RESEARCH PROJECTS

Review of Methods for the Detection of Allergens in Novel Food Alternative Proteins

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Novel food, defined in the retained food regulations as 'food that has not been consumed to a significant degree by humans in the EU before 15 May 1997 (EU Regulation 2015/2283),' is expected to be used to an increasing extent and in a range of food products within the coming decade aiming to feed the growing global population in a more sustainable manner and comprising nutritious forms of protein. The safety aspects of novel foods must be thoroughly assessed before they can reach the market, and this includes assessment of allergenicity risks. FSA have funded this project to review current knowledge of the allergenicity of insect protein and precision fermentation (PF) protein and identify future research needs in this field. This report comprises an unbiased critical literature review (Section 1) coupled with consultations with experts and stakeholders in the field (Section 2) and focussing specifically on insect protein as well as milk and egg protein produced by precision fermentation. Section 3 comprises testing data to determine whether current allergen testing ELISA kits can be used to detect allergens in novel food. The literature review addresses allergenicity considerations of these novel proteins. The expert consultation (allergen testing, innovative methods, protein biochemistry) aimed to gain information on the potential allergenicity of PF and insect proteins, cross-reactivity (insect/shellfish allergens) and to identify knowledge gaps and challenges to recommend future strategies.

1. Executive summary

Safety assessment of novel food proteins is paramount, and allergenicity risk assessment is a critical part of this assessment. Allergenicity prediction is very challenging, and current methodologies involve a weight-of-evidence approach where bioinformatics plays a central role by comparing the sequences of novel proteins to those of known allergens (Naegeli et al., 2017) to give an indication of potential allergenicity. Fernandez *et al.* (Fernandez et al., 2021) have proposed a bottom-up approach for allergenicity evaluation which places greater emphasis on curated allergen sequence databases including additional criteria that would be applied to

rank the clinical relevance of allergens. These criteria may include data such as their proven ability to trigger allergy, the potency of the allergen or the prevalence in the population, among others.

While precision fermentation (PF) is under development for milk and egg protein, it is clear that the allergenicity of PF egg and milk proteins is not being considered separately to that of their dairy equivalents. The potential effect of PF technology on the allergenicity of the protein is not considered in the literature. Future focus should include the fact that PF protein products will differ depending on factors including the specific gene sequence used, microorganism species, culture media and other processing conditions. This may impact the allergenicity of each product.

Regarding the allergenicity of insect, there are a great many studies in this area and there are benefits from considerations of cross-reactivity from pan-allergens. The vast majority focus on predictive analysis of allergenicity, and the potential for *de novo* sensitisation from insect protein must be understood. Perhaps, particularly with reference to the new consumption of insect protein by Western populations, more data regarding allergenicity are required. As discussed throughout this review, much more data are needed relating to human oral exposure, either by clinical trial or case studies for consumers exhibiting symptoms of allergy to novel foods to understand their allergenicity. More definitive data is required regarding the effect of processing on allergenicity with total protein hydrolysis showing potential to reduce and even to remove allergenicity but at the expense of destroying the functional properties of the proteins.

Innovators developing PF and insect protein products are aware of risks relating to allergenicity of these products. PF innovators intend to label foods by declaring the presence of milk allergen. The cross-reactivity of allergens between crustacea and insects is broadly acknowledged among insect protein producers and products are being labelled as posing an allergen risk to crustacea-sensitive consumers.

Research which has demonstrated the possible transfer of allergens from insect feed to the final product, either from the insect gut or from adherence to the insect body must also be considered to manage risk and such transfer has been demonstrated in a small study herein. The method comparison study and the in-house preliminary work in this project go some way to determining the suitability of commercially available ELISA kits, originally developed for allergen detection in ‘conventional’ foods, to detect proteins in products containing milk proteins produced by PF and products containing insect protein. Further work is of course required to validate the performance of these kits in a comprehensive manner.

2. Abbreviations

- ACAF Advisory Committee on Animal Feedingstuffs
- AOP Adverse Outcome Pathway
- β-LG Beta-lactoglobulin
- BSF Black Soldier Fly
- DBPCFC Double-Blind, Placebo-Controlled Food Challenge.
- ELISA Enzyme-Linked ImmunoSorbent Assay
- FAO Food and Agriculture Organisation of the United States
- FARA Food Allergenicity Risk Assessment
- FERA Fera Science Limited
- HDM House Dust Mite
- Ige Immunoglobulin E
- IPFF International Platform of Insects for Food and Feed
- IUIS International Union of Immunological Societies
- Kg kilogram
- LOD Limit of Determination
- LOQ Limit of Quantitation
- mg milligram
- MSDS Material Safety Data Sheet
- NDA EFSA Panel: Nutrition, Novel Foods and Food Allergens
- PF Precision Fermentation
- RCT Randomized Controlled Trial
- >SOC Level detected was above the scope of the standard curve
- SWP Silkworm Pupa
- TAC Threshold of Allergic Concern
- Tm *T. molitor*
- WHO World Health Organisation

- w/w Weight-for-weight
- µg microgram

3. Background to the project

Novel food, defined in the retained food regulations as 'food that has not been consumed to a significant degree by humans in the EU before 15 May 1997 (EU regulation 2015/2283),' is expected to be used to an increasing extent and in a range of food products within the coming decade aiming to feed the growing global population in a more sustainable manner and comprising nutritious forms of protein. The safety aspects of novel foods must be thoroughly assessed before they can reach the market, and this includes assessment of allergenicity risks. FSA have funded this project to review current knowledge of the allergenicity of insect protein and PF protein and identify future research needs in this field. This report comprises an unbiased critical literature review (Section 1) coupled with consultations with experts and stakeholders in the field (Section 2) and focussing specifically on insect protein as well as milk and egg protein produced by precision fermentation. Section 3 comprises testing data to determine whether current allergen testing ELISA kits can be used to detect allergens in novel food. The literature review addresses allergenicity considerations of these novel proteins. The expert consultation (allergen testing, innovative methods, protein biochemistry) aimed to gain information on the potential allergenicity of PF and insect proteins, cross-reactivity (insect/shellfish allergens) and to identify knowledge gaps and challenges to recommend future strategies.

A review of the literature in this area was first conducted relating to the allergenicity of insect protein and protein produced by PF. The latter refers to protein produced by microorganisms which have been genetically modified to express milk or egg proteins within a fermentation chamber. No information was identified in the public domain regarding the allergenicity of PF egg or milk. A contributing reason for this may be that this form of production of milk and egg protein as food ingredients is in its infancy. From discussion with stakeholders in this area, it seems likely that another contributing factor is that allergenicity of these products is assumed due to the presence of milk or egg, which are known regulated allergens, and the assumption is that these products will be labelled as containing these allergens. At present, there is no evidence that consideration is being given to potential changes in allergenicity due to novel production processes. This is likely to be since PF protein production tends to be conducted by innovators with a biotechnology background rather than with a food manufacturing or food safety background. Important considerations regarding the allergenicity of PF proteins are

discussed in Sections 2a and 2b relating to expert consultation with Professor Clare Mills, Professor of Allergology, University of Surrey and Dr Bert Popping, FOCOS Consulting.

Since little literature was available regarding allergenicity of PF proteins, the majority of the allergenicity considerations reviewed in this project relate to insect protein. Insect protein is known to elicit allergy in some individuals amongst populations with high intakes of edible insect (for example in Asia and some parts of North Africa) and insect protein is also known to comprise certain proteins which are established pan-allergens shared (at least in part) with crustacea.

In addition to discussing pan-allergens between crustacea and insects, in the Series of EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) reports on the safety of insects as a Novel Food pursuant to Regulation (EU) 2015/2283 (Turck, Bohn, et al., 2021a; Turck, Castenmiller, et al., 2021a; Turck et al., 2022), the panel also raised concerns regarding whether an insect feed substrate comprised of another known allergen, for example gluten, could pass to the consumer and elicit an allergic reaction. One of the later elements of this current project by Fera Science Limited (Fera) is to determine if gluten can be detected in insects which have been raised on processed white bread. This testing will go some way towards determining whether residues of the rearing substrates (gluten in this case) can be present and detected in insect protein for food. The potential for food substrates to be vehiculated by insects to the final food product has been flagged by other authors (Frigerio et al., 2020; Mancini et al., 2020) and is discussed in more detail in this review. Although this substrate transfer may depend on the type of substrate and the insect species used and will also depend on insect gut purging and insect washing practices, this testing will start addressing the question of whether the substrate used to rear the insects needs to be considered for the allergenicity risk assessment of insect proteins.

4. Section 1. Literature review

4.1. Search parameters for the literature review

The following databases of peer-reviewed literature were searched for applicable articles.

- Web of Science^{TM} Core Collection
- BIOSIS Citation Index^{SM}
- Current Contents Connect®

- FSTA® - the food science resource
- KCI-Korean Journal Database
- MEDLINE®
- SciELO Citation Index
- Zoological Record®

Articles published between 2013 and 2023 were the focus of this literature search. The search terms applied and the number of articles captured by each search are detailed in Appendix 1. Grey literature was also interrogated using a general web search with Google and then by searching of the food trade journals and food regulatory web sites for the terms allerg* and precision fermentation OR insect. Journals included:

- The Dairy Site journal
- The Grocer journal
- Food Manufacture journal
- Beverage Daily journal
- Food Safety News journal
- Seafood Source journal
- All About Feed journal
- The Beef Site journal
- ADHB journal
- Food Standards Agency website

4.2. Introduction to novel foods

It is predicted that the World's population will reach 9.7 billion by 2025 (United Nations Department of Economic and Social Affairs, Population Division, 2022) and there is a serious challenge to ensure access to safe and nutritious food in a sustainable manner, protecting natural resources and using a non-infinite land area. Alternative forms of protein are being investigated (new protein sources and the expansion and diversification of existing ones) to fill the shortfall in predicted food availability, with an additional aim of creating more sustainable food sources. Some of these proteins will be classed as novel foods and therefore, they must be authorised before they can be placed on the market. A novel food is defined as '*food that had not been consumed to a significant degree by humans in the EU before 15 May 1997*' (EU regulation 2015/2283), meaning

that the foods do not have a history of consumption in the EU in the timeframe since the first regulation came into force. The definition of novel foods includes foods that may be currently consumed but are processed using new technologies.

Briefly, some of the forms of protein under investigation to feed the increasing global population are:

- Plant-based protein products (including algae, pulses with protein extracted in novel manners)
- Cultivated meat and seafood (cultivated from cells)
- Protein produced by fermentation
    - Produced by biomass fermentation (cultivated microorganisms form the product, such as the Quorn mycoprotein)
    - Produced by precision fermentation (yeast/microalgae/ bacteria/microfungi are genetically modified to express a protein naturally expressed in a plant or animal e.g. for milk/whey and egg protein).
- Insects

This review focusses on the potential allergenicity of two forms of novel foods, namely PF products (milk and egg) and insect protein products.

Allergenicity of novel foods is of critical concern. Precision fermentation has been used for decades but for the preparation of food additives rather than for foods, for example, for the production of rennet for cheese making. While the development of PF milk and egg as a novel food is in its infancy and allergenicity can only be conjectured due to a lack of data (see consultations in Section 2a and 2b), the incidence of allergic reaction to insects is well-documented and a major consideration for this novel form of protein.

The role of insects in human nutrition is becoming a more vital topic in satisfying the increasing demand for sustainable sources of protein. Previous studies into insect allergenicity have typically focused on occupational health or inhalation allergies, but with the growing prevalence of insects available to buy for human consumption it is vital that studies into food safety, and within that the allergenicity, are carried out. The starting point for this area of research has been the cross-reactivity of known pan-allergens such as arginine-kinase and tropomyosin which are

well characterised as allergens in crustaceans. Further to this, the potential for uncharacterised proteins in insects to cause IgE-mediated reactivity is an area which requires research to ensure consumer safety.

In 2013, the Food and Agricultural Organization (FAO) designated insects as future protein resources to support the feeding of the growing global population in a sustainable manner (FAO, 2013). Various insect resources including Coleoptera, Hymenoptera, Orthoptera, and Lepidoptera are consumed across the globe, either as whole insects or as insect derivatives, with many comprising beneficial nutrition including high-value protein, crude fat, carbohydrate, fibre, fatty acids, minerals and vitamins profiles and health-benefitting antioxidants. Four insect species are currently authorised for consumption in the EU (frozen, dried and powder forms of *T. molitor* larva (yellow mealworm); frozen, dried and powder forms of *Locusta migratoria* (migratory locust); and frozen, dried and powder forms of *Acheta domesticus* (house cricket) (Weimers, 2023) and *Alphitobius diaperinus* (lesser mealworm). The European Food Safety Authority (EFSA) is currently carrying out safety assessments on a further eight insects. These insects are rich in high value protein, fat and fibre and provide a range of vitamins and minerals including omega-3 and omega-6, depending on species. Compared to current agricultural protein production practices, insect farming has less impact on deforestation and soil fertility reduction, water requirement and water pollution (Oonincx, 2017) and benefits from relatively low emissions of greenhouse gases and ammonia compared to traditionally farmed cattle, poultry, fish, and seafood (Poma et al., 2017). Insects also benefit from a high feed conversion efficiency (van Huis et al., 2013), short life cycles and high reproduction rates (Sun-Waterhouse et al., 2016) and can be fed on a wide range of foods, including by-products from food processing and high-impacting waste streams. Insects therefore show the potential to provide larger populations of consumers with sustainable and nutritious food and are becoming increasingly interesting as an alternative nutrient source, in food and feed. In addition to human food, the European Commission (EC) recently regulated the production, transport, and storage conditions of insect-based meal allowed in aquafeed, pig and poultry feeds for certain insect species.

UK regulation and EU regulation 2015/2283 requires that novel foods do not, on the basis of the scientific evidence available, pose a safety risk to human health. The majority (13) of the 14 UK and EU regulated food allergens are proteins. The requirement by the retained EU regulation 2015/2283 for novel foods being considered as safe for consumption requires risk assessment including anti-nutritional factors and allergy, and the benefits of the novel food must outweigh the risks. According to EFSA regulation, a complete allergenicity risk assessment is required for novel

foods, considering route of exposure (e.g., oral), dose of protein exposure, protein properties (e.g., physicochemical properties) and effect on the human immune system (Parenti et al., 2019).

As reviewed recently, (Kopko et al., 2022), changes in dietary habits as a result of globalization can contribute to new exposure to allergens. The introduction of traditional foods from one region or country to another can result in an increase in prevalence of allergy to the new food or even in a new allergy. New protein sources, such as plant-based, meat alternatives and edible insects, while promising to be sustainable protein sources, can also potentially expose allergic and non-allergic consumers to new food allergens. Despite technological and industrial advances, the safety aspects of alternative proteins remain poorly researched. It is currently unknown why certain proteins are allergens, whereas others are not and what characteristics drive a protein to provoke an allergic immune response.

4.3. Weight-of-evidence assessments

As reviewed by Kopko (Kopko et al., 2022), to assess the allergenicity of new protein sources, current guidance relies mainly on a weight-of-evidence allergenicity risk assessment which focuses on the impact of a single protein (or at most a few proteins) on individuals with pre-existing allergies and the potential for sensitisation and cross-reactivity. This approach protects individuals with known existing allergies, but it is not applicable for the prediction of risks of *de novo* (new) sensitization and allergies to novel proteins. Weight-of-evidence approaches were originally developed to assess the safety of new foods such as genetically modified foods, whereby multiple criteria are considered in combination to predict safety risks. The potential allergenicity of a protein is typically determined to be suspect based on its structural similarity to previously studied allergenic proteins. Weight-of-evidence methodology considers relevant literature, taxonomic relationships between the food species with other species containing known allergens, degree of protein sequence homology with known allergens, total protein content and robustness to heat, low pH, simulated gastrointestinal digestion and protein transport across the intestinal barrier as well as its implication on epithelial permeability. As stated by Kopko and collaborators (Kopko et al., 2022), many of these tests are not validated for their predictive capability and there is concern that allergenic risks of proteins may therefore be over- or underestimated (B. Remington et al., 2018).

The main form of immune-mediated allergic reactions to foods is linked to IgE formation against food allergens. In this case, an abnormal immunological response causing an allergic reaction occurs when IgE antibodies are produced and bind to the ingested proteins. Consequently, IgE is bound to the surface of effector cells (basophils or mast cells), and

histamine, leukotrienes and cytokines, are released in the allergic response, either at the sites of allergen contact (e.g., mouth and intestine) or elsewhere in the body, if allergens cross the mucosa barrier into the blood circulation (Valenta et al., 2015).

For a comprehensive assessment of allergenicity risks of novel foods, two different types of allergenic potential must be considered:

(i) cross-reactivity due to similarities between the structure of a protein in a novel food and the allergenic counterpart in other foods (pan-allergens) – this may pose a risk for individuals already sensitised to the other foods.

(ii) *de novo* sensitisers - proteins eliciting allergenic reaction for the first time in an individual. This may be due to exposure to new proteins or, potentially, to the presence of novel allergenic structures within a protein which might occur as a consequence of processing or of new ways of producing a protein such as PF.

Allergic reactions due to cross-reactivity may be induced on the first dietary exposure to a novel food or shortly thereafter. This has been evidenced with insect allergens which are highly similar to those in crustacean, mealworm and house dust mite (HDM) allergens, due to their close taxonomic relationship. Crustacean- and HDM-sensitive consumers must therefore consider this is relation to insect foods. Alternatively, in the case of novel allergens, reactions in predisposed individuals occur after repeated exposure following primary sensitisation (production of specific IgE) induced by the initial exposure to the allergen.

For allergenic risk assessment, cross-reactivity can be assessed using sera from already sensitised donors, while for *de novo* sensitisation this is not possible. Therefore, investigating novel foods for the presence of pan-allergens is a comparatively straight-forward challenge. However, there is a significant challenge in terms of well-defined and predictive cellular assays or animal models to address *de novo* sensitisation. As reviewed by Mazzucchelli and collaborators (Mazzucchelli et al., 2018), for novel foods for which no previous allergic sensitisations are reported, there is a lack of patient sera for use in IgE-based *in vitro* assays although sera are available to screen for cross-reactivity.

Detection of the allergen by human IgE and reaction to skin prick tests would add a higher level of knowledge to a weight-of-evidence approach but they do not necessarily confirm clinical relevance. This type of test, in general, only indicates the presence or absence of sensitisation to a known protein, but not clinical allergy (Frati et al., 2018; Jeong et al., 2017; B. Remington et al., 2018). Studies on the simulated digestion of protein, a method on which strong focus has been placed in the past to predict allergenicity, have also shown that not clear evidence exists that protein digestibility always correlates with allergenicity (K. Verhoeckx et al., 2019).

Therefore, while this review mentions a range of studies which consider allergenicity, only those which involve oral exposure are conclusive. Double-blind placebo-controlled oral food challenge studies are the only way to determine whether humans will demonstrate allergy to novel foods. For this reason, in this review, we would like to highlight that data generated in human exposure studies forms the gold standard approach, while we acknowledge that other approaches are more amenable to researchers and contribute data to a weight-of-evidence approach.

4.4. Allergenicity of insect protein

Insects are a regular part of the diet of 2 billion consumers across the globe (Mason et al., 2018; Quintieri et al., 2023) with farms especially in India, China, Thailand, South Korea, Japan, and is also consumed in Mexico, Brazil, and some African regions. Over 1,900 species of insect are consumed worldwide (FAO, 2013). However, insects are novel foods for Western countries with three species receiving much of the focus (*T.molitor, Gryllodes sigillatus, Schisocerca gregaria*).

As discussed by Pan and co-workers (Pan et al., 2022), there are many questions about the safety of using insects as food, which involve the following three food safety risks: microbiological (for which freezing and cooking/heat treatment can help to mitigate the risk), chemical (mainly heavy metal contamination but also hormones and pesticide pollutants from the environment, which can be mitigated during feeding of the insects by aiming to prevent or minimise the accumulation of toxins, drugs, and other contaminants from the external environment), and issues relating to allergenicity, which is an important consideration related to the widespread use of insect proteins.

Discussing the allergenic potential of yellow mealworm, the Series of EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) reports on the safety of insects as a novel food pursuant to Regulation (EU) 2015/2283 published 2021-2023: Safety of frozen and dried formulations from whole yellow mealworm larva (Turck, Bohn, et al., 2021b; Turck, Castenmiller, et al., 2021a) reached the conclusion that the novel food is safe for most consumers although likely to cause allergenic reactions in some individuals. The group stated that further research should be undertaken on allergenicity relating to insects.

Edible insects are possible new allergen sources (FAO, 2021; Turck, Bohn, et al., 2021b), Many edible insects share equivalent proteins to protein allergens in phylogenetically-related species, referred to as pan-allergens and widely distributed in the different groups of arthropods (mites, insects, crustaceans) and molluscs. Additional allergens which are highly specific to insects alone have also been reported (Barre et al., 2021).Consumption of insects or insect-based products has the potential to induce allergic

reactions in individuals sensitised to crustaceans (Broekman et al., 2016). Other arthropods such as house dust mites have also been reported to be involved in co-sensitisation/cross-reactivity with edible insects (Beaumont et al., 2019; Broekhoven et al., 2016; K. C. M. Verhoeckx et al., 2014). Proteins from molluscs and nematodes also share homology with some pan-allergens found in insects, although for the major mollusc pan-allergen, tropomyosin, the degree of homology with the insect counterparts is around 55–65%, which is lower than the homology that insect tropomyosins share with crustaceans (65–85%) or with mites (75–80%). Cross-reactivity between molluscs and mites or insects has been reported, however this seems to be in conjunction with other allergens, with tropomyosin playing a minor role (S. L. Taylor, 2008). *T.molitor,* the yellow mealworm (Tm), is one of the best characterised insects in terms of allergenicity and multiple proteins have been extracted from Tm that belong to known families of insect allergens and that have homologous proteins in other taxonomic groups (Garino et al., 2020). Oral food challenges have been performed with Tm in humans (Broekman et al., 2016) demonstrating that most shrimp-allergic patients are allergic to mealworm. The data from these oral food challenges were used in the above-cited work of Garino and co-authors to conduct a quantitative allergenicity risk assessment of food products containing yellow mealworm based on the concept of food allergenicity risk assessment (FARA). This approach analyses the data from allergic individuals to statistically calculate threshold doses of allergen able to elicit a reaction in a given proportion of them (shrimp allergic subjects in this case). According to the study, yellow mealworm-based food products represent a major risk for individuals allergic to crustaceans to develop a reaction after consuming a dose lower than an average serving size. Moreover, the study identified other consumer groups that might be at risk based on the information available. The authors discuss that this type of risk assessment provides a tool to better describe the problem and facilitate risk management. However, this is only an early case study, and more clinical data for insect species intended for food are required to be able to apply the FARA concept widely.

4.5. Insect allergens

The main allergens in insects are tropomyosin and arginine kinase. However, many others are proposed, and clinical trials are required for their confirmation. In their recent review, Ma *et al.* (2023) state that potentially, the allergenicity level of tropomyosin depends on the amino acid sequence homology between the different tropomyosin isoforms in different insect species, as well as the amount of allergen-specific IgE present, the route by which the allergen is introduced and the dose of

allergen. The authors argue that more studies regarding the epitopes and secondary protein structures of insect allergens are needed in order to elucidate how these are related to potential allergenicity.

Marzoli and co-workers reviewed articles relating to case reports of humans with allergic reaction following ingestion of *B. mori* (domestic silk moth) and the effect on allergenicity of thermal processing treatments, identifying 16 articles or case reports (Marzoli et al., 2022). Three articles relating to allergic symptomatology following ingestion were identified, with two patients from USA and one from China. Complete details were not included but one of the foods was canned *B. mori* pupae while another was oil-fried pupae. Thirteen separate cases of severe anaphylaxis in Chinese citizens were also reported following ingestion of *B. mori*. To date, five *B. mori* allergenic proteins have been registered by the WHO/IUIS (International Union of Immunological Societies) Allergen Nomenclature Sub- Committee (https://allergen.org/), namely: Bom m 1 (arginine kinase), Bomb m 3 (tropomyosin), Bom m 4 (30 kDa hemolymph lipoprotein), Bom m 5 (30 kDa lipoprotein) and Bomb m 6 (hemolymph lipoprotein 3).

In a systematic review of human studies relating to the effects of insect consumption on human health, Cunha *et al.* reviewed 14 studies (shortlisted from 896 identified studies, of which 14 met the eligibility criteria which included steps taken to reduce bias of data) relating to allergenicity of insect protein. As independent reviewers, we too highlight that the small number of studies of allergenicity deemed suitable for further review by these authors indicates the lack of suitable data regarding the allergenicity of insect protein. Nine of the 14 studies were randomized controlled trials (RCT) and five studies addressed allergenicity: one was a patient study case relating to food-induced anaphylaxis to *T. molitor* (Beaumont et al., 2019), one was an epidemiological study that assessed exposure to insect allergens by skin pricks (Ndlovu et al., 2021) (but with no control group so is not discussed further here), and three were cross-reactivity studies involving patients' sera exposure to extracts containing insect allergens which are covered elsewhere in this review (K. C. M. Verhoeckx et al., 2014), (Kamemura et al., 2019) and (Lamberti et al., 2021).

