tools in risk assessment are not established, especially for new methods employed in protein structural analysis.

For in vitro methodologies, significant advances over the past decade have been made in developing standardised and harmonised in vitro GI protocols considering physiological conditions. However, criteria are needed to identify digestion fragments relevant to assessing the allergenicity and toxicity risks, considering abundance, persistence, molecular size, etc.

For in vivo testing, toxicology studies were originally designed for simple chemicals and remains unclear whether they are useful for the assessment of proteins. Thus, the GMO Panel recommends a stepwise approach in which animal studies are required only when potential hazards have been predicted or when required information cannot be provided (Figure 3). Additional specific targeted in vivo studies needed to complete the assessment should be selected following a hypothesis-driven approach. The GMO Panel is committed to reducing and replacing animal studies whenever possible, while consistently ensuring a high level of consumer protection. This also aligns with the EC roadmap for phasing out animal testing.[10]

[15] https://open.efsa.europa.eu/questions/EFSA-Q-2019-00572; https://open.efsa.europa.eu/questions/EFSA-Q-2019-00394.

Exposure should be more effectively integrated into the overall safety assessment of NEPs especially because the primary focus now is on hazard identification, with limited consideration of exposure.

Post-market monitoring could also be considered in cases requiring risk characterisation, following a hypothesis-driven exercise and be supported by an exposure assessment tailored to specific intended uses. This is important to confirm, over time, the safety assumptions made during the pre-market risk assessment, similarly to the monitoring required for nutritionally enhanced crops in nutritional assessments.

Addressing de novo allergen sensitisation, effect of processing on protein safety, –omics approaches and complex case evaluations, are all critical components of the overall risk assessment of NEPs.

The GMO Panel considers that engagement of advanced expertise across diverse disciplines as well as increased funding support by the EC are critical needs for improving the protein risk assessment, and that achieving these goals requires continued efforts to establish consensus and define criteria for key elements of risk assessments, prioritising those mentioned above.

5 | DOCUMENTATION AS PROVIDED TO EFSA

a. Proposal for a self-task mandate of the EFSA GMO Panel to develop a scientific opinion reflecting on current practice, challenges and future opportunities of protein safety in GMOs. October 2023. Submitted by the Chair of the ESA GMO Panel.[16]
b. Acceptance of the self-task mandate of the EFSA GMO Panel to develop a scientific opinion reflecting on current practice, challenges and future opportunities of protein safety in GMOs. October 2023. Submitted by EFSA Executive Director.[16]

ABBREVIATIONS

2D	two-dimensional
3Rs	replacement, reduction and refinement of animal testing
AAD	amino acid deaminases
AAD-12	aryloxyalkanoate dioxygenase-12
AHAS/ALS	acetohydroxyacid synthase/acetolactate synthase (large subunit)
AI	artificial intelligence
AL	amyloid light-chaiN
ALI	air-liquid interface
AMY797E	thermostable alpha-amylase enzyme
AP	application number
APC	antigen-presenting cell
APH4	aminoglycoside phosphotransferase
APH4	hygromycin B phosphotransferase
APR	aggregating protein regions
AtHB17	Arabidopsis thaliana Homeobox 17
ATP	adenosine triphosphate
Barnase	ribonuclease protein
Barstar	barnase inhibitor protein
BI	bioinformatics
BBM	brush border membrane
BLAST	basic local alignment search algorithm
CompERA	comparative analysis and environmental risk assessment
Cry	parasporal crystal
CspB	cold shock protein B
DB-ALM	DataBase service on ALternative Methods
DIAAS	Digestible Indispensable Amino Acid Score
DMO	dicamba monooxygenase
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FF	food/feed
FT_T	fatty acid thioesterase T
GAP	generalised aggregation proneness
GAT	glyphosate acetyl transferase
GDT	global distance test
GI	gastrointestinal

[16] https://open.efsa.europa.eu/questions/EFSA-Q-2023-00664?search=protein+safety.

GM	genetically modified
GMO	genetically modified organisms
GMO Panel	Panel on Genetically Modified Organisms
GO	Gene Ontology
GOX	glyphosate oxidoreductase
GRAS	generally recognised as safe
HD-Zip II	homeodomain-leucine zipper II
HLA	human leucocyte antigen
HoSU	history of safe use
HPPD	hydroxyphenylpyruvate dioxygenase
IgE	immunoglobulin E
IP	insecticidal protein
IR	Implementing regulation
LC–MS	liquid chromatography–mass spectrometry
LDH	lactate dehydrogenase
MC	molecular characterisation
MSA	multiple sequence alignment
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAMs	new approach methodologies
NCBI	National Centre for Biotechnology Information
NEP	newly expressed protein
NIF Unit	Nutrition and Food Innovation Unit
NMR	nuclear magnetic resonance
NPTII	neomycin phosphotransferase II
NRU	neutral red uptake
OECD	Organisation for Economic Co-operation and Development
OoC	organ-on-a-chip
PAT	phosphinothricin acetyltransferase
PDB	Protein Data Bank
PMI	phosphomannose isomerase
PMM	post-market monitoring
PTM	post-translational modification
QPS	qualified presumption of safety
RMSD	root mean square deviation
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SSM	structural superimposition of macromolecules
TDO	triketone dioxygenase
TM	template modelling
ToR	Term of Reference
TTC	threshold of toxicological concern
Vip	vegetative insecticidal proteins
WG	Working Group
WHO	World Health Organization
WoE	weight-of-evidence
ZMM28	maize gene involved in various biochemical and physiological processes

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REQUESTOR

EFSA self-task activity

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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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Annexes

ANNEX A

Protocol of the activity

ANNEX B

Results of a survey on protein safety launched by EFSA

ANNEX C

Outcome of the public consultation

ANNEX D

NEPs assessed or under assessment by the GMO panel

ANNEX E

Examples of enzymes assessed by EFSA

ANNEX F

Protein aggregation

ANNEX G

Immunogenicity assessment