4.6. Cross-reactivity eliciting allergy to insect protein

The known cross-reactivity due to similarities between crustacean and insect proteins is helpful in that insect foods are currently labelled as being of potential allergy concern for crustacean-sensitised consumers. While the insect food industry advocate for testing to this regard to characterise species-specific allergens and help determine which insect products (if any) different crustacean-sensitised consumers could eat (Section 2d), this

labelling approach is a useful manner to help protect these consumers until more data are available regarding crustacea-insect pan-allergens. Concerns relating to pan-allergy have been raised by the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) reports on the safety of insects as a Novel food pursuant to Regulation (EU) 2015/2283 including (Turck, Castenmiller, et al., 2021a; Turck et al., 2022).

Cross-reactivity is defined as an immune-mediated phenomenon of an IgE antibody recognizing, binding to, and inducing an immune response to similar allergenic molecules (Cunha et al., 2023). These authors remarked that, in addition to direct sensitisation, a crucial aspect of insect allergies that needs to be considered is IgE cross-reactivity between insects, crustaceous, and HDM. Cross-reactivity in individuals allergic to crustaceans and house dust mites can also be triggered after insect ingestion. These pan-allergens, are proteins with highly conserved sequences and structures across many different species (Pfaar et al., 2014), (Romero et al., 2016), (Lange & Nakamura, 2021; G. Taylor & Wang, 2018). Taken from a recent article (Marchi, Wangorsch, et al., 2021), a simplified classification of some of the species for which evidence of cross-reactivity has been exhibited is shown below in Figure 1, although confirmatory patient data is not available in all cases.

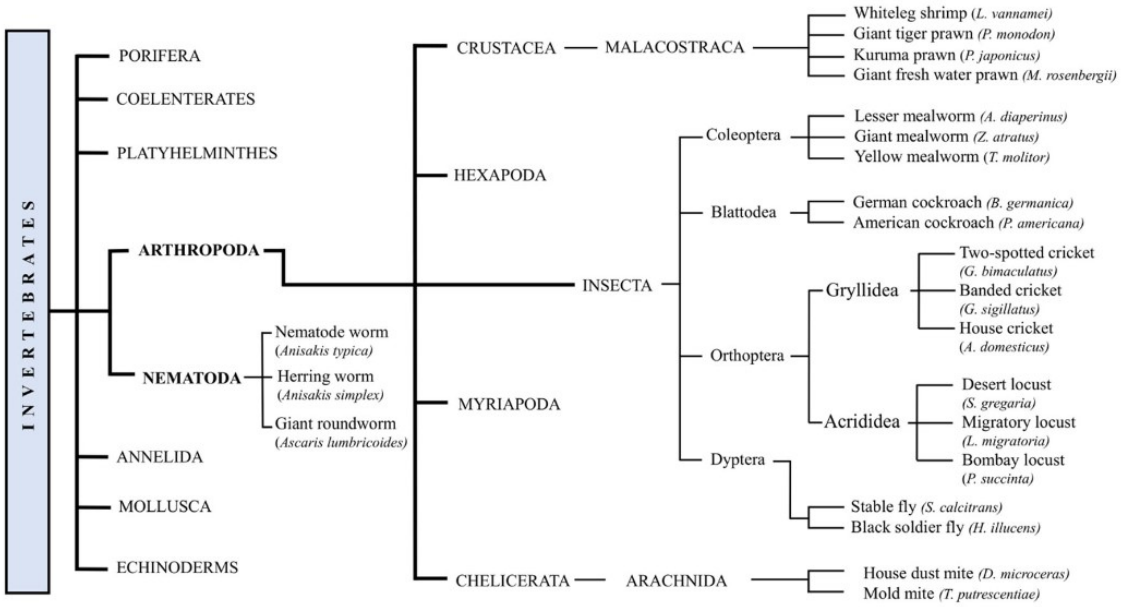

Figure 1. Simplified classification of the insect species for which evidence is available of pan-allergy (Taken from Marchi, Wangorsch, et al., 2021).

Focussing first on articles for which human exposure studies have been the focus, authors have noted the pan-allergy of tropomyosin and arginine kinase allergens cross-reacting between shrimp and mealworm (Broekman et al., 2016) and house dust mite and mealworm (Beaumont et al., 2019).

In work by Pali-Schöll and collaborators (Pali-Schöll, Meinlschmidt, et al., 2019), cross-reactivity data were presented. Protein was extracted from adult *Locusta migratori* (migratory locust) and larvae of *T.molitor* (Yellow mealworm), with the insect legs and wings removed from the main body and extracted separately. Extracts were profiled by SDS-PAGE and immunoblotting was also performed using sera from patients sensitive to crustacean allergy (n=3), house dust mite allergy (n=8) and stable fly allergy (n=1). Immunoblotting of extracts of mealworm, shrimp, cricket legs, cricket body, desert locust legs, body and wings, stable flies or the processed extracts of migratory locust were performed. Additional shrimp allergic patients (n=5) underwent skin prick testing for migratory locust and for mealworm. Cross-reactivity of crustacean IgE to house dust mite IgE and *vice versa* was observed. Cross-reactivity for crustacean-sensitive patients to mealworm was also observed, with tropomyosin the presumed allergen (SDS-PAGE mass profiling analysis). There was cross-reactivity between crustacean-allergic patients and migratory locust, with α -amylase as the presumed allergen which is also cross-reactive between house dust mite and mealworm-sensitive patients. The data showed that crustacean- and house dust mite-allergic patients are at risk for cross-reactions to desert locust and house cricket. Crustacean-allergic patients also intensively reacted to stable flies, necessitating a warning if crustacean-allergic patients would consume flies as edible insects. A stable-fly-allergic patient also showed IgE-binding to legs of cricket, desert locust and migratory locust legs and therefore is probably at risk for cross-reactions to these insects, not only via inhalation (including when handling pet feed such as insect-based reptile feed) but also when ingesting insects such as migratory locust. There was also cross-reactivity between house dust mite-sensitive patients to the leg proteins of desert locust and legs and wings of house cricket. Evidence was also obtained suggesting that some patients may be more sensitive to allergens occurring in the legs rather than in the main body of insects. With the effects of processing on insects not fully understood, the paper's authors call for further studies in the research of enzymatic hydrolysis and heating on the allergenicity of insect proteins.

4.6.1. Tropomyosin

A study of 15 shrimp-sensitive patients in a double-blind, placebo-controlled food challenge (DBPCFC) (Broekman et al., 2016) showed that there is risk of mealworm allergy in patients with shrimp allergy. The study comprised a 47:53 ratio of male: female patients and covered the age range of 19-69 years with a median age of 38. None of the patients had knowingly consumed mealworm proteins although most had inhalant allergies to house dust mite. Thirteen of the 15 patients were confirmed as sensitive to mealworm during this DBPCFC study. This work suggests a link between shrimp, house dust mite and mealworm sensitivity among patients. Most of the shrimp-allergic patients (14 of 15) were sensitized

to either mealworm tropomyosin and/or arginine kinase, and 13 of 15 reacted positive in DBPCFC with mealworm, of whom 11 of 15 developed moderate to severe symptoms. IgE binding was also noted in other proteins which were not identified in this study. The severity of mealworm allergy varied between mild (oral allergy) and moderate (urticaria and gastrointestinal symptoms) to severe (dyspnea). The authors called for more oral food challenge studies, noting that the individual threshold for objective symptoms was 216 mg of mealworm – lower than the serving size of products already on the market.

A study by Beaumont and co-workers (Beaumont et al., 2019) , described a severe food anaphylaxis being induced by the mealworm (*T.molitor*) in a 31-year-old French man sensitised to HDM but not to crustacea (who has safely consumed crustacea several times before and since the event), who consumed one cooked mealworm larva. It was determined by proteomic analysis of his sera that he showed IgE response to hexamerin, tropomyosin and possibly to α-amylase which shares much structural homology with that of HDM, and tubulin, a recognised potential pan-allergen. It must be highlighted that this case study is of a single patient and alcohol and pharmaceutical interactions may have played a part in the reaction to mealworm and it is possible that the patient may have been unwittingly pre-sensitised by consuming mealworm previously, rather than HDM pan-allergy causing the reaction. The authors call for strict regulation concerning food labelling of insect protein.

As discussed below, other authors have reported evidence from non-oral exposure that tropomyosin is a pan-allergen, with several pan-allergenic proteins reported in house dust mite and mealworm in *in vitro* studies (K. C. M. Verhoeckx et al., 2014), tropomyosin pan-allergy in crustacea and mealworm (Broekhoven et al., 2016), tropomyosin in cricket and shrimp (Kamemura et al., 2019), tropomyosin in silkworm and crustacea (Jeong et al., 2016), and tropomyosin in HDM-, insect- and crustacean-sensitive patient sera (Leni et al., 2020). In addition to these *in vitro* studies, structural prediction analysis has also been used to emphasise the likelihood of tropomyosin and arginine kinase pan-allergy in crustacea-, mollusc-, insect- and HDM-sensitive patients (Barre et al., 2019).

The group of Verhoeckx (K. C. M. Verhoeckx et al., 2014) studied proteins extracted in water from yellow mealworm and identified proteins which are known allergens in other species (shown in parentheses) including cationic trypsin (e.g. mites), arginine kinase (mites, crustaceans, insects), ovalbumin-like protein (chicken eggs), α-tubulin (mites) and α-amylase (mites, insects). Proteins found in urea extracts included cationic trypsin, ovalbumin-like protein and tropomyosin (mites, crustaceans, insects). Studying sera from patients allergic to crustaceans and HDM Der p 10 (tropomyosin), the authors concluded that there is a realistic possibility

that patients allergic to house dust mites will react to food containing yellow mealworm protein. Only proteins which were already characterised from other species could be addressed in this work as the protein database does not include more than a few yellow mealworm sequences. Additionally, the authors highlighted the need to challenge patients with yellow mealworm extract by means of a DBPCFC, which were later performed (Broekman et al., 2016), (K. Verhoeckx et al., 2016) and are discussed above.

Three edible mealworm species (*T. molitor, Zophobas atratus* and *A. diaperinus*) were studied by Broekhoven and collaborators (Broekhoven et al., 2016). The study used both raw and processed proteins to challenge patient sera pools from tropomyosin-allergic patients. A strong match between the composition of tropomyosin found in mealworm and other insect species and crustaceans was observed by mass spectrometric analysis. The effect of processing was that frying samples decreased allergenicity (although possibly some protein escaped into oil), while boiling had less of an impact (again leeching of proteins into the boiling water was noticed) and this reduction in allergenicity is speculated to result from comparatively higher temperatures used in frying. The authors call for oral food challenge experiments to provide more information on the allergenicity of mealworm proteins and a wider range of food processing methods, to include fermentation and hydrolysis.

A recent study (He, Li, et al., 2021) identified potential allergens from silkworm based on protein reactivity to IgE from individuals allergic to the insect. The authors used larva, pupa, moth, silk, slough and faeces of domestic silkworm (*Bombyx mori*), extracted proteins and conducted gel electrophoresis (one and two-dimensional) followed by Western blots using pooled blood sera from allergic patients. Following protein identification by proteomics workflow on the reactive bands / spots, they searched the sequences of the 45 potential allergens identified against the AllergenOnline database and obtained percentage of homology to known allergens. Tropomyosin isoform 6, identified in larva and pupa, has 87.9% identity to allergen Aed a 10 of *Aedes aegypti*, a mosquito that causes allergic reactions by biting. According to Aalberse (Aalberse, 2000), identity matches > 70% in the entire protein length between 2 allergens usually means cross-reactivity, whereas matches > 50% mean potential cross-reactivity. Hence, tropomyosin isoform 6 of *B. mori* is likely to cause cross-reactivity with that of *A. aegyptis*. Tropomyosin is a known invertebrate pan-allergen across members of the insect and crustacean groups. The study found > 50% identity to other known allergens including paramyosin, voltage-dependent anion-selective channel isoform and aldehyde dehydrogenase of insect species, malate dehydrogenase of skin-fungus *Malassezia furfur* and, interestingly, glyceraldehyde-3-phosphate dehydrogenase Tri a 34 of *Triticum aestivum* (wheat). The study found

7, 16, 17, 4, 3, 4 IgE- binding allergens in larva, pupa, moth, silk, slough and faeces, respectively, concluding that silkworm may be an important cause of allergic reactions by allergen cross-reactivity, although further work would be needed to explore if these observations correlate with real clinical cross-reactivity.

H. illucens (Black soldier fly, BSF) larvae are considered a promising sustainable source of nutrients, and numerous studies have focused on protein production from BSF. Safety aspects of BSF, including allergenicity were reported recently (Bessa et al., 2021). The allergen investigation in this study aimed to determine if tropomyosin, arginine kinase and myosin (three main crustacean cross-reactive allergenic proteins) were identified in BSF, and whether the feed and method of killing had an influence on the allergens detected. The authors conducted untargeted proteomics to identify peptides within these proteins and used homology to the Drosophila sequences to identify peptide targets in BSF. Tropomyosin was the most abundant protein identified, followed by arginine kinase and very low levels of myosin. They analysed the relative levels of tropomyosin and arginine kinase in samples produced from three different rearing substrates (broiler-based, brewers grain by-product and cereal grain by-product) and subjected to either blanching or freezing during processing. They found that tropomyosin was higher in the blanched samples whereas arginine kinase was equal or higher in frozen samples. There was no evidence of a trend related to the diet. The authors discussed that the increased relative levels of the allergenic proteins under certain treatments may be due to structural changes that make them easier to extract, but that the clinical significance of this is unknown.

Tropomyosin and arginine kinase have been suggested as the most dominant allergens responsible for cross-reactivity between shrimp and insects (Broekman et al., 2016; Broekman, Knulst, de Jong, et al., 2017).The work by Broekman analysed the cross-reactivity of sera from fifteen shrimp allergic patients (also mealworm allergic or sensitised) to proteins extracted from multiple insect species. They tested IgE binding by immunoblot and by basophil activation test (BAT), showing that all the sera reacted to multiple protein bands, most of them including a band that LC-MSMS identified as containing tropomyosin and arginine kinase. The study also analysed sera from primary mealworm sensitised individuals, and these showed reactivity with fewer proteins and more variability across the various insect proteins tested. The pan-allergens tropomyosin and arginine kinase were barely recognised by these sera, in line with previous data from the authors that showed larval cuticle protein plays a principal role in primary mealworm allergy. This indicates that, although tropomyosin and arginine kinase are pan-allergens that may be responsible for allergy cross-reactivity, as in the case of shrimp allergy, there may be other insect proteins that can cause species-specific allergies. Thus, the data presented

in that study suggest that shrimp allergic patients are probably at risk of food allergy to a variety of insects, whereas primary mealworm allergic subjects are unlikely to have the same risk.

In silico studies for the bioinformatic assessment of the degree of homology between insect proteins and known crustacean allergens are often used to help predicting allergenicity. This form of study supports the prediction of the likelihood of cross-reactivity of insect allergy for crustacean-sensitised patients. Varunjikar and collaborators (Varunjikar et al., 2022) described a shotgun proteomics approach for detection of insect species in food and feed. They used analytical flow liquid chromatography combined with high-resolution mass spectrometry (Thermo Q Exactive Orbitrap) and bioinformatics for species identification using spectral libraries and for protein identification using search engines and databases. This approach was proposed as a more amenable option for application in regulatory laboratories compared to the typical non-targeted methods using nano-flow chromatography and high specification mass spectrometers. For detection of known insect allergens, they created a database of 48 allergenic proteins from the Allergen Nomenclature website and searched the data acquired using the approach above (as well as Q-TOF data for comparison) against that database. They detected 37 of those proteins, 32 of them in both datasets. Known allergen such as arginine kinase or tropomyosin were consistently detected across all five species tested. The authors concluded that the combination of standard MS instruments commonly available in control laboratories (Q Exactive being considered one of them by the authors) with freely available databases provides a tool for the untargeted testing of insect food and feed samples, including the identification of known allergens such as tropomyosin.

The novelty of the approach resides in the use of normal flow liquid chromatography, which is an advantage over the use of the less affordable high-resolution nano-flow MS instrumentation. The scope of the study does not cover specific information relevant to allergen detection methods, such as sensitivity, selectivity, potential matrix effect, etc. Although briefly mentioned in the text as a possibility, the transfer of food allergens from the insect substrate to the final insect meal was not investigated.

Silkworm pupa (SWP) is an important cause of food allergy in East Asia where silkworm pupa is commonly consumed after boiling. Crustacean tropomyosin is known for its heat-stable allergenic nature. Jeong and co-workers (Jeong et al., 2017) studied the role of tropomyosin in silkworm allergy. This study showed that silkworm tropomyosin shares 73-92.3% sequence homology with other allergenic tropomyosins (by BLAST bioinformatics amino acid sequence alignment). SWP tropomyosin exhibited up to 92.3% sequence identity to Chi k 10, a chironomid tropomyosin, followed by 90.1% to Per a 7 and 89.4% to Bla g 7 (two

cockroach tropomyosins). SWP tropomyosin also shared 78.5-81.0% identity with mite tropomyosins (Der p 10, Der f 10, Tyr p 10, and Lep d 10) and 73.5% identity with shrimp (Pen a 1) and crab (Hom a 1) tropomyosins. Since shellfish tropomyosins account for over 80% of IgE reactivity in shellfish-sensitive patients, the authors investigated the potential cross-reactivity of these tropomyosins with SWP tropomyosin. Eleven of the 15 SWP-sensitive patients exhibited IgE cross-reactivity to shrimp and crab tropomyosin, of which, six reacted to recombinant tropomyosin from SWP also, indicating potential cross-reactivity between the different tropomyosins. However, these patients showed no symptoms when consuming crab or shrimp. Also, the IgE titre to tropomyosin was very low, and the authors remarked that these tests may not always reflect clinical relevance. The authors noted that a high prevalence of IgE reaction to SWP is detected in Koreans but without clinical symptoms. Therefore, they suggest that there is an urgent need for component-resolved diagnosis of SWP allergy based on molecular studies to improve diagnostic tests for SWP allergy.

Using *in silico* and *in vitro* analyses, the group of Leni (Leni et al., 2020) found that tropomyosin was the most abundant insect allergen identified in lesser mealworm and black soldier fly with cross-reactivity to known crustacean allergens. Cross-reactivity was also observed between the *Gryllus bimaculatus* (cricket) and shrimp in a dose-dependent manner (Kamemura et al., 2019). These authors found a protein of approximately 40 kDa reacted with the positive, but not with the negative sera patients for shrimp-specific IgE and was identified as a high molecular weight (HMW) tropomyosin which is present in both species. Work by Barre and collaborators (Barre et al., 2018) showed parallels between the structure of potential allergens, including muscle proteins (such as tropomyosin) and enzyme proteins (such as arginine kinase) in different species including crustaceans, molluscs, insects and HDM. This work concluded by highlighting the similarities between the tertiary structure of the allergenic proteins and therefore the likelihood of cross-reactivity in sensitised individuals, calling for clear labelling on products to warn consumers. This work does not look at any *in vivo* studies or those using patient sera to draw these conclusions.

4.6.2. Arginine kinase

As discussed above, arginine kinase along with tropomyosin, is considered as one of the major allergens in crustacea and insects (Broekman et al., 2016). In non-oral exposure studies, a study by Mattison and co-workers (Mattison et al., 2020) reported arginine kinase as a pan-allergen for cockroach-, shrimp- and termite-sensitive patients. In this study, the cross-reactivity of Formosan subterranean termite (*Coptotermes formosanus*) arginine kinase with serum IgE from both cockroach- and shrimp-allergic

patients was demonstrated through positive responses in immunoblot testing. The authors reported the recognition of recombinant arginine kinase from *C. formosanus* by nine out of twelve shrimp and/or cockroach patient serum IgE. The authors warned that termite arginine kinase may potentially contribute as an allergic sensitising agent in geographic areas infested with termites. This work does not present any data from *in vivo* testing, such as oral food challenges or look at the effect of heating or fermentation on the allergenicity of these proteins.

In another study, arginine kinase was found to cross-react between *Gryllus bimaculatus* (cricket) and shrimp (Srinroch et al., 2015). Liu and co-workers (Z. Liu et al., 2009) investigated the interaction between arginine kinase from American cockroach (expressed as recombinant protein) and sera from silkworm sensitive patients by *in vitro* testing. The authors concluded that arginine kinase from the B. mori silkworm is a major allergen and cross-reacts with its orthologue in the American cockroach, a species that is already exploited both as food for humans and feed for livestock in China. As mentioned earlier, Barre also noted the theoretical pan-allergenicity of arginine kinase by structural elucidation studies (Barre et al., 2018).

4.6.3. Other potential cross-reactive allergens in insect protein

In addition to tropomyosin and arginine kinases, other possible allergens which cause cross-reactivity have been reported in the literature, again without confirmation by human oral exposure studies. As reviewed by Marzoli and colleagues (Marzoli et al., 2022), cross-reactivities found in non-clinical studies have been reported in the literature with *B. mori* proteins. Araujo (Araujo et al., 2020) speculated about the similarity between *B. mori* vitellogenin and that of *Galleria mellonella*. Zuo (Zuo et al., 2015) did not find Bom m 9 (a silkworm protein which is accumulated in the insect haemolymph) showing cross-reactivity with moth or cockroach in immunoblot inhibition assays; however, the amino acid sequence of this protein had a high similarity with the microvitellogenin of the moth, *Manduca sexta*. Given this sequence homology, it would be interesting to confirm from clinical studies if moth Bom m 9 is in fact an allergen. Zhao (Zhao et al., 2015) observed a homology between the *B. mori* chitinase and a protein from the mite *Dermatophagoides farinae* (24.8 % amino acid identity and 57.4 % similarity), as well as between *B. mori* paramyosin and a protein from the mite *Dermatophagoides pteronyssinus* (62.8 % amino acid identity and 90.0 % similarity). Jeong and co-workers (Jeong et al., 2016) found amino acid similarities among a 27 kDa glycoprotein in *B. mori* and those from other Lepidoptera species, such as *G. mellonella*.

4.7. Insect-specific allergens

Other allergens have been proposed for insects. For example, a study using a proteomic- and bioinformatic-based approach, investigated edible insects such as *B. mori* (silkworm), *Acheta domesticus* (cricket), *L. migratoria* (African migratory locust), *T.molitor* (yellow mealworm), *Rhynchophorus ferrugineus* (red palm weevil), and *Zophobas atratus* (giant mealworm beetle) (Barre et al., 2021). Potential insect-specific (or highly specific) allergens were identified including chemosensory protein, the fatty storage protein hexamerin, and odorant- or pheromone-binding proteins. To a lesser extent, other proteins such as apolipophorin III, the larval cuticle protein, and the receptor for activated protein kinase, also exhibited a rather good specificity for edible insects. These proteins, that are apparently missing or much less represented in other groups of arthropods, molluscs and nematodes, share well conserved amino acid sequences and very similar three-dimensional structures.

The study identified certain peptides within the allergenic proteins analysed that are unique to BSF and that can be used to differentiate BSF from crustaceans.

4.8. The effect of processing on the allergenicity of precision fermentation protein and insect protein

One of the major questions concerning food allergenicity relates to the properties that result in one food showing a higher allergenicity compared to another food. It is known that processing can affect the level of allergenicity of proteins. Ma and collaborators (Ma et al., 2023) highlight that it is important to note that new active sites can be generated during processing which could create new allergens or altered allergenicity. This is a relevant area for consideration in terms of processing of both insect and PF proteins.

Processing techniques can alter the physicochemical protein structure of an allergen, often causing protein unfolding and aggregation. The presence of other food ingredients such as fats and sugars, processing-induced mixing and shearing and the time and temperature of processing will affect the patterns and kinetics of protein denaturation and aggregation. This will alter the way in which it is digested, absorbed and the way the immune system will respond to it. The allergen may consequently be recognised to a different degree once processed. Similarly, due to the alteration of the allergen structure, the level at which testing methods such as ELISA detect allergens in food after processing often alters, due to related changes in epitope recognition and binding of the ELISA antibodies to the allergen.

A review of studies on the stability and allergenicity of processed foods (Besler et al., 2001) gathered evidence obtained for various foods of animal and plant origin subjected to various processing methods. The evidence gathered showed that processing can either have no effect, reduce or even increase allergenicity, depending on the structural modifications induced on the allergen. The molecular changes induced by processing may lead to the inactivation or destruction of epitope structures, the formation of new epitopes or better access to native epitopes, thus changing the allergenic activity of the food. Some of the examples described in the review show that the reactivity of a protein to IgE from allergic patients or to specific antibodies does not always correlate to allergenic potential. For example, lactic fermentation of cow's milk reduced the ELISA response of whey proteins to rabbit antibodies against alpha- and beta-lactoglobulin by over 99%. However, the allergenicity potential of the whey as determined by skin tests was only slightly reduced (Jedrychowski, 1999).

Types of processing include thermal processing, hydrolysis, high pressure treatments and microwave-based processing. Production by fermentation can also be considered as a form of processing, with potential to alter the allergenicity of the protein being expressed. While processing can enhance protein extraction and preservation and provide benefits such as nutraceutical attributes, depending on the conditions applied to the foods or ingredients, changes to the conformation of proteins can occur which can have an impact on their allergenicity as well as on nutritional quality. The characteristics of the processed protein allergen will influence the inherent potency of the allergen itself. Critically, in terms of safety, the impact on allergenicity cannot easily be predicted. This section of the review, therefore, discusses the effect of processing on the allergenicity of the novel foods in question. As highlighted above, given the lack of research efforts relating to precision fermentation safety risk assessment, data are lacking for these matrices. However, data are available for insect protein.

The effect of processing on allergenicity was considered during the series of EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) reports on the safety of insects as a Novel food pursuant to Regulation (EU) 2015/2283 published 2021-2023: Safety of dried, frozen and ground *L. migratoria.* The panel acknowledged that processing treatments such as heating and hydrolysis appear to impact the level of allergenicity and conclude that further research should be undertaken (Turck, Castenmiller, et al., 2021b).

In order to reduce the allergen risks associated with certain foods, many authors have investigated the effect of processing on foods including insect protein, using a range of processing technologies and a range of study types such as *in silico* predictive analysis, studies of resistance to simulated

gastrointestinal digestibility, *in vitro* studies and *in vivo* studies. As highlighted by many authors and in the expert consultation discussed in Section 2b, the interpretation of data and the effect of processing on allergenicity in consumers can only be confirmed by human oral exposure studies. As described above, the data from other forms of study, even those based on *in vitro* IgE exposure or skin prick testing cannot categorically predict allergenicity. Studies using small animal models rather than human models show a lack of reproducibility to human studies of allergenicity, as discussed previously. While discussing a range of studies which interrogate the effect of processing on allergenicity, this review places most gravitas on those which involve human exposure studies. Since the literature concentrates on the effect of processing on insect protein and not on precision fermentation, the latter is considered in Sections 2a and 2b which relates to the expert consultations.

4.8.1. Hydrolysis

In a review by Ribeiro (Ribeiro et al., 2021) the authors discuss the evidence that tropomyosin is a cross-reactive allergen between crustaceans and insects, although this is potentially different for mealworm species. In agreement with information highlighted elsewhere in this report (expert consultation in Section 2b) the authors review the effect of enzymatic hydrolysis followed by heating to reduce the IgE reactivity of some insect species, and in some cases eliminate it. As argued by both parties, hydrolysis of the allergen protein is the only manner in which allergenicity can be reduced with confidence at present. also call for greater reporting of case studies of allergic reactions from insects. This reporting is expected to increase with the increased consumption of insects in Western diets.

While case studies and human challenge studies are lacking, other research techniques substantiate the claim that hydrolysis reduces (and may have the potential to eliminate) allergenicity of insect protein Leni and collaborators (Leni et al., 2020) employed proteomic assessment along with computational models and IgG- and IgE-immunoblotting experiments to consider the sequence similarity of the relevant potential allergenic proteins were *in silico* identified, chiefly among them tropomyosin. *In vitro* testing using serum from crustacean-allergic patients revealed that once the proteins were hydrolysed by the protease enzyme subtilisin from *Bacillus licheniforms* at 60 °C (1% concentration protease), only partial immunoreactivity was retained for black soldier fly, whereas with lesser mealworms, there was a total loss of immunoreactivity. These results are highly promising for creating safer insect products for consumption by the general population, however further studies need to include *in vivo* testing, such as oral food challenges, to confirm the results presented here.

In vitro studies by Boukil (Boukil et al., 2020) demonstrated that the structure of mealworm proteins from mealworm powders prepared from fresh larvae and then roasted at 107 °C can be modified using a combination of high hydrostatic pressure (HHP) and enzymatic hydrolysis. The studies applied hydrolysis with Alcalase® (60°C for 120 minutes, pH 8.5) and/or with pepsin (40C for 240 minutes, pH 2) in combination with and without high pressure treatment. This affects the *in vitro* digestion of allergenic proteins to alter their conformation, and reduced allergenicity has been observed in *A. domesticus* (house cricket), *Schistocerca gregaria* (desert locust), and *T.molitor* (yellow mealworm), (Pali-Schöll, Verhoeckx, et al., 2019). The degree of hydrolysation by pepsin was not increased by application of high pressure during hydrolysis although a pre-treatment prior to hydrolysis did enhance in vitro hydrolysation by pepsin. Interestingly, the degree of hydrolysis was not evident on the SDS-PAGE gels, suggesting that very small protein fragments (less than 10 kDa) may have resulted from hydrolysis but then migrated off the end of the gel (this protein size is outside of the tolerance of the gel) and hence were not visualised. Boukil and co-workers. also stated that other authors reported different degrees of hydrolysis in similar studies and suggested that the effectiveness of high-pressure treatment depends on specific parameters such as substrate: enzyme ratio, pressure level and treatment duration. They suggested that irreversible protein aggregation occurring during the commercial-scale production of mealworm meals could decrease the efficiency of HHP and enzymatic hydrolysis. Cleavage specificities are also important, since Alcalase® has broad specificity, hydrolysing most peptide bonds. It preferentially hydrolyses those containing aromatic amino acid residues whereas pepsin is more specific and cleaves peptide bonds following Phe or Tyr residues, as well as other hydrophobic amino acids. These authors also stated that further research on native mealworm proteins extracted from fresh larvae and subject to minimal heat treatment is necessary to improve the combination of HHP and enzymatic hydrolysis (Boukil et al., 2020). Other authors have also suggested that enzyme cleavage specificity can be important with higher degrees of hydrolysis observed when applying broad-specificity enzymes compared to other more specific commercial enzymes (trypsin, Neutrase, papain, and pepsin) (Dai et al., 2013).

To further the research of their peers, Hall and Liceaga (Hall & Liceaga, 2021) focussed their *in vitro* research on the 37 kDa tropomyosin crustacean-insect pan-allergen, using tropical banded *G. Sigillatus* (cricket) and shrimp (used as a reference) as a source of the protein. The crickets were digested with Alcalase enzyme either with convection heating in a water bath or using microwave-assisted hydrolysis. Both hydrolysates were then pasteurised prior to extraction of the tropomyosin. In desk-based mass spectrometric and bioinformatic analysis, allergens from various species of shellfish, insect and nematode showed over 60% homology

with cricket tropomyosin. In the lab-based work, which was then coupled with bioinformatic analysis, proteins excised from the cricket matched the structure of tropomyosin, as expected, but also to other proteins such as paramyosin, arginine kinase and myosin heavy chain. Shrimp tropomyosin showed higher matches to *Cryptotermes secundus* (termite) and *Blattella germanica* (cockroach) tropomyosin. In contrast, cricket tropomyosin had no matches to cockroach tropomyosin but did show higher matches with *Teleogryllus emma* (field cricket) tropomyosin. The peptides also closely matched those of *A. domesticus* (house cricket). These observations were limited by the lack of databases and characterised insect proteome. Nonetheless, the study identified the proteins near 37 kDa in molecular mass. The likely immunoreactive protein in this study was tropomyosin and/or its fragments and the overall IgE reactivity was suppressed with microwave-assisted enzymative proteolysis, probably due to cleavage of the epitope region. Importantly, new allergenic peptide fragments were not formed during this treatment. The authors stated that protein folding or cross-linking reactions during convection heating likely masked the epitope region, which resulted in retained tropomyosin reactivity when hydrolysed using convection heating. The authors concluded that microwave heating along with enzymatic proteolysis could be effective methods for lowering the concentration of active tropomyosin regions when formulating insect-based food products, although evaluation is required for each individual insect species. The authors highlighted that there is a need to establish specific concentration levels that trigger an allergenic response.

Pali-Schöll and co-workers (Pali-Schöll, Meinlschmidt, et al., 2019) conducted hydrolysis studies to determine the effect of enzymatic hydrolysis on allergenicity. Four different commercially available food-grade enzymes (Alcalase, Neutrase, Flavour zyme, and papain) were used to digest migratory locust (*L. migratoria*) protein extracts according to manufacturers' guidelines (50 °C and pH 7.0 for each enzyme). These enzyme preparations are commonly used in the food industry for the production of protein hydrolysates. Responses of crustacean-allergic patient IgE were only seen on immunoblots and skin prick tests for the untreated extracts rather than in the enzymatically treated extracts. With the effects of processing on insects not fully understood, the authors called for confirmatory *in vivo* oral food challenge testing and the effect of enzymatic hydrolysis and heating on the allergenicity of insect proteins.

Some authors (Hall et al., 2018; Yang et al., 2023) warned that *in vitro* studies have suggested that enzymatic treatment under conditions with a degree of hydrolysis of less than 50% may expose additional epitopes in cricket tropomyosin allergen, potentially leading to an increase in

allergenicity. Again, this would need to be investigated by human exposure studies. As discussed in the expert consultation in Section 2b, high degrees of hydrolysis are required to reduce allergenicity.

4.8.2. Thermal processing

Edible insects are often consumed after thermal processing to improve palatability and microbiological safety. These processes (industrial and domestic) may alter the protein structure and may affect cross-reactivity through the masking/unmasking of pre-existing epitopes or even through the generation of new epitopes, previously not accessible to the patient's IgE (Wal, 2003).

There is some clinical evidence from case studies that thermal processing can reduce the allergenicity of insect protein. It is recognised that the characteristics of the protein allergen will influence the inherent potency of the allergen itself (Kopko et al., 2022). The following aspects have been identified as being important: structural features and characteristics of 2D and 3D epitopes, functional properties of the protein, including properties and activity of enzymes and the influence of post-translational modifications. This has been exemplified in paediatric case studies of Singaporean children as researchers observed increases in peanut allergy, partly due to the exposure to nuts processed by roasting rather than boiling (Liew et al., 2013). It must be highlighted though that, while changes occur due to the boiling of peanuts, it is suspected that these changes reflect the initiation of hydrolysis of the allergen by boiling. In cases where thermal treatment causes the allergen to hydrolyse (rather than simply to alter the protein structure), there is clinical evidence of reduced allergenicity (for further details, see expert consultation in Section 2b).

Broekhoven (Broekman et al., 2016) studied the effect of processing and *in vitro* digestion in the cross-reactivity of three species of mealworm with crustaceans and HDM allergens. They analysed the insect proteins that reacted to IgE from patients and identified tropomyosin, α-amylase, hexamerin 1B precursor and muscle myosin. Heat processing and *in vitro* digestion reduced, but did not eliminate, HDM or tropomyosin IgE cross-reactivity. The authors concluded that individuals allergic to HDM or crustacea might be at risk when consuming mealworms, even after heat treatment.

Research aimed at increasing our knowledge relating to the effect of thermal processing on the allergenicity of insect protein suggests that this is a promising area to focus on with future exposure studies. *In vitro* and skin prick test studies (Pali-Schöll, Meinlschmidt, et al., 2019) suggest that the cross-reactivity and allergenicity of insect proteins can be reduced by processing treatments. This study involved different extraction methods, enzymatic hydrolysis, and thermal processing of insect protein extracts

to determine the effect of processing on allergenicity of migratory locust proteins. The effects were measured by IgE-binding assays with sera from crustacean- and HDM patients. The results showed that the processing treatments employed, reduced significantly the IgE-binding capacity of migratory locust extracts (for most proteins to zero or thereabouts on the immunoblot) and there was no reaction in skin prick tests.

Others (Jeong et al., 2016) however, found increased IgE binding after heat treatment of silkworm pupae protein extracts. In parts of Asia, boiled and seasoned silkworm pupae are a traditional snack food, and allergy cases are common. The study employed SDS-PAGE and immunoblotting using IgE from 15 silkworm-sensitive patients and protein identification by proteomic analysis in an attempt to identify novel heat-stable allergens in *B. mori*. They identified a 27-kDa glycoprotein, which they produced as recombinant protein to conduct *in vitro* studies. The data showed that proteins above 100 kDa (unidentified) and the 27 kDa glycoprotein increased their reactivity to IgE from patients. The authors conjectured that glycation or aggregation of protein by heating (100°C for 5 minutes) may create new IgE binding epitopes, which may explain the increased IgE reactivity. *In vitro* IgE binding to the recombinant 27-kDa glycoprotein was detected in one third (5) of the sera from the 15 silkworm-sensitive subjects. *In vivo* studies would be required to determine if allergenicity was truly increased by thermal processing.

In vitro studies by other authors have suggested that allergenicity of insect protein may be reduced by thermal processing (Fernandez-Cassi et al., 2019; Hall & Liceaga, 2021; He, Li, et al., 2021; Lamberti et al., 2021; Phiriyangkul et al., 2015).

He and collaborators (He, He, et al., 2021) studied the effect of heat (including autoclaving at 120°C), enzymatic hydrolysis and acid-alkali treatment on the molecular characteristics, structure and allergenicity of silkworm pupa protein extract (SPPE). The authors highlight that further research is required to understand the conformational and line epitope changes in silkworm pupa allergens, which will help in understanding the mechanisms to reduce silkworm pupa allergenicity. Following the treatment of the SPPE, allergenicity levels were artificially assessed by the following: measuring histamine release from cultured human basophil cells, assessing IgE-binding capacity by ELISA and IgE binding capability by Western blot. Protein integrity was monitored using SDS-PAGE, and spectroscopy techniques. Heating altered the secondary and tertiary structure of the proteins at 60 °C and above, and allergenicity was reduced. At 100 and 120 °C, the proteins were degraded which further contributed to significant reduction in allergenicity, especially at 120 °C. These results show that heat, enzymatic hydrolysis and acid treatment of SPPE might reduce its allergenicity. The authors did not acknowledge the effect of

the increased atmospheric pressure to which the samples which were autoclaved to achieve temperatures of 120 °C were inherently subjected. The samples treated at 120 °C appear to have lost both the integrity of the allergenic protein at 25-33 kDa and also, critically, the IgE-binding capacity. We conjecture that the effect of increased pressure may also have contributed to the reduction of allergenicity of samples treated at 120 °C compared to those treated at 100 °C. Boukil and colleagues (Boukil et al., 2020) noted the effect of high pressure in apparently reducing the allergenicity in hydrolysed insect protein.

In *in vitro* and *in silico* studies (Lamberti et al., 2021) investigated the effect of boiling and frying on the IgE cross-recognition of patients allergic to shrimp, HDM and mealworm towards five edible insects. They concluded that there was IgE cross-reactivity with the five insect species, but this cross-reactivity was affected in different ways depending on the specific protein and the thermal treatment used. The insects were considered raw, boiled for 5 minutes at 100 °C or fried for 3 minutes at 180°C in sunflower oil prior to grinding to a powder and protein extraction. Raw and boiled samples were extracted in phosphate-buffer (PBS). In the case of fried samples, they were powdered and mixed with hexane to avoid interference from residual frying oil prior to extraction in PBS. Protein was then extracted both with water and with urea and analysed by protein electrophoresis and to immunoblotting (dot blot) using patient sera. The immunoreactive bands were excised from the gels, digested and the proteins identified by LC-MS/MS and interrogation of NCBI protein databases for Tenebrionoidea for mealworm and buffalo worm, *B. mori* for silkworm, and Polyneoptera for cricket and grasshopper. Multiple proteins were identified in the reactive bands, and their allergic potential was predicted using Allermatch™ prediction software. The results showed that Troponin T and β actin cross-recognition was reduced by thermal treatment whereas. Cross-reactivity of tropomyosin appeared to be heat-stable both by boiling and frying between mealworm, buffalo worm, silkworm and cricket. This is in agreement with Broekman (Broekman et al., 2016) but in contrast with Van Broekhoven *et al.* (Broekhoven et al., 2016) who reported tropomyosin immunoreactivity to decrease after frying, probably due to the longer processing time (5 versus 3 minutes. The article of Lamberti concluded that the effect of processing on allergenic potential seems to be protein-, species- and treatment-specific. The authors stated that HDM, shrimp and mealworm allergic patients should be cautious about consuming insects but that further studies are needed to verify the real risk for HDM and shrimp allergic patients who have never tasted insects before of developing allergic symptoms after insect ingestion. They suggested that this could be done by oral food challenge in order to clarify the relationship between the patterns of primary sensitization and the oral food challenge results.

A study of marine invertebrate tropomyosins reported that although tropomyosin's helical structure is disrupted when heated at temperatures above 80 °C, it may reform upon cooling and consequently, its immunoreactivity as well (Ozawa et al., 2011). This suggests that, at least in some circumstances, it may be optimal to combine other processes with thermal processing, such as hydrolysis, to achieve reduced allergenicity. Hall and Liceaga (Hall & Liceaga, 2021) studied the reactivity of tropomyosin hydrolysates obtained through various methods to IgG. They observed that the combination of microwave and enzymatic hydrolysis achieved lower reactivity to IgG than any of the treatments alone. The authors discuss that this trend has also been observed with IgE reactivity but remark that more research is needed to understand allergenicity implications, including human exposure studies. These authors highlight that, while processing methods can reduce allergenicity, this has been shown in studies often on individual species, and evaluation and/or quantification would need to be performed for each individual insect species and processing conditions.

As discussed above, the impact of processing on allergenicity can also vary depending on the specific protein. Moreover, processing methods can also create new binding regions in proteins that have the potential to induce new sensitisation and allergic responses (K. C. M. Verhoeckx et al., 2015).

4.8.3. Other processing technologies: microwaves, high pressure treatment, irradiation, acidification and ultraviolet light

During *in vitro* studies, microwave cooking was shown to have the potential to reduce allergenicity of shrimp tropomyosin, possibly due to changes in secondary structure including the increase in β-sheet structures, and the loss of β-turn structures affecting IgE-binding capability (Na et al., 2014) (Dong et al., 2021). The IgE-binding ability of shrimp tropomyosin was found to be decreased by around 75% by microwave treatment of 700 W, 4 minutes (Dong et al., 2021). Microwave treatment was also found to cause conformational changes in the myosin heavy chain (MHC) allergen protein in crab to reduce allergenicity (Liang et al., 2020). It would be interesting to apply microwave processing to insect protein to determine if these data are reproduced in insects. Microwave-assisted enzymatic hydrolysis was reported to be an effective method used to prepare bioactive peptides from insect proteins to reduce their immunoreactivity in *in vitro* studies (Hall & Liceaga, 2020).

Other forms of processing which have shown reduction of allergenicity in crustacean proteins may be worth considering for insect protein. Methods include irradiation (Khan et al., 2019), (Zhenxing et al., 2007) and acidification of extracted insect protein (He, He, et al., 2021). Irradiation of

foods has been shown to induce changes such as oxidation of fatty acid and undesired smells (Q. Wang et al., 2022) while treating proteins at low pH may create a bitter taste (He, He, et al., 2021).

4.8.4. Glycation and Maillard reaction

Glycation and Maillard reaction have been reported to be effective in reducing tropomyosin allergenicity in crustacea so may be relevant to insect protein. The Maillard reaction has been found by mass spectrometry to impact the α-helix structure within IgE-binding epitopes of tropomyosin isolated from *Scylla paramamosain* (green mud crab). This resulted in a decreased ability to bind IgE in ELISA tests (X. Y. Han et al., 2018). However, caution must be exercised since the allergenicity of (scallop) tropomyosin was increased during the early phases of the Maillard reaction with glucose, ribose, and maltose, but not with maltotriose, which leads to structural changes (Nakamura et al., 2005). As reviewers, it would be interesting to understand more on the effect of the Maillard reaction on insect protein. Insect protein is currently used in protein nutrition sports bars. Since the allergenicity of tropomyosin was increased during the early phases of the Maillard reaction with sugars in shrimp (Nakamura et al., 2005), it would be interesting to learn the effect on the allergenicity of insect protein due to sugar presence in nutritional bars. Again, we advocate for human studies relating to oral exposure to a range of representative final composite foods rather than to individual ingredients.

Glycation with saccharides such as glucose, maltose, maltotriose, maltopentaose, and maltoheptaose reduces the allergenicity of shrimp tropomyosin and inactivates the mast cell allergic response in allergic mice (Zhang et al., 2019), (Zhang et al., 2021). Furthermore, glycation can generate new epitopes and increase the allergenicity of tropomyosin, although this effect is dependent on the reaction conditions (Gupta et al., 2018), (F. Q. Wang et al., 2023). Sonication has been shown to enhance the level of glycation in other foods (e.g. milk) at the original site of the protein and exposes new glycation sites in allergens, which promotes the masking effects of the glycation reaction on epitopes and further decreases the IgE-/IgG binding ability (Shao et al., 2020), (J. Liu et al., 2018). Again, it would be interesting to understand the effect of glycation on insect protein.

4.8.5. Insect rearing practices and allergen considerations

The current regulations (Regulation (EU) No 2015/2283) state that insects raised as novel food must be gut-purged prior to culling and preparing for application to a product. Research has shown that purging will reduce the nutritional value of the larva (Egnew et al., 2021). Studies to ascertain the time needed for gut purging has shown that this may be in excess of 72-96 hours for some species which raises welfare concerns. Welfare is

currently being considered relating to gut purging with a possibility that the requirement for purging may be removed or that insects may be fed on an alternative diet such as a non-waste or vegetable diet for their final days of rearing. Researchers have warned of allergen risks associated with a lack of purging and/or washing.

Frigerio and collaborators (Frigerio et al., 2020) applied DNA barcoding analysis to investigate the species detected in thirteen commercial insect-based products sourced via e-commerce in Europe, namely flour (n=3), pasta(n=3), crackers(n=2), protein bars (n=4) and pet food (n=1). They detected various plant species in the products which were not declared among the ingredients including sweet clovers (*Melilotus* species), radish (*Raphanus* species), beet (*Beta vulgaris*), rye (*Secale cereale*), fennel (*Foeniculum vulgare*). Rye is an allergen, being a cereal containing gluten. The method identified plant ingredients and vegetal traces belonging to insect forming or possible adulteration events and argued that the method was applicable to act as an early warning strategy for the occurrence of undeclared allergens in insect products. While a DNA-based approach detects the origin of species known to be of concern due to allergenicity, a protein detection method such as ELISA would be required to confirm the presence of the actual allergen proteins.

In other work to evaluate the possibility of insects vehiculating the feed substrate into the food product, Mancini (Mancini et al., 2020) detected gluten in mealworm (*Tenebrio molitor)* larvae raised on brewery spent grains, wheat flour, whole grain bread, white bread and also puffed rice and corn (the latter two being negative controls). Gluten was detected in the larvae in a direct relation to the gluten content of the feed substrates. Washing of insects is often included in processing with the aim of reducing microbiological contamination. Other research studies reported the lack of effect of the washing step on the microbiological loads of the larvae, thus the effectiveness of this procedure depends on the fixed goal (Mancini, Fratini, et al., 2019; Mancini, Paci, et al., 2019; Wynants et al., 2017). In the 2020 study by Mancini *et al.*, washing decreased the level of gluten detected to below 20 ppm which is classed as gluten-free (relating to the internationally agreed maximum level of gluten considered safe for those suffering from coeliac disease), suggesting that much of the gluten was adhered to the outside of the insects, with 5-50 ppm gluten detected in the wash water, depending on species. Purging procedures of a 48-hour duration also resulted in less than 20 ppm gluten being detected, and very low levels of gluten were detected from the gut in unpurged, washed insects (8 ppm) (Mancini et al., 2020).

4.9. Gaps in knowledge in allergenicity

An outcome of work of an EU-FOR A Series 5 fellowship programme (Liguori et al., 2022) was to review, assess and identify gaps in the current strategies for predicting allergenicity of novel foods and new alternative protein sources. The fellowship focussed on the allergenicity assessment of novel *foods in silico, in vitro* and *in vivo* in a case study. The authors highlighted that, importantly, several pieces of information and experimental data are needed for allergenicity assessment. To obtain sufficient evidence to predict allergenicity, advances are needed in areas such as threshold doses of food allergens, integration and standardisation for *in vitro* / *in vivo* tests and protocols, and modernisation of the *in silico* tools and databases, as well as clinical data. Threshold doses for traditional foods have recently been provided by FAO/WHO, World Health Organization, World Health Organisation International, "Ad hoc Joint FAO/ WHO Expert Consultation on Risk Assessment of Food Allergens - Part 2: Review and establish threshold levels in foods of the priority allergens" (FAO & WHO, 2022). However, given the above limitations, careful allergenicity assessment is still challenging.

The COST (pan-European Cooperation in Science and Technology) Action ImpARAS was conducted as a 4-year networking symposium to better-understand the mechanisms of allergy and develop new ways to assess the allergenicity of novel proteins in order to improve allergenicity assessments. ImpARAS is the acronym for COST Action on Improved Allergenicity Risk Assessment Strategy (project reference FA1402). The project was based on the premise that, to protect consumers from allergy, the introduction of novel food protein requires a multidisciplinary approach based on an improved understanding of what determines the relative allergenic potency of proteins, novel testing and assessment methodologies, harmonized decision-making criteria, and a ranking of the level of allergenicity of a novel protein. The project highlighted the importance of assessing the *de novo* sensitisation potential of novel and processed proteins. Such an assessment would complement allergenicity risk assessments with regard to potential allergenic cross-reactivity and permit a complete prediction of allergenicity for novel foods. Representatives from industry, academia, risk assessors, regulators and clinicians from 30 European countries were involved during the 4-year project. The project comprised four working groups addressing, (i) physicochemical properties of proteins impacting allergenicity; (ii) *in vitro* methods to predict sensitisation; (iii) *in vivo* methods to predict sensitisation; (iv) risk assessment and dissemination. The outcomes of this COST action are reported by the group of Verhoeckx (K. Verhoeckx et al., 2020). The report highlighted that knowledge on novel techniques of food processing and their impact on allergenicity is incomplete. Similarly, there are knowledge gaps in how food ingredients will interact with novel

ingredients in terms of allergenicity. The group also highlighted the lack of harmonisation of allergen testing protocols and standardised IgE-binding reference assays plus the lack of reference proteins for IgE binding assays as an issue.

As an independent review, this project highlights the complexities surrounding our understanding of the elicitation of allergy and the considerable gaps in knowledge relating to 'what makes a protein more or less allergenic than another'. Existing tools and tests are capable of predicting potential cross-reactivity and research is available regarding predicting cross-reactivity for complex novel foods (K. Verhoeckx et al., 2016). However, as discussed throughout this review, until human oral exposure studies are completed, predictions regarding allergenicity are by no means confirmed and prediction mechanisms have been shown to have drawbacks (K. Verhoeckx et al., 2019),(B. Remington et al., 2018),(Frati et al., 2018). Furthermore, the availability of methods to predict and understand *de novo* sensitisation in addition to elicitation due to cross-reactivity between species is a significantly larger challenge and an area of considerable knowledge deficit.

An interesting outcome of the ImpARAS project was the use of protein pairs (a combination of an allergenic and a homologous non/weak allergenic protein) in order to develop potential comparators for future allergenicity assessment. Using well-characterised patient cohorts, the applicability of the tropomyosin protein pair from shrimp (allergenic) and chicken muscle (non/weak allergenic) and the protein pair of beta-parvalbumins (allergenic) and alpha-parvalbumins (non/weakly allergenic), respectively was investigated. Both pairs were used in immunoassays and proved suitable (at the level of cellular testing) as a potential novel approach in allergenicity testing of potentially cross-reacting novel foods. The authors stated that, in the future, this protein pairs approach may help to inform the current knowledge gap and facilitate the development of methodologies concerning the lack of systematic data to rank known allergenic proteins according to their allergenic potency to inform decision making on allocation of funds for future clinical study. It will be interesting to determine whether other authors adopt this approach.

Mazzucchelli and co-workers (Mazzucchelli et al., 2018) reviewed the limitations of determining the allergenicity of novel foods. The authors highlighted limitations of current studies including giving more consideration to methods of protein extraction prior to analysis. Downfalls of extraction must be considered, for example the maximum profile of proteins present in a food or protein preparation should be present in an extract so extraction methods should be optimised. Consideration should be given to the fact that proteins, once extracted, may no longer be in their native form or may have lost relevant isoforms. Enzymes in the extract may

hydrolyse the protein before allergen characterisation studies can take place or structural modifications may occur during the extraction process. Similarly, recombinant proteins, developed for allergy research, may differ from their native counterparts due to differences in the biochemistry of the cells (prokaryotic versus eukaryotic) producing the proteins. Nonetheless, there are numerous examples of recombinant proteins that react with sera from patients (Azemi et al., 2021; Jeong et al., 2017; Z. Liu et al., 2009; Mattison et al., 2020), illustrating that recombinant proteins produced by microorganisms might have the same allergenic potential as their natural counterparts. This is also relevant in the context of allergenicity of proteins produced by precision fermentation, supporting the cautious assumption that egg and milk PF proteins may be allergenic.

As also described in the consultation with Prof. Mills, work by Mazzucchelli and collaborators highlighted that purified proteins must be checked for their equivalence to native allergen equivalent in terms of purity (when considering further immunisation or sensitisation studies) along with post-translational modifications, biological activity and the 3D structure of the protein. All of these factors could alter the accuracy of the data of a study regarding the allergenicity of a protein. In line with ImpARAS and the expert consultation in Section 2a, these authors also highlighted limitations in allergenicity assessment studies relating to the quality of available databases, which lack harmonisation and some are not curated or updated. As reviewed by the authors, by combining crystallographic and NMR-based approaches with mass spectrometry (MS) analysis, progress has been made in detailed allergen characterization. MS facilitates the sequencing of proteins and, coupled to sequence database search, allows the unambiguous identification of allergenic proteins and can be used to detect allergen presence in food. MS therefore facilitates the study of (a) the primary protein sequence of a target allergen, (b) post-translational and post food processing modifications, (c) molecular interactions, and (d) structural studies (including the structural elucidation of the areas of the allergen which interact with IgE antibodies (the B-cell epitopes) and can be used to identify differences between native and recombinant proteins (Mazzucchelli et al., 2018).

When considering the allergenicity of all foods, including novel, alternative protein foods, it is important to determine an eliciting dose, a threshold dose of allergenic protein which would elicit allergenic response. An eliciting dose takes into consideration the amount of allergenic protein in a product and also considers what would comprise a reasonable serving size for that food type. Eliciting doses have been recommended for other allergens by the Joint FAO/WHO Expert Consultation (FAO & WHO, 2022) and this might also be relevant for insect and PF proteins. Using data generated by Broekman *et al.* (Broekman et al., 2016), Garino *et al.* (Garino et al., 2020) statistically calculated threshold doses of tropomyosin protein

able to elicit a reaction in 5, 10 and 20% of the shrimp allergic population. They considered different scenarios corresponding to various foods containing varying levels of tropomyosin protein. Their computations indicated that the amount of tropomyosin proteins able to elicit a reaction in 5% of a shrimp allergic population (ED05) can vary between 63 mg and 147 mg, depending on the statistical distribution employed. In the scenario of a protein bar containing 10% tropomyosin protein, the portion of serving that would be tolerated by 95% of the shrimp allergic population would be 9.3g. The authors state that the maximum value calculated as ED05 (147 mg) is about half of the ED05 calculated by Remington and collaborators (B. C. Remington et al., 2020) for shrimps, based on 75 allergic individuals. More work is required in order that WHO/FAO could agree and publish eliciting doses for insects.

It is apparent from the research reviewed above that standardisation of research design is lacking. The EU-FOR A Series 5 fellowship programme, (Liguori et al., 2022) highlighted the need for integration and standardisation for *in vitro* / *in vivo* tests and protocols, and modernisation of the *in silico* tools and databases, as well as clinical data. The author highlighted that a careful allergenicity assessment is difficult. A standardisation of study approach would go some way towards aligning the data and better understanding the implications of, for example, different processing technologies. A 2018 review (de Gier & Verhoeckx, 2018) called for greater standardisation of testing, noting that either extracts or recombinant proteins are used in most studies, while there was limited use of purified natural proteins. Additionally, these authors highlighted the importance of testing for allergens in the processed state in which they will be sold and ingested as studies have shown variously increased, decreased and unaffected IgE binding. As stated throughout this report, as reviewers, we would also advocate that conducting human exposure studies of insects in this processed state would provide the most meaningful data. As reviewed by de Marchi and co-workers, the effects of heating protein extracts are not the same as when the whole food matrix is treated, and other food components may interact with the allergens and affect IgE epitopes (Marchi, Mainente, et al., 2021). As these authors highlight, much research relating to processing has been conducted with the dietary behaviours of Eastern countries considered, where insects are ingested raw or with low levels of processing. In Western countries, insect protein tends to be thermally processed and/or included in more complex and more processed matrices such as protein bars, pasta and sausage. It cannot be assumed that studies relating to processing treatments on extracted proteins will be directly transferrable to our knowledge of the effect of a processing treatment on the allergen(s) in a composite food matrix. There is a consideration that other components in complex food matrices may interact to impact on allergenicity, changing the solubility and structure of IgE epitopes and susceptibility to digestion.

In their work to learn more about the effect of hydrolysis on allergenicity, Boukil and co-workers (Boukil et al., 2020) stated that other authors reported different increases of degrees of hydrolysis in similar studies and suggested that the effectiveness of processing treatments such as high-pressure treatment depends on specific parameters such as substrate: enzyme ratio, pressure level and treatment duration.

Studies investigating the potential of peptic digestion to alter the allergenicity of proteins have been hindered by a lack of a standard peptic digestion protocol. As highlighted in the review by Mazzucchelli (Mazzucchelli et al., 2018) there is a lack of harmonisation in hydrolysis studies, with authors using different experimental conditions such as pH, pepsin:protein ratio, purity and chemical environment. This has resulted in a lack of correlation between digestibility and allergenicity. The authors also argue that allergenicity studies are also hindered by the degree of sensitivity used in different studies, with proteins presumed harmless in some non-immunological studies but found to exhibit IgE reactivity in others (Mazzucchelli et al., 2018). The authors highlight the importance of challenging reactivity to proteins in the final (e.g., cooked or pH-treated) product, to gain an accurate assessment of the allergenicity of a product. While a harmonised protocol has been proposed (Minekus et al., 2014), at the time of the 2018 review, this had not been used to characterise correlations between digestibility and allergenicity.

It is important to highlight that, in addition to determining allergenicity of alternative proteins, research is required to develop and/or optimise testing technologies for allergens in alternative proteins. Concerning insect allergy testing for example, Bose and collaborators (Bose et al., 2021) determined that allergen extraction efficiency can vary depending on the extraction method, the food processing method and even the insect species (Yi et al., 2013 in Bose et al., 2021) and highlighted that aspects of protein extraction such as defatting should be considered to achieve reproducibility of extraction.

Data generation from a wide range of study designs may be helpful in highlighting the complexities relating to the effect of processing and different matrix and species dependant effects. However, it is evident that some harmonisation methods may be beneficial to begin to align the data. Finally, improvement and standardisation of research approaches for diagnostic testing would be beneficial. These will help us understand incidence and increase our confidence on the ingredient which elicited each allergic event that could, consequently, be expanded across to all foods, including novel foods.

4.9.1. *De novo* sensitisation to novel foods: Gaps in knowledge

In addition to considerations of allergenicity by cross-reactivity, exposure to new proteins expressed in edible insects can cause *de novo* sensitisation, with or without clinical allergy (Delgado et al., 2022). Consumption of insects has been reported to cause primary allergy in individuals with no previously known allergies or allergic to house dust mites. This needs to be considered, in addition to potential cross-reactivity to known allergens, for a comprehensive risk assessment of allergenic potential of insect proteins.

Relating to whether prior exposure to inhalant allergens such as house dust mite or cockroach allergens can sensitise patients, more research is required in this area as studies have been inconclusive. However, there is evidence that inhalant allergy does not necessarily pre-sensitise patients to food allergy (Barre et al., 2019; Broekhoven et al., 2016; Broekman, Knulst, de Jong, et al., 2017; Broekman, Knulst, den Hartog Jager, et al., 2017).

As stated by various authors, while current methodology is suitable for assessing the allergenic potential of new proteins for cross-reactivity, there were limited options to assess the hazard and potential risks of new proteins causing *de novo* sensitisation (EFSA GMO Panel, 2017; Liguori et al., 2022; Mazzucchelli et al., 2018; B. Remington et al., 2018), as there is currently no single test or parameter available that may provide sufficient evidence to predict *de novo* sensitisation. Mazzucchelli argued that the development and validation of methods such as dendritic cell activation assays or mouse models to discriminate between allergens and non-allergens will help to inform risk. While there is not a huge and urgent need for improving tools and methods for assessing cross-reactivity with known allergens, there is a considerable requirement and challenge for improving and developing tools for the evaluation of *de novo* sensitisation to assess the potential risk of inducing a new and severe allergy (Mazzucchelli et al., 2018). Kopko and co-workers (Kopko et al., 2022) acknowledged progress in our understanding of *de novo* sensitisation, for example the understanding of the Adverse Outcome Pathway (AOP) for the *de novo* sensitization (Strategy for novel food proteins, ImpARAS, 2015) and there is consensus that a better understanding of AOPs could guide the development of better *in vitro* and *in vivo* testing methods to assess protein allergenicity (K. Verhoeckx et al., 2020). The group of Kopko also discussed the derivation of a threshold of allergic concern, which may allow the exclusion of proteins for assessment that remain below this threshold. Again, ultimately, case study data is required to inform our understanding of *de novo* sensitisation and how it may relate to novel foods such as insect and PF protein.

4.9.2. Requirement for human oral food challenge studies

The lack of clinical studies focused on insect consumption and of reliable data about the prevalence of allergy to insects and levels of exposure makes the current risk assessment of insects as food a complex challenge. The principles and guidelines of the Codex Alimentarius for the safety assessment of foods derived from modern biotechnology published in 2003 serve currently as basis for allergenicity risk assessment (FAO & WHO, 2009). As allergenicity cannot be predicted from a single piece of information or experimental method, the main approach for the allergenicity assessment is based on a "weight-of-evidence", where different types of information are considered (Delgado et al., 2022) and where bioinformatics plays a central role by comparing the sequences of novel proteins to those of known allergens (Naegeli et al., 2017) to give an indication of potential allergenicity. Although the Codex Alimentarius and EFSA guidance documents successfully addressed allergenicity assessments of GM applications, new developments in the field of novel proteins call for a modernisation of the risk assessment (Mullins et al., 2022). Information about clinical relevance, route of exposure and potential threshold values of food allergens should be included, and updated *in silico* tools should be used with more targeted databases and better integration and standardisation of test materials and *in vitro* / *in vivo* protocols. A bottom-up strategy that defines a priori the specific risk assessment needs for the investigation of any given novel protein's cross-reactive allergenic potential has been proposed (Fernandez et al., 2021). This approach places greater emphasis on curated allergen sequence databases including additional criteria that would be applied to rank the clinical relevance of allergens. These criteria may include data such as their proven ability to trigger allergy, the potency of the allergen or the prevalence in the population. Regarding *in vitro* protein digestibility, it is currently considered that additional investigations are needed before providing any further recommendations in the form of guidance (Mullins et al., 2022). Verhoeckx and co-workers advised that there is no rationale for a clear readout that is predictive for allergenicity and suggested to omit the digestion test from the allergenicity assessment strategy for now (K. Verhoeckx et al., 2019).

In terms of allergenicity assessment, comparison of human exposure studies with other forms of study demonstrates the dangers of considering studies which involve other animal models. For example, (S.-R. Han et al., 2016) performed a 90-day toxicity study on rats which found no evidence of hypersensitivity to powdered yellow mealworm when measuring IgE and histamine concentrations. However, human exposure studies by showed sensitivity among patients. Similarly, toxicity and food safety studies have been conducted with migratory locust (*L. migratoria*) on rats, feeding with

doses of 750-3000 mg/kg/day of the freeze-dried insect (S. Y. Kim et al., 2023). No toxicological changes including increases in serum IgE were observed for 13 weeks, the time period stipulated in OECD guidelines. The conclusion of the study was that the no-observed-adverse-effect level was at least 3000mg/kg *L. migratoria*/day. However, this data appears to conflict with that of human data from (Lamberti et al., 2021) who reported cross-reactivity of IgE for shrimp- and house dust mite-allergic patients with *L. migratoria.* As discussed in more detail elsewhere in this review (Section 2b), small animal studies are much less applicable to understanding allergenicity than to understanding other types of toxicology to humans and human exposure studies are categorically needed to determine whether humans will show hypersensitivity to foods. Garino *et al.* (Garino et al., 2019) considered the current methods employed by EFSA for risk assessing products for allergenicity and raised the issue that more direct testing of samples needs to be carried out, either *in vitro* using patient's sera or *in vivo* via oral food challenges on top of weight-of-evidence approaches. Other authors have highlighted the need for human oral food challenge studies to understand allergenicity and the effect of processing such as heat treatment on allergenicity (van der Fels-Klerx et al., 2018).

4.10. Further challenges to understanding allergenicity

Allergenicity risk assessment in novel foods is complex, since the presence of an allergenic protein in one food type does not directly correlate with its allergenicity (or severity of allergic reaction) in another food due to potential small differences in the proteins between species (Mazzucchelli et al., 2018). We conjecture that differences in the protein structure due to other factors such as methods of production (for example, in PF proteins) or to food processing methods may also affect allergenic potential. The case of the muscle allergenic protein tropomyosin, in which the non-allergenic tropomyosin of vertebrates differs to the allergenic tropomyosin equivalent in shrimp by only 12 amino acids is highlighted by the authors. However, the authors underline the complexity of allergenicity, emphasising that the mere structural relationship or degree of amino acid sequence homology does not seem to explain the differences in allergenicity of proteins, stating that amino acid identity and structural homology alone are weak predictors in allergenicity risk assessment of novel foods, and additional tests are needed to assess their allergenic potential.

Other considerations of allergenicity were highlighted in work by Marchi and collaborators (Marchi, Wangorsch, et al., 2021). The authors compared patient sensitivities to digested *A. domesticus* (cricket) and *Litopenaeus vannamei* (shrimp). Tropomyosin fragments showed IgE-binding patterns

differed depending on the subject, thus highlighting a strong inter-individual variability, probably as a consequence of the sensitisation to different proteins. The authors stated that it was possible that the co-sensitisation to other allergens, such as house-dust mites, might contribute to the variability of the IgE-binding profiles.

The group of Marzoli warned that many incidences of insect allergy to ingestion may be unreported and concluded that more data was required to fully understand the allergenicity of silkworm (Marzoli et al., 2022). We conjecture that much allergen incidence goes unreported for all food allergies or is not recorded centrally (with individual hospitals holding patient information with no centralised reporting system). Our understanding of allergy would be much improved should allergen incidence be recorded centrally. Since the incidence of certain allergies differs across the globe, it would seem that allergenicity risk assessments would benefit from data sharing or centralised reporting of patient allergen incidence, however transient or serious, to better understand incidence, triggering species method of processing. In support of our understanding of novel food allergens, instigation of centralised reporting may be of benefit not only in countries where insect ingestion is currently common and where consumption of PF protein is regulated, but also in UK and wider Europe, to understand allergenicity of these novel foods in the digestive systems of Western populations.

4.11. Detection of allergens in insect protein and precision fermentation protein

To support consumer safety, it is important that methods are available to test for allergens in foods and currently various methods including ELISA and mass spectrometric methods are available to test for milk and egg allergens in traditional foods. No literature was identified in this review which addresses the detection of allergens in PF milk and egg. As discussed in this review, it appears that innovators in the PF field are assuming the allergenicity of their products, are in line with traditional dairy products. Later in this project, the applicability of current allergen detection methods for dairy products will be tested on PF products.

Literature was scarce regarding the detection of insect allergens in food. Later in this project, the applicability of testing methods which currently target crustacean allergens in traditional foods will be tested to determine the suitability to detect pan-allergens in insects. Limited work is also documented in this area using a commercial crustacean allergen ELISA kit to successfully determine the presence of pan-allergens in black soldier fly (Bessa et al., 2021). In this study, the authors analysed samples of black soldier fly larvae by ELISA using the RIDASCREEN®FAST Crustacean kit (R7312; R-Biopharm), specific for crustacean allergens, mainly

tropomyosin. The ELISA results showed levels of reactivity above 1600 ppm (above the LOQ of the standard curve) in all samples, in agreement with the peptide sequence homology between insect and crustacean species. However, the authors remark that this cross-reactivity does not necessarily imply clinical cross-reactivity, and therefore, whether individuals allergic to crustaceans would react to BSF protein would need to be tested. The study identified certain peptides within the allergenic proteins analysed that are unique to BSF and that can be used to differentiate BSF from crustaceans. The authors suggest that this might be useful when testing unknown samples (as it will discriminate between BSF and crustaceans) and that the results from cross-reactivity are technically inaccurate.

In order to verify labelling claims and inform potential presence of allergens, it would be beneficial to develop robust methods to detect presence of insects (while not necessarily insect allergen) in food. A recent article from Villa *et al.* (2023) describes the development and validation of a real-time PCR method for detection and quantification of yellow mealworm (*T. molitor*) and suggests that the method can be used to monitor this insect as an allergenic food in processed and complex matrices. The study found that matrix and processing conditions affect the sensitivity of the method and the authors remark the importance of appropriate calibration models. The method shows a Limit of Detection of 1 ppm and Limit of Quantitation of 0.1 ppm in autoclaved sausages and baked biscuits, respectively. The applicability of the method was tested on protein bars containing various amounts of *T. molitor* larva flour and chocolate containing dehydrated *T. molitor* larvae, with results in agreement with the product labels. This real-time PCR method targets the cytochrome b gene, which is a generic marker for the presence of *T. molitor*. As such, the method enables quantification of this insect in food, but it does not provide information about the allergenic proteins, neither in terms of identity nor in terms of quantity. Several proteins in this species of insects have been identified as allergenic, including tropomyosin, α-amylase, arginine kinase and hexamerin (Barre et al., 2019). The ability to measure specific allergenic proteins in food would facilitate allergy management for allergic individuals. Tramuta and colleagues (Tramuta et al., 2018) also reported a multiplex PCR method to detect nine species of insects in raw and heat-treated insect products. This assay targeted 16S ribosomal RNA gene (16S rRNA) and the mitochondrial Cytochrome Oxidase I (COI) gene.

As discussed above, success in identifying insect species in commercial insect-based foods including crackers, pasta, protein bars and dog food by DNA barcoding has also been shown (Frigerio et al., 2020).

An LC-MS proteomic method has also been proposed by Barre *et al.* (2021) to determine insect-specific proteins in ready-to-eat *T. molitor* (yellow mealworm), *A. domesticus* (house cricket), *B. mori* (silkworm) pupae,

Zophobas morio (giant worm) and *Hynchophorus ferrugineus* (palm worm) larvae. This method shows potential to be further developed for extraction of insect protein from composite food products prior to identifying (non-allergen) insect-specific proteins. This will then enable confirmation of the presence of insect in food to support labelling claims relating to species authenticity and potential allergen risks. Francis *et al.* (2020) also determined some insect species by proteomic analysis with further development required to expand the scope of insect species.

4.12. Final Conclusions of the literature review

Safety assessment of novel food proteins is paramount, and allergenicity risk assessment is a critical part of it. Allergenicity prediction is very challenging, and current methodologies involve a weight-of-evidence approach where bioinformatics plays a central role by comparing the sequences of novel proteins to those of known allergens (Naegeli et al., 2017) to give an indication of potential allergenicity. Fernandez and co-workers (Fernandez et al., 2021) have proposed a bottom-up approach for allergenicity evaluation which places greater emphasis on curated allergen sequence databases including additional criteria that would be applied to rank the clinical relevance of allergens. These criteria may include data such as their proven ability to trigger allergy, the potency of the allergen or the prevalence in the population, among others.

While precision fermentation is under development for milk and egg protein, and products containing PF milk proteins are permitted in regulation and available for consumption in the USA and Israel, it is clear that the allergenicity of PF egg and milk proteins is not being considered separately to that of their conventional (dairy) equivalents. The potential effect of PF technology on the allergenicity of the protein is not considered in the literature. Future focus should include the fact that PF protein products will differ depending on factors such as specific gene used for the protein expression, microorganism species, culture media and other processing conditions. This may impact the allergenicity of each product.

Regarding the allergenicity of insect, there are a great many studies in this area and there are benefits from considerations of cross-reactivity from pan-allergens. The vast majority focus on predictive analysis of allergenicity and the potential for *de novo* sensitisation from insect protein must be understood. Perhaps particularly with reference to the new consumption of insect protein by Western populations, more data regarding allergenicity are required. As discussed throughout this review, it is clear that much more data are needed relating to human oral exposure, either by clinical trial or case studies for consumers exhibiting symptoms of allergy to novel foods to understand their allergenicity. Other types of predictive study such as protein digestibility analysis, *in silico* allergenicity prediction or *in vitro* testing of allergen-sensitive patient sera IgE binding capacity are very

informative, although not definitive, regarding the allergenicity of novel foods. More definitive data are required regarding the effect of processing on allergenicity with total protein hydrolysis seeming the only current approach to reduce and even to remove allergenicity across food matrices, although this would be at the cost of nutritional value so is untenable for consideration for most food matrices. Research which has demonstrated the possible transfer of allergens from insect feed to the final product, either from the insect gut or from adherence to the insect body must also be considered to manage risk. During Section 3 of this project, research was conducted to determine whether allergens can be detected in alternative protein products using currently available commercial allergen testing kits. Milk allergen ELISA kits were used to screen for allergens in PF milk products. Given the presence of similar allergens in insects to crustacea, as described above, crustacean allergen ELISA kits were applied to screen for insect allergens. Finally, insects raised on gluten and soya-containing diets were tested using commercial kits which are sensitive to gluten (gliadin) to determine whether gluten can be detected, either from the insect gut or gluten adhered to the body of the insects.

5. Section 2. Consultation

5.1. Section 2a. Expert 1. Consultation with Dr Bert Popping, FOCOS strategic food consulting company: Allergenicity and allergen testing considerations for Precision Fermentation protein and insect protein

5.1.1. Introduction

Dr Bert Popping is the Chief Executive Officer of the strategic food consulting company FOCOS. His company advises food manufacturers, start-up companies, not-for-profit organisations, investors, laboratories and governments on strategic food safety solutions and emerging technologies. Bert previously worked as Chief Scientific Officer and Director Scientific Development and Regulatory Affairs for multi-national contract laboratories in global roles. Bert also serves on numerous standardisation committees and government working groups related to food allergens, including FAO/WHO Food Allergen Expert Consultations, CEN TC 275 WG 12, BRCGS Gluten working group and USP Food Chemical Codex Food Ingredients Committee. He previously served as AOAC General Referee for food allergen methods. Bert is a member of the FAO/WHO ad hoc expert committee for risk assessment of food allergens, which – *inter alia* - assessed the Codex Alimentarius priority allergen (CXS 1-1985 rev

2018) list. He authored over 75 peer-reviewed publications on food safety, food allergens, food authenticity, food analysis, validation and regulatory assessments. Bert served on the board of directors of AOAC International and is a member of the editorial board of J. Food Additives and Contaminants and J. Food Analytical Methods. He is co-editor of the recently published reference book "Present Knowledge in Food Safety – A Risk-based Approach Through the Food Chain" (ISBN 978-0-12-819470-6). Bert also consults for start-up companies which are developing new innovations to detect allergens in foods. Dr. Popping's contribution will be to highlight new innovations and emerging technologies which may have applicability to detecting and quantifying allergens in novel foods.

5.1.2. Questions

Q1. Can you describe the concerns over the possible allergenicity of Precision Fermentation (PF) protein and insect protein?

There are many unknowns relating to potential new allergens which may potentially result from preparing novel foods. Considerations around the potential allergenicity of novel foods include prediction. Common tools to predict the potential of a protein to trigger allergic reactions are:

In silico tools: In the field of allergenicity assessment, *in silico* methods serve as an initial means of determining potential similarities between a (novel) protein and a recognised allergen. These computational approaches are employed prior to more resource-intensive confirmatory procedures, such as *in vitro* and/or *in vivo* investigations. Nevertheless, the *in silico* approach solely provides information regarding the ability of a protein to exhibit cross-reactivity with IgE antibodies that are specific to a recognised allergen. If a significant level of sequence similarity is detected between a protein of interest and a known allergen, as defined by the FAO/WHO in 2001 (requiring a sequence identity greater than 35% over a minimum of 80 amino acids), it would be appropriate to conduct serum IgE binding studies. These studies would involve the use of sera from individuals who have a specific and relevant type of allergy, as outlined by the Codex Alimentarius between 2003 and 2009. The selection of allergen sequence databases utilised for sequence comparison significantly impacts the results of the *in silico* research. The existing allergen sequence databases utilised for assessing the risk of allergenicity lack consistent information regarding the allergenic potential of entries. Furthermore, the inclusion criteria employed by these databases typically vary. The presence of disparities in both the quantity and quality of entries across various databases serves as documented evidence indicating a lack of consensus regarding the criteria for inclusion in the construction of a dependable database. The potential for divergent viewpoints may arise from variations

in the database employed for conducting sequence identity searches, as well as the resources accessible for the curation and upkeep of data. Commonly used databases for this approach are:

- AllerBase
- AllerCatPro
- Allergome
- COMPARE
- AllFam
- IEDB
- SDAP

A recent addition to this list is the AllerDet[1] database, a free online resource using deep learning.

- *In vitro* tools: The weight-of-evidence strategy for assessing allergenicity incorporates several *in vitro* methods, such as the pepsin resistance test and immunological assays like immunoblots, assuming serum samples are accessible. The pepsin resistance test is routinely used, despite multiple studies indicating a weak association between resistance to pepsin digestion and allergenicity.
- The use of human data in allergenicity assessment: The sera of allergic patients include human-specific immunoglobulins E (sIgE) that can serve as molecular markers for identifying allergenic proteins in products for human consumption, e.g. novel proteins. Nevertheless, it should be noted that this tool is not intended for primary screening purposes.

There are no 'silver bullet' solutions to determine the allergenicity of these proteins, instead various forms of data must be collected and considered jointly.

In the past, weight-of-evidence strategies have been implemented to predict allergenicity of new foods, for example when genetically modified crops were introduced. Additional caution on top of weight-of-evidence approaches must be applied since unexpected novel allergens may occur in PF and insect proteins. A recent example of a novel allergen is that of

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1 AllerDet, 2022. Allergen Detection Web APP

citrin[2] which occurs in citrus seeds and can be a contaminant of pectin. Citrin cross-reacts with pistachio and cashew and has been shown to elicit allergic reactions in patients. Since we can't completely predict the allergenicity of PF proteins, monitoring of those populations consuming such products is highly advisable.

EFSA produced a document in 2021 stating that there is a requirement for the development of a roadmap to harmonise approaches for allergenicity determination and the use of data[3].

There are also known concerns with cross-reactivity of allergens, for example between cockroach protein and shellfish protein. Manufacturers must give consideration to the potential prevalence of cockroaches in their factories and prevent them from entering storage facilities and processing areas as cockroach presence is known to elicit pan-allergy in shellfish-sensitive consumers.

There are limitations in that we require better diagnosis of food allergy. We need to be able to determine with confidence which of the ingredients which a patient has consumed in the lead up to an allergic reaction has provoked the reaction. Improved testing methods are required for this. There is a considerable challenge here to support the gathering of accurate data to improve our understanding of allergenicity in general, especially for products where there is no history of safe use.

It must be considered that different cultures and populations exhibit varying abilities to digest different ingredients, relating to the genetics of the consumers. Coeliac disease for example is a genetic disorder, affecting approximately 1% of individuals worldwide although can go undiagnosed for several years so this figure is likely an under-representation (NICE, 2020). Similarly, as reported in Science journal (Curry, 2021), humans thousands of years ago consumed milk but were incapable of fully digesting it. Mutations occurred and gradually the majority of European consumers have become able to digest milk. However, due to a lack of lactase production in the gut of certain populations (for example, in Northern Asia lactase production declines by adolescence), at least 70% of those consumers have a reduced ability to digest lactose after infancy (Evershed et al., 2022). We therefore cannot assume that consumption of

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2 Konstantinou et al. 2023. Citrin: a novel food allergen in citrus seeds and citrus-derived pectin that shows cross-reactivity with cashew and pistachio. Annals of Allergy, Asthma and Immunology. 131(6), e3. doi: 10.1016/j.anai.2023.08.603

3 EFSA Panel on Genetically Modified Organisms, 2022. Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology. 20(10), e07044. doi: 10.2903/j.efsa.2022.7044

insect which is relatively well-digested and relatively well-tolerated from a safety point of view in Asian countries (although some incidences of insect-elicited anaphylaxis do occur), will not result in allergy issues in European consumers and it could potentially require many generations of consumption and genetic mutation prior to Western populations adapting to correctly digest novel proteins such as insect or PF protein.

Q2. Can you describe the new and emerging technologies in allergen detection and quantitation you are aware of, particularly relating to the detection of allergens in egg and milk and relevant to insect protein?

The majority of current allergen testing in foods uses ELISA technology which is a sensitive and fairly specific technology with only a few considerations for cross-reactivity. Most of the emerging allergen testing technologies apply to a range of allergens and are aimed at faster sensitive detection and application to multiple allergens in a single test. Such tests include the use of aptamers rather than the antibodies required for ELISA, smart phone technology using Surface Plasmon Resonance (Ross et al., 2018), dye encapsulation with antibodies, new lateral flow and other paper-based tests. Recent additions are the electrochemical sensing strategies for food allergen detection, which deploy various detection techniques, including constant potential amperometry (CPA), chronoamperometry (CA) and cyclic voltammetry (CV), to name but a few. The detector molecules can be molecular imprinted polymers (MIPS), antibodies, nucleic acids (aptamers) or nanomaterials. A recent review describing these techniques was published by Antonella Curulli[4]. However, more relevant to the requirements of this project (detection of milk, egg and insect allergens), a LAMP assay[5] , as well as a paper-based biosensor approach were developed by the technical University of Beijing and collaborators[6]. In addition, a multi-milk chip based on LAMP was developed by the Chinese Academy of Science, also in Beijing[7]. The LOD for cows milk

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4 Curulli, 2022. Recent Advances in Electrochemical Sensing Strategies for Food Allergen Detection. 12(7):503. doi: 10.3390/bios12070503

5 F. Q. Wang et al., 2023. Integration of in-cassette lysis, purification, and lateral flow strips-based sensor for rapid and on-site detection of yak milk adulteration. Sensors and Actuators B: Chemical. 394(134309). doi: 10.1016/j.snb.2023.134309

6 Jiang et al., 2019. A novel electrochemical mast cell-based paper biosensor for the rapid detection of milk allergen casein. Biosensors and Bioelectronics. 130:299-306. doi: 10.1016/j.bios.2019.01.050

7 Yu at el., 2021. Multiple authentications of high-value milk by centrifugal microfluidic chip-based real-time fluorescent LAMP. Food Chemistry. 351:129348 doi: 10.1016/j.foodchem.2021.129348

is stated to be 0.05 mg/kg (0.05ppm). Specifically for the detection of cow's milk, Nehra *et al.* describe nano-biosensing platforms[8], and for lysozyme, Melinte *et al.*[9] describe aptasensors.

Q3. Do you conceive any special considerations for detecting egg and milk allergens in PF products compared to animal-origin milk and egg products?

Considering PF protein, since this is a novel production method, no HACCP or food safety knowledge about this form of production of milk and egg is in place for many start-up companies. It appears that innovators are currently focussing on developing production and scale up and safety issues are being considered later in the innovation process. Once guidance is put in place by regulators, innovators will have more information about the safety aspects to consider.

The environment in which a food allergen is present must be considered, for example, within a fermentation chamber and within fermentation media. When testing for an allergen in a new environment (within a new food matrix or fermentation medium) there must also be considerations regarding possible impacts of the matrix on the test method, such as non-specific binding which prevents the detection of antibodies binding with the target protein.

The possibility of mutations occurring in the microorganisms – specifically in the inserted sequences - used to express the protein must also be considered since, if the conformation of the resulting protein is altered, detection of the allergen may no longer be possible by existing methods such as ELISA, LC-MS or lateral flow.

At present, methods for an allergen ingredient group are designed to detect different allergenic proteins within the food, so it must be ascertained which proteins are expected to be present in the PF product so that an appropriate testing method can be applied. For example, some current methods to detect milk allergens target the allergenic protein casein which comprises around 80% of milk protein, while other methods detect β-lactoglobulin which is found in the milk's whey fraction only which comprises around 10% of milk proteins. Since in some cases it may not be known which allergenic proteins from the commodity are present, it is important that screening methods are being deployed, ie. methods targeting several allergens from the commodity.

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8 Nehra et al., 2019. Nano-Biosensing Platforms for Detection of Cow's Milk Allergens: An Overview. Sensors. 20(1):32 doi: 10.3390/s20010032

9 Melinte et al., 2021. Aptasensors for lysozyme detection: Recent advances. Talanta. 226:122169. doi: 10.1016/j.talanta.2021.122169

Another consideration when accounting for allergen testing of PF products is the possibility of post-translational modifications (PTMs) which may occur in dairy products but will not occur in PF milk and egg (since the latter will not have the natural PTMs found in conventional proteins). An example is post-translational glycosylation[10]. *In silico* analysis of proteins is not sufficient to predict the possible presence of PTMs. Should it turn out that current allergen detection methods rely on the presence on PTMs, these will not be applicable for the testing of PF products. It may be that the use of Artificial Intelligence (AI) may provide support in this area and is already being used to predict the allergenicity of genetically modified crops. An example in this area is the publicly available ALLERDET tool (Garcia-Moreno & Gutiérrez-Naranjo, 2022).

In summary, there are a great many unknowns relating to the potential for novel foods to contain new allergens and precautions must be in place to consider this.

Q4. Can you describe any concerns relating to the allergenicity of insect protein?

The development of insect protein is more advanced than that of PF protein but consideration must be given to the consumption of chitin in insects which is present in insects at a high proportion of the total insect mass. While also present in crustacea, chitin in the shellfish exoskeleton is separated from the edible shellfish protein before consumption so is less of an issue in terms of eliciting an immune response, unlike in insects where the entire insect is often consumed or milled into a powder for inclusion in products. Although a carbohydrate and not an allergen, chitin has been implicated in adverse immune responses.

Q5. Can you describe any potential knowledge or capability gaps you have identified in relation to allergen testing, particularly in relation to PF products?

One knowledge gap is the incongruence of the outputs when using the different databases to predict the allergenicity of a protein. The databases apply different data and the tools are designed in varying ways which impacts on the outputs. It may be that AI or Deep Learning could be applied in the future to streamline the allergen prediction databases.

10 Mazzucchelli, et al., 2017. Current (Food) Allergenic Risk Assessment: Is It Fit for Novel Foods? Status Quo and Identification of Gaps. Molecular Nutrition and Food Research. 62(1):1700278. doi: 10.1002/mnfr.201700278

Allergenicity co-factors and their impact must also be determined, in order to understand the potential allergenicity of a product rather than simply of individual proteins.

In the past, a weight-of-evidence approach has been applied to perform the risk assessment on new food types such as when genetically modified crops were introduced. However, there is so little research and data concerning PF products, the dataset is too small to make meaningful conclusions regarding safety. Patient data are required for patients who have consumed PF protein, with an emphasis on human studies rather than studies concerning the reactivity of the sera of smaller animals such as dogs and rats to novel foods.

The composition of products containing PF protein will be important in order to prepare a meaningful risk assessment. Factual information is required on the level of PF protein within a product. For example, will a product contain high levels of PF protein or will it contain low amounts, perhaps diluted with other protein sources? This, plus information regarding the product type and thus the expected portion/daily intake mass, are important data so that calculations can be made to predict the allergen trigger level of a given PF food product.

A list of current gaps can be found in the publication by Mazzucchelli *et al*[11] as shown in Table 1 below:

Table 1. List of current knowledge gaps as published by Mazzucchelli, et al., 2017

Methods & Tools	Features & Limitations	Recommendations for further research
Allergen databases	Different databases provide different levels of information; some of them are not regularly updated/curated, and therefore relevant information is missing or available information outdated Inclusion criteria for allergenic proteins vary for individual databases	Linking of existing (allergen) databases; harmonization of inclusion criteria for allergens Experimental studies in B- and T-cell epitopes and implications on cross-reactivity Improving predictive algorithms for sensitizing potential of proteins linked with and without clinical relevance
Analytical methods	Highly sensitive and advanced methods available for protein characterization Sample preparation especially for complex food extracts is	Harmonization of method protocols; improvements in sample preparation; generation of scientific

11 Mazzucchelli, et al., 2017. Current (Food) Allergenic Risk Assessment: Is It Fit for Novel Foods? Status Quo and Identification of Gaps. Molecular Nutrition and Food Research. 62(1):1700278. doi: 10.1002/mnfr.201700278

Methods & Tools	Features & Limitations	Recommendations for further research
	sometimes difficult (lack of harmonized protocols)	evidence of certain structural determinants (glycosylation, aggregation, etc.) linked with increased allergenicity, which is currently lacking
IgE binding assays	Well standardised reference assays including reference proteins are missing. In case of novel proteins, no reference material is available; if sIgE is not available, animal derived antibodies can be used	Identification and generation of suitable reference proteins
Digestion assays	Different protocols for protein digestion are available; however, harmonized protocols are needed; lack of guidance on how to interpretate data, and lack of reference material; evidence of linking protein stability and de novo sensitization is missing	Development of reference materials and harmonized protocols Performance of harmonized digestion assays in ring trials with reference materials Animal studies on comparative digestion and de novo sensitization
Food processing techniques	Knowledge on food processing and its impact on allergenicity is incomplete on a qualitative and quantitative level. Limited knowledge about the most effective methods (combinations), including novel processing techniques	More data on processed food proteins and their allergenicity required; to identify the most important (combination of) processing techniques with an impact on allergenicity
Food matrix	Analytical methods are established—but limited data are available showing a link of food matrix components to allergenicity; limited knowledge available about food components and their interaction with allergens	Studies required on food matrix composition and interaction with individual food proteins in model systems; identification of relevant immunomodulating food matrix components
Biological assays	Cellular and animal models are established but reliable assays for detection of de novo sensitization are lacking	Method development to assess protein ligand binding and impact on innate and adaptive immune responses; identification of biomarkers for de novo sensitization

Q6. Can you describe any potential knowledge or capability gaps you have identified in relation to allergen testing, particularly in relation to insect protein?

A small number of allergens have been confirmed (meaning identified in human clinical trials) in insects for the seven insect species permitted for consumption under retained EU regulation. Data generated regarding insect allergens from other types of studies such the use of sera from rats or dogs or protein studies by other means do not confirm the identity of allergens. More research is required and data from different study types must be considered in unison to formulate a firm prediction of allergenicity. The digestibility of the proteins in the human digestive tract must be considered (Naegeli et al., 2021) along with considering matrix effects. Research methods must be standardised so that reliable and comparable data can be considered from studies.

Q7. Please discuss the challenges you consider in terms of allergenicity and allergen testing of PF and insect protein products for our consumers.

At present, the global scientific and medical communities are struggling to fully understand the established allergens which have been known for some time for traditional foods and to develop sensitive multiplex methods to detect the allergens in foods. The introduction of novel foods is adding a layer of complexity to this allergenicity research. Our FAO/WHO *ad hoc* expert group also recommended in our first report that pulses, insects and other foods such as kiwi fruit be included in a “watch list” in terms of their allergenicity.

Furthermore, manufacturers producing clinical testing methods applicable to traditional foods are unlikely to produce new methods which may only be applicable to a small number of novel foods. This lack of testing will impact on clinical diagnosis so will slow down the analytical identification of novel food allergens as well as the development of our understanding of allergens and allergenicity of novel foods.

While AI may have a significant positive impact on the identification of novel allergens when combined with information from the various databases mentioned above, care must be taken to only apply AI appropriately to predict allergenicity and control the outcome. Currently PF proteins are only consumed by a small number of consumers and therefore any datasets relating to consumption and subsequent illness are small. Applying AI on small datasets can lead to skewed data which should be viewed as providing guidance only with careful consideration in place. Instead, it may be applicable to focus the efforts of AI on predicting the structure of allergens.

5.1.3. Conclusions

Many unknowns exist regarding the allergenicity and potential allergenicity of novel foods such as insect and PF products. In the case of PF proteins, data relating to allergenicity are not reported in the public domain and we conjecture that little or no allergenicity testing has been conducted since these known allergens will be marked as such on the product ingredients label. The effect on allergenicity of the fermentation process can currently only be estimated due to lack of case study data. Regarding whether testing methods are available to detect allergens in PF protein, ELISA-based studies planned for later in this project will go some way as to beginning to inform in this area.

In the case of insect protein and its allergenicity in Western populations, the possible cross-reactivity of pan-allergens provides data relating to potential allergenicity, alongside other types of study which provide information for inclusion in weight-of-evidence assessments. Consideration must also be given to potential *de novo* sensitisation of insect proteins. The gold standard to our understanding of the allergenicity of insect protein requires clinical trials and case studies of patients who have displayed symptoms following consumption of this novel food.

There is no simple guaranteed solution to determine the allergenicity of novel foods within a short timeframe. A scenario is foreseen whereby consumption occurs by Western consumers (as is permitted at present under regulation in Israel and USA for PF protein and across Europe for four species of insect) which will provide a growing dataset against which to assess allergy risks to inform future regulation and current weight-of-evidence procedures. As consumption grows, clinical data will be gathered and clinical studies can occur. In the meantime, data can be gathered using allergen prediction tools and digestibility studies to provide risk assessments to regulators. The applicability of current testing methods on currently available novel foods must be determined in order to inform regulators according to our testing capabilities in this area. It should be noted that, as novel proteins are further developed, testing capabilities must develop in line. For example, PF proteins and their production processes will differ between each product on the market depending on the specific gene used for the protein expression, microorganism, culture media and other processing conditions. This will impact the allergenicity of these products.

5.2. Section 2b. Expert 2. Consultation with Prof. Clare Mills Professor of Molecular Allergology, University of Surrey: Allergenicity considerations for Precision Fermentation protein and insect protein

5.2.1. Introduction

Prof. Clare Mills, Professor of Molecular Allergology, University of Surrey, is a member of the FSA Advisory Committee on Novel Foods and Processes and was involved in the recent FAO/WHO Expert Consultation on Food Allergens. She led the EU integrated projects iFAAM (integrated approaches to Food Allergen and Allergy Management) and EuroPrevall (the prevalence, cost and basis of food allergy across Europe) and coordinated the European Food Safety Authority Project ThrAll *(Detection and quantification of allergens in food and minimum eliciting doses in food allergic individuals)* and leads the UK Food Standards Agency project PAFA (Patterns and Prevalence of Adult Food Allergy). Clare is also a partner in a recently awarded project from EFSA led by EuroFIR on allergenicity prediction. Her personal research interests are focused on structure-function relationships in food proteins particularly with regards what makes some proteins, and not others, become allergens, including the effects of the food matrix and processing on resistance of food proteins to digestion and the role this plays in determining the allergenicity of foods. Prof. Mills is currently also performing a review of potential allergenicity risks for an FSA-funded project led by Fera that studies the safety of currently non-permitted waste streams to be used for rearing insects for feed.

5.2.2. Questions

Q1. Please discuss concerns about the allergenicity of PF products, especially compared to traditional dairy/egg products.

The areas which food safety committees will consider when advising on the safety of PF proteins include benchmarking the new technology against similar well-established technologies in the food industry. Similar to PF, fermentation processing aids based on microbial hosts have been used for many years and in well-characterised foods including cheese. There is no evidence to date that the use of a microbial host has caused a safety issue. To be sure of safety however, exposure studies are required.

Consideration must also be given to the intended level of the novel protein in products and whether the level is high enough to reach an eliciting dose for egg/milk-sensitive patients.

Q2. Please discuss concerns about the allergenicity of insect protein products for animal feed.

A project is currently ongoing with FSA and Fera Science Limited to assess the safety of non-permitted waste streams for rearing insects for feed (FS900220, Assessing the safety of currently non-permitted waste streams for rearing insects for feed) and Prof. Mills is performing a literature review as a partner in this project. The literature review is showing that much insect feed currently being used is cereal-based and often chicken feed is being used and its composition is not completely transparent. The literature review to date suggests that allergenicity relating to insect feed (with concerns relating to both ingested feed and feed adhered to the insect bodies) is being considered to a minimal or zero degree at present and research is required to verify the allergenicity of the insects depending on the feed matrix and whether insect washing processes are sufficient to remove any adhered allergen from the insect prior to use in food. In line with some of the interests of this project, a publication has been identified which raises insect diet as an allergy concern for consumers (Mancini et al., 2020; Mancini, Fratini, et al., 2019). Prof. Mills highlighted that the review she is currently conducting is highlighting a fundamental absence of evidence relating to the use of various insect feed substrates and that more research is required. While at very low levels and (we assume) at least partly digested by the insect, the possibility of allergy to insect gut content must be investigated.

Q3. Please discuss concerns about the allergenicity of insect protein products for food.

Professor Mills highlights the requirement for more human clinical research and human sera studies in this area and argues that other analytical approaches provide data but the true knowledge relating to allergenicity of consuming insects will come from human reactivity only. There is sparce evidence relating to the allergenicity of insect protein due to the lack of human clinical study. One example of such a study applying human sera to determine allergy to yellow mealworm (*T. molitor*) was conducted by Verhoeckx *et al.* (2014) but other data are lacking. As stated in the EU-funded GIANT LEAPS project (Gap resolutIon in sAfety, NuTritional alLergenicity and Environmental assessments to promote Alternative Protein utilization and the dietary Shift) which provides risk assessment scenarios, Prof. Mills highlighted that animals (dog, rat) are very poor models for research studies in the case of allergy. This is partly due to the fact that allergenicity depends on exposure levels and the

exposure that a human would undergo from consuming a certain amount of a food product cannot necessarily be replicated in a small animal. Also, many aspects of a human diet do not suit the digestive system of a small animal and it is therefore difficult to make accurate toxicological interpretations from animal studies. Prof Mills stated that the only case in which animals are useful models for allergenicity is in the case of assessing the allergenicity of (highly) hydrolysed infant formula since the hydrolysed protein passes through the gut in much the same way in animals as it would in humans. In all other studies of the allergenicity of foods, clinical studies are required for a proper understanding.

Q4. Please discuss the state of knowledge relating to differences in allergenicity and differences in the types of biochemical changes to allergenic proteins which could be anticipated with PF and insect proteins?

Prof. Mills stated that it is unlikely that PF proteins will be more allergenic than their dairy equivalents, but there may be other concerns. There are many unknowns in this area and changes will depend on the organism and process. The host organism is critical here in terms of potential post-translational modifications which could alter the protein structure and thus possibly the allergenicity. Depending on whether the host is a prokaryote or eukaryote, whether a prokaryote has been engineered to assemble the protein or whether it will be a secretory vector or enter an inclusion body will impact on how the PF protein is folded and how this structure compares to the dairy equivalent, which impacts on allergenicity. Downstream processing and purification mechanisms will also impact on protein structure and allergenicity. It is likely that milk casein protein will be difficult to produce by fermentation and may be easier to express in plants (soya) instead and that milk proteins such as lactoferrin may be more accessible by PF.

Q5. What factors and strategies relating to allergens should be considered before PF products enter the market?

Prof. Mills highlights that the only type of processing for which there is evidence that it reduces allergenicity is the (extensive) hydrolysis of insect formula. There is no evidence of food challenge studies that other forms of processing reduce allergenicity. There is evidence in the public domain for peanut that thermal processing, in the form of six hours of boiling, reduces allergenicity but in fact, after six hours of boiling, hydrolysis of the protein has begun to occur and is the likely cause of reduced allergenicity.

Some researchers have stated that, if a child is first sensitised to baked egg (for example, in a muffin), the child will later react to a lesser degree to scrambled egg. However, these studies are flawed since there is far less egg in a muffin than in scrambled egg so the level of exposure is incongruous between the two scenarios.

A useful benchmark when considering the acceptability of novel foods is the EFSA opinion on 'ice structuring protein' (ISP) as a novel ingredient (EFSA, 2008). ISPs are widely distributed in nature in cold water fish, vegetables, grains and bacteria and were accepted as a novel ingredient to add to edible ice (0.01%, weight/weight) when expressed in a genetically modified yeast and produced by fermentation. When expressed by the yeast, the ISP became unexpectedly glycosylated, due to post-translational modification. This suggests that possible post-translational modifications of PF proteins and the impact on allergenicity must be considered. This would require clinical trials, the cost of which may prove preventative for the development of PFs. An example of unexpected outcomes when changing the diet of an organism was cited when insects were fed to fish which resulted in fish exhibiting the piscine equivalent of ulcerative colitis. Prof. Mills argues that this is a warning that humans must comprehensively assess products when investigating possible safety issues of novel foods.

Q6. What factors and strategies relating to allergens should be considered for insect protein?

There are two areas to consider here:

(a) unintended allergy, potentially stemming from a reaction to the feed the insect is feeding on and (inherently) standing in. Due to welfare concerns, insect guts are always not purged prior to culling and any undigested allergenic feed such as bread, soya bean or sesame seed could theoretically cause allergy in the subsequent human consumer. It must also be ascertained that current insect washing processing are sufficient to remove any feed from the insect carcass prior to production of insect protein products. It should be possible to manage these concerns by an appropriate allergen risk assessment, comprising factors including the monitoring of the feed and understanding the washing medium and length of washing time.

(b) known pan-allergy of the insect itself, including to tropomyosin and arginine kinase allergens amongst other allergenic proteins. Hydrolysis is the only manner at present which is known to decrease the allergenicity of these proteins. There is also a requirement to ascertain the level of insect protein which would be required to cause a reaction in a crustacean-sensitive patient upon consumption of insect products. Again, improving our understanding of exposure in clinical trials is imperative.

Understanding the elicitation doses of these allergens is important. Possible mitigation strategies for allergenicity of PF and insect protein could include mixing/diluting these novel proteins with other proteins and ingredients so that the elicitation dose is not reached. A small 10-patient study by Broekman *et al.*[12] on allergy to mealworm made headway towards determining elicitation dose and larger studies are now required.

c) Insect composition includes the carbohydrate chitin in the exoskeleton. Unlike in shellfish consumption prior to which the exoskeleton is removed, insect chitin is consumed. Carbohydrates such as chitin are not allergens since they are unable to induce a specific IgE responses. Such humoral responses are only generated by proteins or haptens. While chitin cannot be an allergen, it is a pathogen-associated molecular pattern (PAMP) molecule and has potent immune modulatory effects on both the innate and adaptive immune system. It is broken down by chitinases into fragments which can also act as PAMPs. Such effects have been demonstrated in animal models and human cell lines and there is a plausible link to the clinical reactivity to chitin in chronic fungal lung infections and it may exacerbate asthma. There are some recent data published last year in mice pointing to chitin causing gastric distension which in turn triggered a type 2 immune response (D. H. Kim et al., 2023).

Q7. What are the main concerns which should be addressed by stakeholders in food supply concerning the allergenicity of PF and insect protein?

Human clinical data are required relating to the allergen safety of PF proteins and this is imperative. Insect stakeholders must consider *de novo* sensitisation of consumers to new insect proteins while also acknowledging the known risks for crustacean-allergic individuals. There is a strong chance that allergenicity will differ between insect species and more research is required in this area. The potential role of chitin as an immune response modulator in *de novo* sensitisation must also be considered.

Prof. Mills highlighted that finding alternative forms of protein is imperative for feeding the world in a nutritious and sustainable manner within the next two decades. At the current stage of Alternative Protein (AP) innovation and development, a large suite of alternative protein types is needed (which can also include novel plant, meat and algae proteins) in order to investigate and assess in terms of safety to ultimately be in a position to select APs to feed the growing global population.

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12 Broekman et al., 2017. Allergenic risks of mealworm and other insects. Allergenic risks of mealworm and other insects (uu.nl)

5.2.3. Conclusions

Prof. Mills highlighted that introducing alternative forms of protein is imperative to feed the world's population a nutritious diet which is safe.

Regarding the allergenicity of PF protein, there is no evidence to date that the use of a microbial host has caused a safety issue. To be sure of safety however, exposure studies are needed. The true knowledge relating to allergenicity of consuming insects will come from human reactivity only. There is sparce evidence relating to the allergenicity of insect protein due to the lack of human clinical study. Animals (dog, rat) are very poor models for research studies in the case of allergy other than for highly hydrolysed infant formula (since the hydrolysed protein passes through the gut in much the same way in animals as it would in humans).

Concerning the allergenicity of PF protein, it is unlikely that PF protein will be more allergenic than its dairy equivalent. However, there are many unknowns in this area and changes will depend on the organism and process. The host organism is critical here in terms of potential post-translational modifications which could alter the protein structure and thus possibly the allergenicity. Downstream processing will also impact on allergenicity. It is therefore important to consider all relevant clinical data which will be generated when consumption of alternative proteins increases.

Concerning the allergenicity of insect protein, the only type of processing for which there is evidence for reducing allergenicity is extensive hydrolysis. There is a strong chance that allergenicity will differ among insect species and this requires study. Understanding elicitation levels for novel foods is important as these forms of protein could be mixed with foods at levels below the elicitation level to protect consumers. Consideration must be given, not only to the allergens in the insects themselves but also to the potential for cross-contamination with potential allergens present in the insect feed substrate.

5.3. Section 2c. Consultation with representative of Advisory Committee on Animal Feedingstuffs (ACAF) regarding future considerations for the inclusion of novel proteins in feed: Allergenicity considerations for Precision Fermentation protein and insect protein

5.3.1. Summary of consultation

This stakeholder engagement is included in the project to provide initial insight into the considerations when a new product or novel ingredient is proposed for inclusion in animal feed. This consultation provides some detail of the areas considered by authorising committees. In the case of new additives to animal feed, these are considered by The Advisory Committee on Animal Feedingstuffs (ACAF). In the case of protein being used as a bulking product however, as may be the case for insect and PF protein, bulk protein products are not regulated products and do not require a Committee-based approval in the same way that feed additives, novel foods (for humans) or food additives (for humans) would. In the case of bulk proteins being used in feed, this requires addition of the protein bulk product to the Catalogue of Feed Materials which is a more 'light touch approach' than when considering new feed additives. Due to the newness of novel proteins however, it is not unforeseeable that ACAF may be invited to discuss, evaluate and provide opinion to FSA on the safety and efficacy of new feed material.

ACAF is an independent scientific committee, sponsored by the Food Standards Agency, that advises on the safety and use of animal feeds and feeding practices, with particular emphasis on protecting human health, and with reference to new technical developments. ACAF review Feed Additive Authorisation dossiers relating to new animal feeds to advise as to whether full safety assessments have been completed and whether the feed is safe to be regulated, according to EFSA guidance documents. Most of the dossiers submitted to ACAF at present tend to be applications relating to feed additives.

As a first phase, proposals for new feeds must provide evidence that the identity of the feed (or additive) provide Material Safety Data Sheets (MSDS) for all ingredients and prove (with testing certificates) that it complies with regulations. For example, relating to trace element content and microbiology. In the case of genetically modified ingredients, the DNA sequence information must be detailed in the dossier. Toxicology studies

must have been completed and reported and safety must be demonstrated in terms of users handling the feed (manufacturers, farmers etc.), the target animals and for humans consuming and also handling those animals.

The second phase in determining suitability for authorisation relates to environmental impact. It must be demonstrated that safety for the environment has been thoroughly considered (with testing data, as appropriate) and reported. This includes considering the level of breakdown in the animal digestive tract and the form in which the consumed feed will reach the environment in faeces and urine and whether any elements are present which will persist in the soil or waterways.

Efficacy trials must have been completed and detailed in the dossier if ingredients are present which have not been previously authorised. Where appropriate, for example for the inclusion of additives to increase the level of vitamins or nutrients in the feed, the function of these additives must be proved.

Given the inherent novelty of the insect protein and PF protein discussed in this project, examples of these forms of protein have not been submitted for consideration by ACAF to date. The decision by FSA as to which parties to invite for opinion and evaluation of safety will be decided on a case-by-case scenario.

5.4. Section 2d. Stakeholder interviews

5.4.1. Introduction

In addition to the above experts in allergen science, other stakeholders from the food industry and alternative protein sectors were consulted. One interviewee representing many contributors in the UK food supply sector felt that they could not comment as the insect protein market in the UK is very small and PF novel foods are yet to be approved in the country, therefore the knowledge and experience in this sector is currently lacking. Three additional stakeholders were interviewed in person and one other responded to the questionnaire in writing. Their answers are reported below.

5.4.2. Questionnaire responses from Respondent A (Trade Organisation, Insect Protein)

1. Please give an overview of your business

The organisation is a trade association bringing together and representing companies in the UK involved in insect protein supply, including their representation to the Food Standards Agency. Currently representing

approximately 17 companies involved in the supply of insect protein in the UK, including insect farmers, product producers and retailers. Six members responded to contribute their responses in this questionnaire.

2. Please provide an overview of your work/supply relating to precision fermentation and/or insect protein

Representing companies in the UK involved in insect protein supply, including their representation to the Food Standards Agency. Comprising insect farming, product preparation and retail.

3. At what stage is this development of alternative proteins?

Insect protein products are well-developed globally and product development in the UK broadly reflects the fundamentals of products produced elsewhere internationally, such as bars and snacking products. Our members have sold millions of products containing insects within the UK for many years with consistently high approval ratings.

4. Have you considered the allergenicity of your protein or product?

Yes, there is an assumption across the industry that crustacean-sensitive consumers may be sensitive to insect proteins and products are labelled as such. The production of insect protein is well-developed due to very high consumption rates in certain areas of the world.

5. Have you considered how allergens will be labelled on your products?

Yes. Label will state that the product contains insect and may cause sensitivity/allergy in consumers with a sensitivity to crustacean. The International Platform of Promoting Insects for Human Consumption and Animal Feed (IPIFF) which is the EU trade body for insect protein production provides guidance for labelling and members of the organisation adhere to this.

6. If your business already trades in precision fermentation (overseas) or insect protein, what allergen labelling is used on those products?

The labelling states: “People who are allergic to molluscs, crustaceans or dust mites may have an allergic reaction to crickets.”

7. Are you able to describe your manufacturing procedure? We realise that there may be concerns about IP here, so please discuss in as much or as little detail as appropriate. The intention is to gain information regarding conditions that might impact on the structure, and potentially on the allergenic potential of the proteins. Are you aware of this?

Insects undergo a period without feeding to purge any feed-derived gluten from their guts (possible allergy- and coeliac-risk to sensitive consumers) prior to freezing. Most are then boiled or roasted. There are Intellectual Property considerations which apply which prevent full disclosure of all production methods. Interviewee was not aware of members currently targeting to reduce allergenicity via their production methods.

8. Do you have an allergen management regime for alternative protein products and prevention of cross contamination

Responses to this question (from the cohort of 6 members) fell under one of three categories:

a) The manufacturer who produces our products manages risks of cross-contamination within their facility according to relevant protocols.
b) All raw materials are contained and concealed in sealed packages (to prevent cross-contamination).
c) Much effort is applied to clean the production lines to mitigate cross-contamination risk.

9. Have you conducted any allergen testing of your product? Which method? And which allergenic proteins have you tested for?

No, allergenicity is assumed due to known cross-reactivity of insect products for crustacean-sensitive consumers and products that are labelled as such.

10. If conducting allergen testing, what is the testing regime? What factors do you consider when selecting which method(s) to use for testing?

Not applicable, see response to Question 9.

11. Do you perform your own testing or outsource? What factors do you consider when selecting a subcontractor for allergen testing? What is the LOD/LOQ?

Not applicable, see response to Question 9.

12. Have you encountered challenges in allergen testing of your product?

Not applicable, see response to Question 9.

13. What challenges do you anticipate in the allergen testing of your product or any finished product made with it (if product is an ingredient)?

Not applicable, see response to Question 9.

14. Are there any other challenges you face when considering the possible allergen content of your products?

The cross-reactivity of insect protein with crustacean allergens is currently assumed. This reduces the size of the insect consumption market by ruling out the consumption of insects by crustacean-sensitive consumers. The insect protein market would prefer that further research is conducted to understand the extent of cross-reactivity among consumers and that an insect-specific allergen test for food testing is developed to confirm whether all crustacean-sensitive consumers must abstain from all insect protein. Also, if manufacturers could carry out a simple and rapid test for the presence of insect protein in other food products they produce, they would be more willing to manufacture our members' products which could open the market significantly.

15. Do you have any thoughts about what would help allergen management in your sector of alternative proteins?

See response to Question 14. The UK insect protein sector requires more research on cross-reactivity between insect and crustacean allergens and on the potential reduction of allergenicity via various production methods.

16. Are you aware of any other risks/considerations relating to allergy in precision fermentation, insect protein and other alternative proteins?

Concerns relating to farmers becoming sensitive to allergy due to inhalation during farming.

17. Are you aware of any precision fermentation egg protein /products on the market or likely to be introduced soon?

Question not applicable to this stakeholder.

18. Are you aware of any egg precision fermentation products that have been withdrawn from market?

Question not applicable to this stakeholder.

19. Are you aware of why the market of milk precision fermentation products is more advanced than the egg sector?

Question not applicable to this stakeholder.

20. Do you have any points that you would like to raise, or further comments?

No, thank you.

5.4.3. Questionnaire responses from Respondent B (Research and Technology, Precision Fermentation)

1. Please give an overview of your business.

An independent research and technologies organisation, working with companies to develop their products and processes. Taking them closer to commercialisation, including fermentation products, with a focus on agri-food and sustainable materials as well as formulation in the topic of interest here. Operating at TRL4-7 and up to 8 in the 10KL demonstration fermentation plant.

2. Please provide an overview of your work/supply relating to precision fermentation.

A service provider - fee-for-service, collaboration projects, providing range of bioprocess technologies for upstream/fermentation/downstream including extraction and formulation. Having capability and expertise in non-industry-standard cell lines for fermentation, so not concentrating only on yeast or Escherichia coli etc.

3. At what stage is this development of PF proteins?

Expertise in proteins having been prepared for feed, at the stage of submitting to feed trials. Also, in terms of PF for food - at process development stage for companies. Supporting many start-up companies in this area to support their understanding of the regulatory aspects of their product.

4. Have you considered the allergenicity of your protein or product?

Colleagues working in technical roles at the company are mindful of allergens. Allergens are a consideration for their customers and this stakeholder can work with/advise customers relating to this including referring to experts. Currently have laboratory scale food certified facilities, but not the pilot and demonstration plant. However, is currently working on getting the pilot plant upgraded to food. Have a dedicated quality manager and food team lead who are responsible for reviewing processes and carrying out a Hazard Analysis and Critical Control Point (HACCP) if a food or feed project requires it; i.e. for onward testing. Products are provided with a disclaimer that products are meant for research purposes only, so risks of allergen exposure due to consumption are mitigated by this. All products produced with the stakeholder then go on for further processing elsewhere, so not dealing with final products, but important the production of the product at the stakeholder meets the right standards where needed.

5. Have you considered how allergens will be labelled on your products?

Not within stakeholder's remit since the final products are a consideration for their customers and stakeholder is involved in the development. However, if stakeholder were to produce product for the market this would be in line with expected standards and knowledge of any allergens as appropriate. Stakeholder works with customers on allergen labelling. Products prepared at the stakeholder facility are certified with a disclaimer that they are for research purposes only so risks of allergen exposure due to consumption are mitigated in this way.

6. If your business already trades in precision fermentation (overseas), what allergen labelling is used on those products?

Not yet determined.

7. Are you able to describe your manufacturing procedure? We realise that there may be concerns about IP here, so please discuss in as much or as little detail as appropriate. The intention is to gain information regarding conditions that might impact on the structure, and potentially on the allergenic potential of the proteins. Are you aware of this?

Although all PF products currently prepared on the premises are marked as for research purposes only, products are prepared in food-grade laboratories which hold FSSC 22000 scheme documents in Food Safety Management and works to Hazard Analysis and Critical Control Point (HACCP) principles. Not all products prepared by the stakeholder are PF products, but most processes involve fermentation and isolation with a suite of post-production processes, such as drying facilities. Products may require further processing after preparation at the stakeholder. Most processes use customer methodologies and protocols, but with some input from stakeholder. Many of the processes are protected by Intellectual Property. Other processes are co-developed by the stakeholder with a customer or collaborator as part of a funded project. The stakeholder can collect product data for the customers that may be included for novel food applications.

8. Do you have an allergen management regime for alternative protein products? Prevention of cross-contamination?

It is not entirely relevant, as allergenicity is the concern of the customer post-development. However, the stakeholder ensures they operate with the right procedures being utilised. Nevertheless, food manufacturing company-type processes are in place as far as possible as these are the ways in which customers will operate once their product is developed fully. To prevent cross-contamination, clean-in-place and other hygiene processes are followed to reduce cross-contamination risks. Products are certified with a disclaimer that they are for research purposes only so risks of allergen exposure due to consumption are mitigated by this.

9. Have you conducted any allergen testing of your product? Which method? And which allergenic proteins have you tested for?

It is not currently a requirement, as this is mainly a customer's concern, but would likely outsource such testing. Works with customers to help understand the requirements and allergens are likely.

10. If conducting allergen testing, what is the testing regime? What factors do you consider when selecting which method(s) to use for testing?

Not directly applicable to the stakeholder, consideration only for stakeholder's customers once product processing is completed elsewhere, or if a customer asks the stakeholder to deliver allergen testing as part of the work. Would be outsourced if this were the case.

11. Do you perform your own testing or outsource? What factors do you consider when selecting a subcontractor for allergen testing? What is the LOD/LOQ?

Not directly applicable to the stakeholder, consideration only for stakeholder's customers once product processing is completed elsewhere, or if a customer asks the stakeholder to deliver allergen testing as part of the work. Would be outsourced if this were the case.

12. Have you encountered challenges in allergen testing of your product?

Not directly applicable to stakeholder, consideration only for stakeholder's customers once product processing is completed elsewhere.

13. What challenges do you anticipate in the allergen testing of your product or any finished product made with it (if product is an ingredient)?

Ideally would have fast turnaround times so as not to delay preparation of a next batch.

14. Are there any other challenges you face when considering the possible allergen content of your products?

Not directly applicable to stakeholder, consideration only for stakeholder's customers once product processing is completed elsewhere or if customer asks stakeholder to deliver allergen testing as part of the work. Would be outsourced if this were the case. However, as previously noted, if stakeholder does generate products that would enter the market, ensuring the appropriate methodologies and certifications were achieved would be paramount.

15. Do you have any thoughts about what would help allergen management in your sector of alternative proteins?

Fast allergen testing turnaround times and removal of cost limitations. Allergen testing will be required in order to gain product approval from FSA etc. once the products are finalised. Since most of the stakeholder's customers are innovators and hold IP, time to market will be important, so getting certified products fast will be essential. Collaboration in allergen testing for example could potentially provide a cost saving (sharing cost of multi-sample testing), but this is unlikely to occur unless IP protection is guaranteed, or products produced in academic labs are used as testbeds. PF products can differ from batch to batch so validation would be required, testing several PF batches of a product to check for allergenicity- requires cost effectiveness.

16. Are you aware of any other risks/considerations relating to allergy in precision fermentation?

No. Allergens could be as a result of ingredients or by-products.

17. Are you aware of any precision fermentation egg protein products on the market or likely to be introduced soon?

No.

18. Are you aware of any egg precision fermentation products that have been withdrawn from market?

A high number of the companies in the alternative proteins area are very good at marketing and it is conjectured that they may have over-promised the 'ready-for-market' nature of their products. Hence why not so many products are in the market yet. It is conjectured that products which were marketed as 'available' or 'available soon' may in fact still be a way from reaching market and funds or capability may have slowed progress. Would be great to see products in the market to build confidence though.

19. Are you aware of why the market of milk precision fermentation products is more advanced than the egg sector?

It is conjectured by the stakeholder that, since milk tends to be included in a higher number of products compared to egg, that more research effort will be applied to developing PF milk protein compared to egg protein. The production of PF milk also would benefit lactose-intolerant consumers so there may have been a high drive for innovation for milk compared to egg.

20. Do you have any points that you would like to raise, or further comments?

There is a need for regulation and for sustained public funding to drive UK innovation in fermentation products. The sector would benefit if the process to gain approval was faster to create genuine traction in this sector in the UK. Currently, UK innovators are taking their innovations to other countries e.g., the Netherlands where development is perceived as more supported due to the large pots of dedicated Government funding and there is a central hub for such innovations in food. This stakeholder feels that it is important that the UK provides a supportive, connected, sustained environment for the development of novel foods to develop, produce and market alternative protein and to maintain manufacturing capability in the UK.

5.4.4. Questionnaire responses from Respondent C (Regulatory Advisor)

1. Please give an overview of your business

A scientific and regulatory advice to help alternative protein / novel foods companies obtain regulatory approval in the global market. We deal with the allergenicity aspects of the dossier. Interestingly, other countries (Singapore, US, Australia, New Zealand) are more conservative regarding allergenicity risks of alternative proteins. They have more questions about this than EFSA. They are more concerned about understanding thresholds for allergenicity (mainly in relation to cultivated meat currently, but applicable to others) and levels of exposure. In Europe, labelling as “may contain” seems acceptable, but those other countries want to understand triggers and overall risk on population (increased sensitisation in population). A good example of this would be kiwi fruit - it was introduced as a new fruit, and over time people were sensitised and started showing allergy to it.

2. Please provide an overview of your work/supply relating to precision fermentation and/or insect protein

Not applicable

3. At what stage is this development of alternative proteins

Customers are currently producing at pilot scale, generating small batches for their analytical requirements for dossier and working on scaling up in parallel.

4. Have you considered the allergenicity of your protein or product?

Yes. They follow a regulatory road map and advise early on about allergenicity risk assessment. Advise to use a tiered approach, starting with a literature review, followed by bioinformatics sequence analysis and homology to known allergens, then in vitro digestibility tests and ELISA if any potential cross-reactivity to known allergens found.

5. Have you considered how allergens will be labelled on your products?

Yes. Products containing PF egg / milk proteins will be labelled as containing egg / milk. Insect products will be labelled as containing insect protein and the potential to cause allergic reaction in individuals with allergy to crustacean. However, the more problematic aspect is the potential presence of unknown allergens in these products, which may be derived from host cells or components in the culture medium.

6. If your business already trades in precision fermentation or insect protein, what allergen labelling is used on those products?

Not applicable

7. Are you able to describe your manufacturing procedure? We realise that there may be concerns about IP here, so please discuss in as much or as little detail as appropriate. The intention is to gain information regarding conditions that might impact on the structure, and potentially on the allergenic potential of the proteins. Are you aware of this?

PF proteins produced in bacteria will lack the normal post-translational modifications (PTM) found in the animal-derived counterparts. Fungi and yeast, as eukaryotes, will produce more PTM, but they may still not be the same, and this can influence the allergenicity potential of a protein. However, the main concern about PF are allergens from the host cells or the medium. This is because usually the protein is not produced as a purified protein but instead, separation techniques such as filtration are used to obtain a suitably enriched fraction. High value products such as milk oligosaccharides will be highly purified, but for egg / milk proteins this is difficult (it is very expensive and reduces yield). PF proteins can be secreted into the medium or accumulated intracellularly. In the first case, the medium will be filtered to remove host cells, but proteins in the medium other than the PF protein will be present. Where the PF protein is intracellular, the cells will be lysed and then filtered, and host proteins

will likely be present in the final product, at least in the first generation of protein. When doing the allergenicity assessment, they review all the ingredients used for production and flag any potential risks, for example, if the medium contains ingredients produced in soya, they advise testing for soya residues. For insects, contamination of insect protein with potential allergens from the substrate.

8. Do you have an allergen management regime for alternative protein products? Prevention of cross-contamination?

We advise customers of risks from all the inputs. Companies manage these risks via HACCP and quality management processes.

9. Have you conducted any allergen testing of your product? Which method? And which allergenic proteins have you tested for?

Not directly. The approach that is recommended to customers is a tiered approach, starting with a literature review, followed by sequence analysis and homology to known allergens, then in vitro digestibility tests and ELISA if any potential cross-reactivity to known allergens found. Full proteomics analysis is useful for companies for their own development activities, but it produces large volumes of data that can be difficult to interpret, so it is not suitable for inclusion in dossiers, at least while there is no clear guidance on it. An alternative and more focused approach involves using blood serum from allergic patients, although this is expensive and only applicable when the allergy / cross-reactivity in question is known. In vitro digestibility studies can give an indication of the potential for a protein to cause allergic reaction, therefore, it is useful as part of the tiered approach. Performing proteomics analysis of the non-digestible fraction can help identify potential allergens.

10. If conducting allergen testing, what is the testing regime? What factors do you consider when selecting which method(s) to use for testing?

Not conducting testing, but some advice to customers, should their product warrant testing for potential cross-reactivity to known allergens, would be to select a method that has been validated in a relevant matrix for which LOD and LOQ have been established.

11. Do you perform your own testing or outsource? What factors do you consider when selecting a subcontractor for allergen testing? What is the LOD/LOQ?

Not directly applicable. But most customers have a testing lab in mind, but it is difficult to find a lab that can do all that is needed for each specific case, right ELISA tests, right matrices, validated methods, etc.

12. Have you encountered challenges in allergen testing of your product?

Customers are at early stage, not in testing phase yet. Compositional and purity data is the priority at this stage.

13. What challenges do you anticipate in the allergen testing of your product or any finished product made with it (if product is an ingredient)?

Finding the right partner to do not just the test, but to help with interpretation of results and devise strategy to progress. Lack of validated methods is a challenge, as is the fact that there are not set approaches established by regulators. It is up to the company to decide on the approach and the expense of the evaluation of allergenicity. Lack of reference materials can be a problem for certain proteins/products.

14. Are there any other challenges you face when considering the possible allergen content of your products?

Knowing the levels of concern (thresholds for allergic reactions) would be very helpful. It is challenging to know how to label products, for example, if there is a small percentage of an allergen in a protein ingredient, and the ingredient is used at a small proportion in a finished product, how to understand if this is still an allergy risk.

15. Do you have any thoughts about what would help allergen management in your sector of alternative proteins?

More guidance from regulators. More work on thresholds. It is easier to label with “may contain” as testing can be very laborious and a big burden.

16. Are you aware of any other risks/considerations relating to allergy in precision fermentation, insect protein and other alternative proteins?

Cultivated meat - cells may express different proteins in culture.

17. Are you aware of any precision fermentation egg protein /products on the market or likely to be introduced soon?

Several companies working on it, but not ready to apply for approval. Every company in US have got GRAS status for one product, it will be interesting to see if they submit a dossier for approval in Europe.

18. Are you aware of any egg precision fermentation products that have been withdrawn from market?

No.

19. Are you aware of why the market of milk precision fermentation products is more advanced than the egg sector?

Not sure. It may be to do with technical reasons, i.e., egg proteins may be more difficult to produce by PF.

20. Do you have any points that you would like to raise, or further comments?

No further comments.

5.4.5. Questionnaire responses from Respondent D (Research Laboratory, Precision Fermentation)

1. Please give an overview of your business.

The organisation is Europe's leading Contract Research Organization in the safety assessment of microbial food and feed products. It helps new sustainable food & feed solutions get approved and reach the European market. Our professionals are vastly experienced in whole-genome sequencing and our pipeline is optimized to fulfil the safety assessment requirements of EFSA. We have extensive expertise and experience in what the EU, the European Commission and EFSA demand of the safety of industrial microbiology products. Our consulting team give advice on a wide range of topics on food safety. The laboratory services include antimicrobial susceptibility testing, production of antimicrobials, cytotoxicity testing, absence of cells and DNA in fermentation products.

2. Please provide an overview of your work/supply relating to precision fermentation and/or insect protein.

Concerning precision fermentation, the organisation serves the customers, who already have the products, in multiple ways to help their products on the markets within EU and globally. It starts from gap analysis (what is

required) and includes whole genome sequence analysis, many laboratory analyses, literature searches, and a whole dossier of preparation and submission to the authorities. Much of its work also includes consultation during the R&D phases.

3. At what stage is this development of alternative proteins?

Our customers have pilot plant products for which analyses are carried out for the preparation of dossiers to be submitted to the authorities.

4. Have you considered the allergenicity of your protein or product?

Yes, this is something we always do for every product.

5. Have you considered how allergens will be labelled on your products?

No, so far this has not been requested.

6. If your business already trades in precision fermentation (overseas) or insect protein, what allergen labelling is used on those products?

Not relevant for the organisation.

7. Are you able to describe your manufacturing procedure? We realise that there may be concerns about IP here, so please discuss in as much or as little detail as appropriate. The intention is to gain information regarding conditions that might impact on the structure, and potentially on the allergenic potential of the proteins. Are you aware of this?

We occasionally deal with manufacturing procedures. Our focus in such cases is to evaluate if potential allergens are introduced in the final product during processing. We are aware that downstream processing can have an impact on the allergenicity of a protein.

8. Do you have an allergen management regime for alternative protein products? Prevention of cross contamination?

Our laboratory deals with the customer's samples with utmost care, and we have had no cases of allergy from alternative protein products.

9. Have you conducted any allergen testing of your product? Which method? And which allergenic proteins have you tested for?

Our primary approach is bioinformatics, which we recommend to our customers already at the R&D phase. Depending on the case, we run the allergenicity analysis on a particular protein or the whole genome-derived proteome using multiple allergen databases and international guidance (CODEX). We also carry out literature searches on the production organism and on the individual proteins, considering route of exposure, the way the allergenicity was verified etc.

10. If conducting allergen testing, what is the testing regime? What factors do you consider when selecting which method(s) to use for testing?

See the answer above.

11. Do you perform your own testing or outsource? What factors do you consider when selecting a subcontractor for allergen testing? What is the LOD/LOQ?

Our bioinformatics team carries out the analysis, complemented with expert assessment for reporting the results.

12. Have you encountered challenges in allergen testing of your product?

The main challenge is to assess the whole genome-based proteome for allergens, because the outcome of the bioinformatic analysis is often a long list of hits (potential allergens). Another challenge is the interpretation of the results, when it comes to the effects of downstream processing to the allergenicity of the protein. This is mainly due to the lack of data/ knowledge. Also, the actual way of use of the protein can influence allergenicity but this is mostly out of our competence.

13. What challenges do you anticipate in the allergen testing of your product or any finished product made with it (if product is an ingredient)?

No comments.

14. Are there any other challenges you face when considering the possible allergen content of your products?

See above.

15. Do you have any thoughts about what would help allergen management in your sector of alternative proteins?

No comments.

16. Are you aware of any other risks/considerations relating to allergy in precision fermentation, insect protein and other alternative proteins?

All components introduced in the fermentation and downstream processing need to be assessed for allergenicity, including the fermentation medium. Depending on the genetic construct, the final product may or may not contain impurities such as other proteins from the production organism. If the genetic construct contains tools for affinity purification of the specific protein, it can be collected as a rather pure product. The number of other proteins also depends on whether the protein of interest is intracellular or secreted from the cells. We usually carry out SDS-PAGE analysis to find out the protein profile of products, although this gives only rough estimation of the purity / protein composition.

17. Are you aware of any precision fermentation egg protein /products on the market or likely to be introduced soon?

Yes, but it depends on the definition of “soon”. However, they are in the strong developmental phase.

18. Are you aware of any egg precision fermentation products that have been withdrawn from market?

No.

19. Are you aware of why the market of milk precision fermentation products is more advanced than the egg sector?

We see both fields progressing. The difference may be accidental and related to the interest of the companies. We see no technical reason for it.

20. Do you have any points that you would like to raise, or further comments?

Not for the moment.

5.4.6. Summary of key aspects highlighted by stakeholders

- Innovators and contract research laboratories are aware of allergenicity risks
- In the absence of comprehensive guidance regarding allergenicity assessment for novel foods application, the recommended approach is a tiered process that includes different types of data: literature, bioinformatics to analyse protein sequence homology to known allergens, *in vitro* digestibility, IgE reactivity where possible. This is a very lengthy and expensive undertaking, and for the proteins considered in this project, since allergenicity is assumed, the current position of innovators is to label the products as “contains milk or egg protein” or “contains insect protein and may cause sensitivity/allergy in consumers with a sensitivity to crustacean”.
- Producers are aware of cross-contamination issues and generally existing HACCP and hygiene protocols are used in manufacturing to minimise risks.
- The stakeholders consulted are not currently carrying out any allergen detection test, as the products will be labelled as containing allergen. However, when asked about choosing methods, aspects that would be determining include validation in the right matrices, certification, LOD/LOQ, cost and speed.
- For insects, universal cross-reactivity with crustacean is assumed, and it has been remarked that more research is needed into this, and species-specific methods should be developed as it is plausible that sensitive individuals will not be allergic to all insect species, and hence, such testing ability would make the market less restricted.
- Fit-for-purpose tests for insect detection in factories are required as the risk of cross-contamination makes potential end producers reluctant to work with insect protein and is currently a barrier. Such tests would enable confirmation of absence of insect protein in areas where other products may be manufactured.
- Details of processing conditions that might affect allergenicity of the proteins are lacking due to IP considerations. However, from the literature review and allergen expert consultations, it can be concluded that this would not be very informative as there is no generic trend regarding effects of processing and each protein / product may be differently impacted.

- In addition to the allergenicity potential of the novel protein, by-products must be considered. These include all the inputs for fermentation and proteins from the host cells in the case of PF, and residual material from the substrate that insects have been reared on.
- Further research is needed into both, understanding allergenic potential and detection methods.
- Further regulatory guidance and sustained funding are required to support these developments.

5.5. Final Conclusions to Consultation Section

The introduction of alternative proteins is imperative to feed the world's increasing population. The safety of these alternative proteins is paramount and allergy forms a part of the risk assessment. There is no simple and rapid solution to determine the allergenicity of alternative proteins. As consumption in the West grows, clinical data will naturally be generated to feed into risk data. In the meantime, there is a lack of clinical data and clinical trials are needed on human subjects.

Concerning PF products, we can only estimate the effect of fermentation, including the effect of the microbial host, the culture medium and processing conditions on aspects such as post-translational modifications to the protein which could affect allergenicity. While it is unlikely that PF proteins will be more allergenic than their conventional dairy counterparts, this requires testing. Due to the nature and variability of the production process, the allergenicity of PF products, and also the detectability of allergens in PF products, is product-dependent and data will be required for each individual product.

Regarding insect protein, over time, as consumption increases, weight-of-evidence data will become available regarding the risks relating to known insect allergens and *de novo* allergens. There is no simple solution to reducing the allergenicity of insect protein other than complete hydrolysis which will impact on the nutrition of the product. Alignment of allergy for insect consumers is assumed with that of crustacean-sensitive consumers, with cricket protein for example in the West being labelled as "People who are allergic to molluscs, crustaceans or dust mites may have an allergic reaction to crickets." The trade body for insect protein would welcome testing to determine which crustacean-sensitive consumers are likely to be sensitive to which insects and would welcome more research into reduction of allergenicity by processing methods. Prior to marketing, producers are required to risk assess all ingredients involved in production. This comprises a tiered approach including literature review,

bioinformatic sequence alignment of ingredients with that of known allergens, *in vitro* digestibility studies and ELISA testing of cross-contaminants.

Although not an allergen, the insect and crustacean carbohydrate chitin is involved in human immune response and needs to be better understood. Although present in crustacea, chitin is not usually consumed as it is removed in the exoskeleton before consumption. However, chitin in insects is not so easily separated from the protein and is expected to be consumed.

Stakeholders highlighted that speeding up the approvals process for novel protein and regulatory support and advice, including information on validated methods for allergen testing and regarding allergen elicitation levels would drive innovation in the alternative protein field. Stakeholders also highlighted that it is much easier to label novel foods with 'may contain' allergen phrases rather than undergo the laborious and burdensome testing. Finally, stakeholders felt that it is important that the UK provides a supportive, connected, sustained environment for the development of novel foods to develop, produce and market alternative protein and to maintain manufacturing capability in the UK.

6. Section 3. Testing section of report for the general scientific audience

6.1. Sourcing precision fermentation and insect protein products

Testing methods are available to detect allergens in conventional foods. The aim of the remainder of this project was to gain insight regarding:

- whether methods currently applied to detecting allergens in dairy milk products could be applied to detect milk proteins produced by PF in commercial products.
- whether allergens in insects which are homologous to many allergens in crustacea can be detected using methods currently applicable to crustacean foods.

Alternative protein products were sourced to later test to determine whether milk allergen proteins or insect allergens in alternative protein products could be detected by available allergen testing methods developed for allergen detection in conventional foods. The project aimed to source products containing milk and egg proteins made by precision fermentation, and also to source insect protein products, for allergen testing. When the project was designed, several products containing milk

and egg protein made by precision fermentation were being marketed online. However, when sourcing of these products was attempted, it became evident that some, although having an online presence, had been withdrawn from sale or were never in fact on sale but rather were still in the development stage but with product marketing in place. It appears that other products, particularly those containing PF egg protein, had never been commercially available despite marketing activities. No products containing PF egg protein were available to source but products containing PF milk protein were sourced as detailed below.

6.2. Sourcing of products containing milk protein produced by precision fermentation

Following an online search of internationally available products containing PF milk protein, three products were available. These were sourced, as detailed in Table 2 from USA. The sourcing of raw PF material from PF innovators was also attempted since it was intended to spike products with these for quality control purposes, to spike into the test samples in order to gain insight into the limit of detection (LOD) of the kits and matrix interferences. However, innovators were not forthcoming in providing this raw material.

Table 2. Table detailing the precision fermentation milk products sourced for testing

Sample descriptor	Solid/Liquid	Source country
Milk beverage, strawberry flavour	Liquid	USA
Cake mix, vanilla	Solid, powder	USA
Brownie mix, chocolate	Solid, powder	USA

6.3. Sourcing of products containing insect protein

Regarding insect protein, a much wider range of products was available and nineteen products were sourced from commercial sources in Malaysia, UK and Fera Science's Insect Rearing Unit (FIRU). The range of products was chosen to maximise the number of species of edible insect and to maximise the types of processing (roasted, blanched, salted, dried, whole, powdered, solo or incurred in other food matrices, flavourings added, etc.) in order to challenge the testing methods later in

the project with a wide range of insect protein types, types of processing and potential matrix interferences. Processing methods are known to have a positive or negative impact (depending on the individual kit) on the performance of ELISA kits (Grundy et al., 2022) hence the inclusion of a wide range of processing types to challenge the kits. A range of insect species were sourced. Since crustacean allergen testing methods respond

in different manners to different crustacean species, requiring a conversion factor to convert the data yielded to apply to a species if known, it is hypothesised that the methods may show different suitability depending on insect species. A range of insect species was therefore sought for testing. While four insect species are permitted to remain on the market in Great Britain from 1st January 2024 following EU Exit, namely *T. molitor* (Yellow mealworm), *A. domesticus* (House cricket), *H. illucens* (Black Soldier Fly) and *Gryllodes sigallatus* (Banded cricket) ,only the first three of these species were available to purchase at the time of sourcing, with Banded cricket products showing as out of stock from retailers. The products sourced are detailed in Table 3.

Table 3. Insect samples sourced

Fera Reference number	Sample name	Species	Type of processing, as detailed on label or company website
S24-000961	Egg Flavour Whole Roasted Larvae	Black Soldier Fly, *H. illucens*	Roasted
S24-000968	Lightly salted locusts, crickets & mealworms	*L. migratoria, Acheta domesticus, T.molitor*	Freeze dried
S24-000969	Salt & vinegar mealworms	Yellow Mealworms *T.molitor*	Freeze dried
S24-000970	Maple wood smoked crickets	House Cricket, *Acheta domesticus*	Smoked over maple wood
S24-000971	Whole roasted locusts	Migratory Locusts, *L. migratoria*	Roasted
S24-000972	Barbecue Crickets	House Cricket, *Acheta domesticus*	Freeze dried
S24-000973	Teriyaki crickets	House Cricket, *Acheta domesticus*	Freeze dried
S24-001591	Black Soldier Fly larvae, fed on regular 'chick starter crumb*' diet (FIRU)	Black Soldier Fly, *H. illucens*	Blanched, 80°C for 3 minutes
S24-001592	Yellow mealworms, fed on regular 'chick starter crumb*' diet (FIRU)	Yellow Mealworms, *T.molitor*	Blanched, 80°C for 3 minutes
S24-001593	Black Soldier Fly larvae, fed on white bread diet (FIRU)	Black Soldier Fly, *H. illucens*	Blanched, 80°C for 3 minutes
S24-000960	Cricket Protein Penne	House Cricket, *Acheta domesticus*	Vacuum dried
S24-000962	Crickets	House Cricket, *Acheta domesticus*	No information available
S24-000963	Mealworms	Yellow Mealworms, *T.molitor*	No information available

Fera Reference number	Sample name	Species	Type of processing, as detailed on label or company website
S24-000964	Whole Natural Roasted Crickets	House Cricket, *Acheta domesticus*	Roasted
S24-000965	Cricket protein bar containing cereal, nut and fruit	House Cricket, *Acheta domesticus*	Freeze dried
S24-000966	Just Crunchy Locusts	Migratory Locusts, *L. migratoria*	Dried
S24-000967	High Protein Cricket Powder	House Cricket, *A. domesticus*	Freeze dried
S24-000974	Cricket cookie mix	House Cricket, *A. domesticus*	Freeze dried
S24-000975	High Protein Cricket Pancake Mix	House Cricket, *A. domesticus*	Freeze dried

*Chick starter crumb was a commercial feed comprising wheat, wheatfeed, dehulled soya meal, full fat soya, calcium carbonate, mono-calcium phosphate, soya oil, sodium chloride, sodium carbonate (no compositional levels provided) plus vitamins.

7. Section 4. Experimental Work

The experimental work involved two stages which ran concurrently. One stage (Section 4a) involved an ELISA kit comparison study during which four different kit providers received PF milk protein and insect protein samples for testing and applied their milk-sensitive and crustacean-sensitive allergen testing kits to determine whether milk and insect allergens could be detected. The other stage (Section 4b) involved in-house testing of the PF milk protein and insect protein products with a range of test kits.

Allergen testing can be performed using a range of technologies with ELISA being the most established and PCR and mass spectrometry also being used along with various other methods which are in various stages of development, as reviewed previously (Grundy et al., 2022). ELISA technology, based on the detection of allergenic proteins by antibodies, is the most popular form of testing for most allergens, including milk and crustacean allergens, due to the unrivalled sensitivity, relative low cost, ease of use and requirement for relatively low-cost apparatus involved. ELISA technology, along with the high cost and less sensitive mass spectrometry methods for allergen detection, is the preferred technology at present for the future (for example compared to PCR) since this technology has the current capability or future potential (depending on the advancement of a given kit) to report the level of allergen in 'mass of allergenic protein per kg of food.' This format of reporting is the preferred format according to the FAO/WHO guidelines since it is more informative when considering the risk of undeclared allergens contaminating foods to allergen sufferers. Many kits currently report in 'mg of allergenic food

per kg food' (meaning, 'mg of peanut per kg of product) and other kits report in 'mg of food protein per kg of food,' meaning, for example, 'mg of peanut protein per kg food,' but not relating to solely to the proteins which elicit allergy. Neither of these reporting formats are as informative to the food industry as 'mg of allergenic protein per kg food'. The health risk to allergic individuals in consuming any food (be it a conventional food or an alternative protein food) with undeclared presence of allergens is severe, in the worst cases resulting in death. The data yielded by ELISA kits manufactured by different providers for a given allergen can vary widely, in part due to the variation in the antibodies which form the basis of the kits. Currently available ELISA kits, developed for the detection of allergens in 'conventional foods', were tested to determine their suitability to detect their counterpart alternative proteins. If successful, this suitability would potentially provide methods to determine the presence of potential allergens in alternative proteins for future use in the UK.

PF milk products contain one or a selection of proteins present in dairy milk, such as β-lactoglobulin (β-LG) and casein, and ELISA kits are available to detect these proteins in conventional dairy products. Tropomyosin, arginine kinase, myosin light chain, larval cuticle proteins and paramyosin are proteins in crustacea which are known to elicit allergic response in sensitive consumers. These proteins also exist in other invertebrates including insects. ELISA kits for crustacean allergens are likely to detect insect protein based on amino acid sequence homology and the observed cross-reactivity to IgE between crustacean and insect tropomyosin.

7.1. Section 4a. ELISA kit Comparison Study

Several ELISA test kit providers were invited to participate in the ELISA kit Comparison Study. Those whose kits were capable of reporting according to the FAO/WHO preferred format discussed above were invited along with other ELISA kit manufacturers. Four ELISA test kit providers took part in the ELISA kit comparison study and tested the samples detailed in Tables 4 and 8.

7.1.1. ELISA kit Comparison Study: Testing of products containing precision fermentation milk protein

The four participant laboratories which took part in the ELISA kit Comparison Study were invited to test the PF milk protein samples using their kits which are sensitive to milk allergens, alongside provided positive and negative Quality Control (QC) samples. Such kits can include ELISAs sensitive to β-lactoglobulin allergen alone, or casein allergen alone and 'entire' milk kits which tend to comprise antibodies which are sensitive to both casein and β-lactoglobulin allergens.

7.1.2. Precision fermentation milk samples for the ELISA kit Comparison Study

The products which contained PF milk protein, along with positive and negative quality control samples, were prepared for testing. Solid (powder) and liquid products were mixed and then aliquoted into sub-samples for dispatch to the participating laboratories and also for later in-house testing at Fera Science. The content of all samples and QCs was anonymised prior to dispatch to the participating laboratories, providing only sample reference numbers. Participants were requested to analyse the samples in duplicate and to include their own positive and negative QC standards (in addition to those anonymously provided) when performing the analyses for performance assurance reasons. As described above, the data yielded by the test kits of different manufacturers can yield widely varying data, depending on variables including the antibodies used. While we were in possession of data generated by some of these kits when used in Fapas® proficiency testing rounds, we did not have data for all kits. It was therefore challenging to provide test materials which were applicable for each test kit, without participants needing to perform pre-screens to determine whether dilutions of samples were required prior to re-testing. The ELISA kit Comparison Study aimed for participant laboratories only to require to perform one set of tests rather than requiring pre-screens to reduce time requirements in order to help to maximise uptake in the participation.

The samples dispatched for testing are detailed in Table 4.

7.1.3. Results – ELISA kit comparison study of PF milk products

The results for each of the four laboratories are shown in Tables 5 – 7 (Appendix 3). The laboratory names, test kit identifiers, LOD and LOQ details have been removed to maintain the anonymity of the kits. Data are included in the table as provided by the participants, for example, in terms of whether levels were detected below LOD or below LOQ. Laboratory 2 did not participate in the 'entire' milk ELISA testing as that particular laboratory do not perform the 'entire' milk assay on site. All laboratories correctly reported the data for the positive and negative quality control samples (detected or not detected) in all instances.

For the casein-sensitive ELISA tests, all four laboratories detected casein in sample S24-000588. This was somewhat surprising since whey protein (which includes β-LG) was declared on the label for each of the PF samples but not casein. Further investigation, possibly using mass spectrometry, would be interesting to identify which proteins are present to begin to understand whether they are interfering with the test kit and why.

Regarding possible matrix interferences for the casein-sensitive kits, all four laboratories detected milk from the dairy milk component of the positive QC reference material in the matrix-matched quality control sample. While the LOQ have been anonymised in this report, from the data received, it is apparent that, at least for one of the laboratories, there has been a possible matrix effect which has resulted in a reduced yield for the matrix-matched sample of S24-000589 by 56%. Further study, in addition to the current small study, is required to determine any matrix effects of the PF samples on the performance of the test kit, but these data suggest possible matrix interferences.

For the β-LG-sensitive ELISA tests, all four laboratories detected β-LG at above the LOQ of the kit and, as would be expected from this result, above the LOQ for the matrix-matched samples. These kits detect β-LG in PF milk products. Further study is required to determine any matrix effects of the PF samples on the performance of the test kit.

For the ELISA kits which are sensitive to 'entire' milk, three laboratories participated in this study. All laboratories reported detection of milk proteins at above the LOQ of the kit for the PF samples and for the matrix-matched sample. Given that these kits are sensitive to both β-LG and casein, and given the data above showing that β-LG, and in some cases, casein, was detected, this was expected. A full validation study would be required to determine any matrix effects of the PF samples on the performance of the test kits. However, matrix interferences were tested using an 'entire milk' kit in a small study by Fera Science, as described in more detail later (including Table 11 (Appendix 3)). The outcome of this small study was that matrix interferences seemed to be very low level for two of the samples just above the 20% tolerance for the remaining sample. A larger validation study would be required to provide more data. This was outside of the scope of this project.

Table 4. PF milk products tested by the participating kit manufacturer laboratories

Sample	Material sample number	Material type (not disclosed to participants)	Sample type
1	S24-000589	PF milk beverage, strawberry flavour	Liquid
2	S24-000587	Cake mix containing PF milk, vanilla	Solid
3	S24-000588	Brownie mix containing PF milk, chocolate	Solid
4	S24-000624	Negative Quality Control Sample	Solid
5	S24-001599	Positive Quality Control Commercial Reference Material, Cake Mix, containing dairy milk powder	Solid
6	S24-001984	Matrix-matched sample: milk beverage, spiked with a known level of Reference Material	Solid plus liquid

7.1.4. Conclusions to ELISA kit comparison study of PF milk protein products

Kits are available which have the capability to detect PF milk proteins in finished products. Further study is required to determine any matrix effects of the PF samples on the performance of the test kits. Unexpectedly, casein was detected in at least one of the PF milk products, namely sample S24-000588. It would be interesting to investigate this result by an alternative technology such as mass spectrometry, to investigate whether the casein-sensitive ELISA kits are yielding a false positive result for this sample. As discussed above and as expected following previous research (FSA Project FS900246), the data from different kits varies widely, for example by a factor of 30 for some of the β-LG kits' data. It would be interesting to understand the reason that the kit used by Laboratory 4 reported positive data for casein in all three samples. One possible explanation is that the antibody which forms the basis of the kit was raised to a casein extract which contained trace amounts of β-LG and that it is in fact the β-LG which is being detected in these samples.

7.2. ELISA kit Comparison Study: Testing of products containing insect protein

The same four laboratories participated in the ELISA kit comparison study for insect protein products as did for PF milk products. As discussed above, due to the homology of many allergenic proteins between insects and crustacea, and due to the lack of current development of kits specific for insect allergens, insect protein samples were analysed by ELISA kits which are sensitive to crustacean allergens. The samples tested during the ELISA kit comparison study are shown in Table 8.

7.2.1. Insect protein samples for the ELISA kit comparison study

Commercial samples containing insect protein were analysed during the ELISA kit comparison study. In addition, to gain some insight relating to the effect of food matrices on the outcomes of testing, and into the LOD of the kits, incurred positive control samples were prepared, as detailed below. While a raw PF material was not available for preparation of incurred milk products, insect material was available for this purpose.

7.2.2. Preparation and extraction of in-house quality control materials incurred with insect protein

Incurred food samples were prepared to replicate the levels of insect protein in the sample that could be found in baked goods products on sale. Research suggests that the level of insect flour in baked goods may be as low as replacing 5-15% of the wheat flour with an insect flour such as that of *T. molitor* (yellow mealworm) or *A. domesticus* (house cricket) in baked goods. Depending on the type of baked good, at higher levels of insect protein, the texture of the product can be negatively affected (González et al., 2019), (Kowalski et al., 2022), (K. Khuenpet, 2020).

Incurred food samples were prepared to replicate cookies in which 5% and 15% of the wheat flour was substituted with insect flour. This equates to a final insect level of 1.7% and 5.2% (w/w). Four edible insects are permitted in UK since 1st January 2024 (*Tenebrio molitor* (yellow mealworm), *Acheta domesticus* (house cricket), *Gryllodes sigillatus* (banded cricket) and *Hermetia illucens* (black soldier fly). To replicate a baked good product which might be available in the UK, and given the availability of house cricket flour to purchase, the insect flour used in the cookies was *A. domesticus* (house cricket, sample reference S24-000967). A further sample was prepared to challenge the limit of detection of the test kits, comprising 0.4% insect protein (w/w) along with a negative matrix-matched sample containing no insect flour. The cookie recipe for which wheat flour was substituted with insect flour is shown in Appendix 2. As described, the insect protein was folded into the raw dough of each cookie. For this reason, when cookies were analysed, the whole cookie was crushed and weighed, and the entire mass of cookie was extracted to avoid any potential homogeneity issues that might have occurred should a sub-sample had been taken. Since much smaller sub-samples are usually extracted for allergen testing (e.g. 1.0 gram), the volume of extraction buffer used was increased relative to the mass of the cookie, to standardise the ratio of sample:extraction buffer in line with the kit instructions.

While insect protein was detected at Fera Science in the incurred sample containing insect protein at 0.4% level weight-for-weight (w/w), it was detected above the LOD but below the LOQ which was 20.0 mg/kg ‘crustacean’.

Table 8. Insect protein products tested by the participating kit manufacturer laboratories

Sample	Material sample number	Material type (not disclosed to participants)
1	S24-000960	Cricket in penne pasta with sauce
2	S24-000962	Crickets
3	S24-000963	Mealworms

Sample	Material sample number	Material type (not disclosed to participants)
	Commercial insect protein sample	
4	S24-000964	Roasted crickets
5	S24-000965 Commercial insect protein sample	Cricket protein bar containing cereal, nut and fruit
6	S24-000966	Roasted locusts
7	S24-000967	Cricket protein powder
8	S24-000974 Commercial insect protein sample	Cricket cookie mix
9	S24-000975	Cricket pancake mix
10	S24-002047 Incurred Positive QC	Positive Quality Control material, cookie incurred with approx. 5% of the flour as cricket flour
11	S24-002048 Incurred Positive QC	Positive Quality Control material, cookie incurred with approx. 15% of the flour as cricket flour
12	S24-002046 Negative QC	Negative Matrix-Matched cookie Quality Control material
13	S24-002051 Incurred Positive QC	Incurred cookie, 1% cricket flour

7.2.3. Insect samples included in the ELISA kit comparison study

Some of the insect samples were only available for purchase in limited amounts (single packs of 12-20 grams) in one of the online shops. This meant that it was not possible to provide each participant laboratory with the minimum quantity of material required, and therefore, these products were only tested at Fera (Stage 5b) rather than in the ELISA kit comparison study. Also, only commercially available insect samples were included in the ELISA kit comparison study. Insects reared at FIRU were not dispatched to participants due to extra safety considerations in handling insects which have not been prepared for consumption and may contain dusts. The insect samples analysed in the ELISA kit comparison study are detailed in Table 8. Solid products were milled and then all samples (powders and liquids) were mixed by hand and aliquoted into sub-samples to dispatch to the participating laboratories and also for in-house testing at Fera Science.

Regarding the aims of this part of the study, samples were analysed at the standard dilution of the kit to determine if crustacean-equivalent proteins were detected. The incurred sample comprising 0.4% insect protein was analysed to gain some insight relating to the LOD of the kits for insect protein. Participants were invited to perform further dilutions of the 0.4% incurred cookie sample for this reason. However, as shown later in the results section, the participant kits did not demonstrate the capability to detect this low level of insect protein.

7.2.4. Results – ELISA kit comparison study of insect protein products

The data (detected or not detected) have been anonymised in terms of the ELISA kit details (including kit name, product number, LOD and LOQ) and are shown in Table 9 (Appendix 3).

All four laboratories detected crustacean protein or tropomyosin in all commercial insect samples and the samples supplied by FIRU. Despite Fera Science detecting insect allergen in the incurred cookie samples comprising 0.4-5.2% insect protein (with a different test kit used compared to the participants), three laboratories did not detect crustacean-equivalent protein in the incurred cookie samples. Laboratory 3 detected crustacean-equivalent protein in the incurred samples containing insect protein at the 0.4% and 5.2% levels but, surprisingly, not in the sample containing 1.7% insect flour. From the analysis at Fera Science, the level detected in the cookie comprising 0.4% insect protein was around the LOQ of Crustacean Kit A. For some of the kits, it is difficult to compare limits of detection as they have different reporting limits, reporting in either 'mg per kg crustacean' or 'mg per kg tropomyosin allergen (muscle protein)' and the concentration of tropomyosin in crustacea will differ significantly to that in insects due to the larger proportion of muscle in crustacea. Given the values that the four laboratories reported for the cricket flour used in the incurred cookies (S24-000967), some of the participant laboratories would not have been expected to detect insect protein in the cookies containing 0.4% and 1.7% insect as this would have fallen below their limits of detection, but it would have been within the detection limits of the kits to detect insect protein in the cookie incurred with 5.2% insect flour (data not included to protect the anonymity of all participants and related kits). It is not possible to establish the reasons for the lower sensitivity of the kits to insect proteins without further investigation. The most obvious reason is that these kits have been developed against crustacean proteins, and any response to insect proteins is due to cross-reactivity, which is expected given the high degree of homology between the crustacean and insect proteins of interest. Other potential factors such as matrix interference, or the effect of heat treatment, might have contributed to the cricket flour protein (reference S24-000967) in the cookies not being detected by these kits. According to the label, this insect flour had been freeze-dried, but not heat-treated, during manufacture but baking during cookie preparation may have altered to detectability of this protein by some kits. Full validation studies would be required to gain more confidence in the LOD and to provide a more detailed set of data on matrix interferences, which are outside of the scope of this project. It is promising to determine that these kits, for which the antibodies have been raised against allergens in crustacea, detect proteins in insect samples.

7.3. Section 4b In-house testing of the alternative protein samples

The samples detailed in Tables 2 and 3 were tested at Fera Science with a range of commercial ELISA kits to determine whether allergens could be detected. It had originally been intended to gain some insight into the LOD of the kits by preparing matrix-matched samples containing known levels of the alternative protein. However, since raw PF milk ingredients could not be sourced as hoped from PF innovators, this was not possible for the milk testing kits.

7.3.1. In-house testing of milk products for milk allergens

Following dispatch of the ELISA kit comparison study samples, information was noted regarding the milk in each of the PF milk products. The milk protein expressed during precision fermentation as declared on the manufacturer labels was declared as whey protein in each of the three samples, meaning that the samples may contain β-lactoglobulin but not casein. In agreement with the customer, the PF milk products were therefore tested with a range of kits; two kits sensitive to β-lactoglobulin and two kits sensitive to a mixture of β-lactoglobulin and casein proteins. The initial intention was not to test these PF milk samples by kits sensitive to casein protein alone since casein was not declared on the label. However, once the results of the ELISA kit comparison study were received and one laboratory reported detection of casein in all three PF milk samples for which casein was not declared, and all three laboratories reported casein in Sample S24-000588, one casein sensitive kit was used to test the samples in-house (results shown in Table 12 (Appendix 3)). A different manufacturer's casein-sensitive kit to that used by the inter-laboratory which reported casein in all three PF samples was used.

The kits were selected as far as possible (information provided by manufacturers permitting) to be based on different target proteins and using polyclonal antibodies to broaden the scope of the analysis, e.g. selecting kits raised against more than one allergen protein or kits based on polyclonal antibodies rather than monoclonal to maximise the scope of possible detection. A range of kits was used to further maximise the chances of success of determining kits which showed the capability to detect allergens in PF milk protein. All three samples were tested in duplicate, alongside positive and negative quality control samples plus the same matrix-matched sample as included in the ELISA kit comparison study. Insect protein samples which declared milk on the label were also included. Finally, insect protein samples for which no milk was declared on the label to test for false positive reporting for each kit included.

The methods used were according to the manufacturer's instructions and varied for each individual kit. Similarly, the LOD and LOQ of each kit differed and this data is not included in this report so as to anonymise the identity of the kits.

The results of the testing for milk proteins are shown in Table 10 (Appendix 3), showing that milk proteins were detected in all three PF milk samples and in each of the insect samples for which milk was declared on the label.

An additional study was performed to gain insight into the effect of the matrix of the PF samples on recovery of milk protein. Using Milk Kit C, PF milk samples were analysed alone and then spiked with a given ratio of positive control sample, determined as containing 14.1 mg/kg milk protein with this kit. The expected yield of milk protein (taken from the yield when measuring the sample, plus the yield of the amount of positive control sample added to the sample) was compared to the yield for the matrix-match spiked sample to determine the effect of the matrix on recovery. Please note that, due to the high yield of milk protein in the liquid milkshake sample (S24-000589) this sample required a 1+9 dilution prior to analysis in order for the yield to fall within the tolerances of the standard curve. As shown in Table 11 (Appendix 3), the matrix effect (change in recovery) in this study was 1.9% (negligible) for sample S24-000587 and was less than a 10% reduction in yield for sample S24-000588. The level of matrix effect in S24-000589 was at 23.6%. Further replicates would require testing to confirm these results of this small study. The accepted level of matrix effect (recovery) for ELISA is 80-120% (Lugos, 2019). While further study would be required on a larger number of sample replicates to fully validate the effect of matrix on recovery, these values do not show too large a change in yield to cause concern for the data in this small study. In addition to the matrix-match data generated in this study, seven insect samples for which no milk was declared on the label were included in this analysis. No milk was detected in any of these samples, so the false reporting result of this study was 0%. Again, the false reporting rate requires testing on a larger number of samples in a full validation exercise.

Table 12 (Appendix 3) shows the yield of casein protein determined for each sample using Kit D. Casein protein was not declared in any of the three PF products. Only whey protein was declared (PF milk ingredient). However, casein was detected by each of the four ELISA kit comparison participants in PF milk sample S24-000588. Casein protein was determined in-house in this sample also, using Kit D. The casein test results from the inter-laboratory ELISA kit comparison and from in-house testing were positive for sample S24-000588. A possible explanation for this is included in the discussion below. The casein testing results for samples S24-000587 and S24-000589 were negative.

Using this casein-sensitive test kit, a recovery study was also conducted to determine matrix interferences for a casein-sensitive kit. As shown in Table 12 (Appendix 3), for sample S24-000588, the casein yield was 108.8% of that expected which is in line with the acceptable tolerance for recovery (Lugos, 2019). However, for samples S24-000587 and S24-589, the recoveries were 251.5% and 45.6% respectively, which are outside of the tolerance and suggests matrix interferences. These are preliminary data and further investigation regarding the performance of the test kit is required in a comprehensive validation exercise for the suitability of ELISA kits for detection of allergens in PF foods. As expected, casein was not detected in the two samples of insects raised at FIRU (S24-001591 and S24-001592) which were included to investigate false positive reporting of the kit, so there were no falsely reported data for this very small study.

7.3.2. In-house testing: Discussion of milk results

Milk protein was detected in all PF milk samples using kits sensitive to β-LG and to 'entire milk (which detects the totality of β-LG plus casein). Milk could also be detected using these kits in insect protein products for which milk was declared on the label. These data are promising and show that milk can be detected in a background of insect matrix. Since no information is provided on the label concerning the level of milk in these products, no conclusion can be drawn relating to the accuracy of the milk levels detected or the effect of any matrix-interferences, and this could be determined in future work.

The casein ELISA responded in a positive manner, reporting low levels of casein in both the ELISA kit comparison study and the in-house testing for casein which was not declared in sample S24-000588. This sample was sourced from USA by our specialist international sourcing team and was not available to purchase directly from UK. Undeclared casein was also detected in the other two samples containing PF milk by one of the collaborating laboratories. It seems unlikely that PF innovators would be setting out to prepare PF casein in addition to PF whey since this would double their efforts. Further work to explain these data is required. It may be that this product reacts in an unexpected manner to the range of casein-sensitive kits tested during this project. Additional LC-MS/MS study to determine the proteins present which may be interfering with the kit would be interesting. No false positive data were yielded from any of the kits tested when challenged with samples which did declare any milk protein.

7.4. In-house testing of insect products for insect allergens

It was investigated in-house whether crustacean allergen-sensitive ELISA kits respond to insect protein which is known to contain similar allergens. The insect products tested in-house by crustacean-sensitive ELISA kits included the entire range of nineteen products sourced, as detailed in Table 13, alongside each of the positive and negative incurred cookie QC samples shown in Table 8 to provide information relating to the LOD of the kits. The insect protein samples were tested by two commercial ELISA kits which contained antibodies to crustacean tropomyosin and one kit which was sensitive to a mixture of tropomyosin and other crustacean proteins. The methods used were according to the manufacturer's instructions and varied for each individual kit.

The samples tested at Fera are listed in Table 13. The commercial packaging and the supplier website were inspected to determine as much information as possible relating to the level of processing of the products during manufacture, as also detailed in Table 13, since processing can impact detectability.

Table 13. Table showing insect samples and QCs tested in-house

Material sample number	Sample name	Species	Processing detail provided
S24-000961	Egg Flavour Whole Roasted Larvae	*H. illucens,* Black Soldier Fly	Roasted
S24-000968	Lightly salted locusts, crickets & mealworms	*L. migratoria, Acheta domesticus, T.molitor*	Freeze dried
S24-000969	Salt & vinegar mealworms	Yellow Mealworms *T.molitor*	Freeze dried
S24-000970	Maple wood smoked crickets	House Cricket, *A. domesticus*	Smoked over maple wood
S24-000971	Whole roasted locusts	Migratory Locusts, *L. migratoria*	Roasted
S24-000972	Barbecue Crickets	House Cricket, *A. domesticus*	Freeze dried
S24-000973	Teriyaki crickets	House Cricket, *A. domesticus*	Freeze dried
FIRU samples	Black Soldier Fly larvae, regular 'chick feed' diet (FIRU)	*H. illucens* , Black Soldier Fly	Blanched, 80°C for 3 minutes
FIRU samples	Yellow mealworms, regular 'chick feed' diet (FIRU)	Yellow Mealworms, *T.molitor*	Blanched, 80°C for 3 minutes
FIRU samples	Black Soldier Fly larvae, white bread diet (FIRU)	*H. illucens* , Black Soldier Fly	Blanched, 80°C for 3 minutes
S24-000960	Cricket Protein Penne	House Cricket, *A. domesticus*	Vacuum dried
S24-000962	Crickets	House Cricket, *A. domesticus*	No processing information available

Material sample number	Sample name	Species	Processing detail provided
S24-000963	Mealworms	Yellow Mealworms, *T.molitor*	No processing information available
S24-000964	Whole Natural Roasted Crickets	House Cricket, *A. domesticus*	Roasted
S24-000965	Pineapple & nut protein Bar	House Cricket, *A. domesticus*	No processing information available
S24-000966	Just Crunchy Locusts	Migratory Locusts, *L. migratoria*	Dried
S24-000967	High Protein Cricket Powder	House Cricket, *A. domesticus*	Freeze dried
S24-000974	Cricket cookie mix	House Cricket, *A. domesticus*	Freeze dried
S24-000975	High Protein Cricket Pancake Mix	House Cricket, *A. domesticus*	Freeze dried

7.4.2. Results of in-house testing for insect allergens

The results are summarised in Table 14 (Appendix 3). The crustacean allergen-sensitive kits responded with positive data to each sample containing insect protein. Data were negative for all samples for which insect protein was not declared and thus the false reporting rate for this small study was 0%.

None of the three kits investigated provided reliable quantitative data for the incurred samples, with the levels reported for example using Crustacean Kit A all being very similar (30.1, 31.56 and 23.91 mg (equivalent crustacean tissue)/kg cookie and not showing the expected increased yield when comparing the 1%, 5% and 15% cookies). These detected levels are above the LOQ of Kit A so a quantitative response had been anticipated. Tropomyosin was detected in all three positive incurred cookies using Crustacean Kit B, above the limit of detection but below the LOQ for the cookies containing 0.4 and 5.2% insect flour. Surprisingly, for the cookie containing only 1.7% insect flour, the level was detected at a higher level than that of the sample containing 5.2% insect flour, this time within the tolerance of the LOQ of the kit. The levels detected for each of the incurred samples using Kit C were above the LOD but below the LOQ. Further investigation would be required to determine whether the response of the kits is according to a quantitative response when challenged with other incurred foods prepared containing various levels of insect protein as these unexpected patterns in the data cannot be explained at present.

While the kits showed a reaction to insect protein, given the above results, the accuracy of the levels of allergen (mainly tropomyosin) detected in Crustacean Kits B and C should be treated with caution. Tropomyosin is present in insects at much relatively lower levels than in crustacea and

these data are possibly evidence of matrix and/or processing interferences. However, LOD studies were pursued, conducted using Kit B. Crustacean Kit B detected crustacean-equivalent tropomyosin in the incurred sample of the 1.7% insect protein cookie, diluted 1 in 10 (which, calculated from levels of insect protein presence in these incurred samples as detailed in Appendix 2, contained 1,730.5 mg cricket flour per kg of incurred cookie at this dilution) but not in the same sample diluted 1 in 100 (which contained 173.05 mg/kg cricket flour) so the LOD of the kit seems to be between 1,730.5 and 173.05 mg of cricket flour per kg of baked cookie. Without conversion factors to convert from mg/kg insect flour to mg/kg insect tropomyosin, the LOD in terms of tropomyosin cannot be stated. In crustacea, the conversion factor from level of dry crustacea to level of tropomyosin is approximately x0.17, but varies with species. While these LOD levels are high (for example, crustacean kits can have an LOD of 0.4-2.0 mg/kg), it must be considered that crustacea meat contains very high levels of muscle (tropomyosin is a muscle protein) compared to insects which contain high levels of exoskeleton rather than muscle and therefore the levels of the muscle protein tropomyosin will be much higher in crustacea. The data from the kit reported that, when diluted 1 in 10, the final yield (accounting for the dilution) of crustacean-equivalent tropomyosin was 70.03 ppb tropomyosin. While these crustacean-sensitive kits have been shown to show a reaction to insect protein, it may be that development of insect-specific kits is required to gain acceptable limits of detection when testing for insect protein. A more comprehensive validation study is of course required to confirm LOD, which should also be interrogated for a range of insect species, especially given that the values for the kit require a conversion factor depending on the crustacean species analysed, and thus it is reasonable to assume that these levels may vary depending on insect species.

7.4.2. In-house testing: Discussion of insect allergen detection results

A positive result was obtained with the crustacean allergen ELISA kits for each sample containing insect protein. A negative result was obtained for each sample for which insect protein was not declared and therefore the false reporting rate for this small study was 0%. None of the three kits investigated provided data in a quantitative manner for the incurred QC samples. These results show that the antibodies used in these kits to detect crustacean allergens seem to react with the counterpart target proteins in insects. However, further investigation is required to understand whether the kits can respond in a quantitative manner to insect protein and also to understand the conversion factors for different insect proteins. The conversion factors are expected to be different to the crustacean factors, since insect flours contain a large proportion of exoskeleton and less muscle protein compared to shellfish foods. Without provision of

quantitative data, the protein measurements from these kits (which provide quantitative data for crustacean foods) cannot be relied upon as accurate for insect protein.

7.5. In-house testing of insect products for allergens relating to insect feed

An interesting question regarding allergens in insect protein is whether allergens found in insect diet feed could be present in the insect product when later consumed by humans. Due to current considerations concerning insect welfare standards and the prospect that insects used in insect protein in the future may not be 'gut clear' of their food substrate prior to harvesting, there is concern that some allergenic feed in insect guts may be intact / undigested and therefore may present allergen risk in sensitive consumers. Similarly, since insects can stand or lie on their feed when feeding, allergens may adhere to the insect bodies which could, in theory, be transferred to human food. As discussed in the literature review above, recent work has suggested that allergens from the feed matrix may be detected in insects (Mancini et al., 2020). Work was conducted in this project to determine if test kits which are sensitive to gluten or gliadin, an allergen found in cereals, detected gluten in insects fed on a gluten-rich diet. Later in the project, kits sensitive to soya allergen were also applied to insects for which the diet contained soya, in an extra piece of work.

7.5.1. Preparation of test material

The samples used for this study were insects reared in FIRU, comprising *H. illucens* (Black Soldier Fly) larvae. Insects are raised as far as possible to reflect industry practices, including industry standards which have been released by the International Platform of Insects for Food and Feed (IPFF). Given that insects can be raised on food waste and that different producers will use different substrates (often bakery production waste), commercially reared insects are often fed cereal-containing diets. It was anticipated that gluten would only be detected if the insects were raised on a diet comprising a very high proportion of gluten-containing substrate (bread) as the bread would be undergoing digestion in the insect gut. It is anticipated that this digestion would disrupt much of the gluten protein and disrupt (reduce or eliminate) its allergenicity. Alternatively, it may result in the epitope in the gluten protein to the antibody being disrupted so that gluten present is no longer detected by the antibody.

A gluten-rich bread substrate was sought. A mass-produced white bread was selected since these breads tend to contain higher levels of gluten than, for example, artisan breads or mass-produced wholemeal breads. Since bread alone is too low in moisture and bread wetted with water dries out quickly under rearing conditions, the bread was blended at a

50:50 ratio with vegetables (mainly carrot/cucumber), to provide the 30:70 dry matter to moisture content required to provide the insects with the best opportunity for optimum growth. The insects were reared on this substrate for days 0-11, then a diet of 25:75 (vegetables:bread) for days 12-14. At Day 14, the larvae were blanched in a boiling water bath, ensuring the temperature is above 80 °C once the insects have been added to the water, incubating for 3 minutes at a temperature above 80 °C. The insects were then stored frozen prior to freeze-drying to a constant mass and, as with many commercial insect protein products, were crushed to a powder prior to testing. This powder was used for testing for gluten by two ELISAs according to the kit manufacturers' protocols.

To provide additional insects for the study, a batch of Black Soldier Fly larvae and a batch of yellow mealworm larvae were raised on a diet comprising chick starter crumb which is used in poultry production and is also used in industry for insect rearing. This diet contains gluten (in wheatfeed) and also soya as the two main ingredients, although the relative levels are not declared on the label. As an extra to the project, black soldier fly larva were tested for soya by ELISA in addition to testing for gluten.

While the larvae had been blanched as described above, there were concerns that any allergens present may be present due to still being adhered to the outer surface of the insects. For this reason, in addition to testing insects which had been homogenised (crushed to a powder), in another small study, other insects were taken while intact and these were incubated and agitated in the ELISA extraction buffer and then underwent the extraction process for soya allergen and for gluten protein. The intact insects were then removed and the buffer was analysed to determine if the kits reacted to the extracts from the intact insect bodies. Again, it was anticipated that very low levels of allergen would be detected and, for the studies of intact insect bodies, a x4 higher ratio of sample to extraction buffer was used. Due to the testing kits available in a short timeframe for this small extra study immediately prior to the conclusions of this project, only intact samples of Black Soldier Fly were included and not mealworm samples.

7.5.2. Testing methods

The technology used were:

a) RIDASCREEN® FAST Gliadin Sensitive ELISA (Article number R7051) for which the extraction method using a patented extraction buffer (Article number R7006 105 mL /R7016 1000 mL) is the official R5-Mendez method according to Codex Alimentarius and Association of Analytical Chemists (AOAC). LOD of the kit is 20 mg/kg gliadin (equivalent to 40 mg/kg gluten). LOQ is 1.25 mg/kg gliadin (equivalent to 2.5 mg/kg gluten).

b) AgraQuant® Gluten G12 ELISA test kit (Article number 10001994) which is an AOAC official method, based on the G12 antibody method and is approved by the Cereals and Grains association (formerly the American Association of Cereal Chemists. LOD of the kit is 2 mg/kg gluten. LOQ is 4 mg/kg gluten.

c) RIDASCREEN® FAST Soya ELISA (Article number R7102). LOD of the kit is 0.15-0.32 mg/kg soya protein (depending on the matrix) and the LOQ is 2.5 mg/kg soya protein.

The methods were applied according to the manufacturers' instructions. Since this is not a direct requirement of the tender, only a low amount of time and funds were allocated to these small studies. Tables 15, 16 and 17 show the samples tested.

7.5.3. Results for the detection of dietary allergens in insects

Gluten was detected in all of the homogenised insect samples raised on an enriched diet of bread and was detected at a level above the scope of the standard curve. This level is also above the internationally agreed maximum level considered safe to consume for people with coeliac disease (20 mg gluten/kg food). Later in the project, in order to gain more information relating to whether the allergen was detected from inside the gut or from allergens adhered to the outside of the insects, intact insects were incubated in allergen extraction buffer and the buffer tested for allergens, using the G12 kit and the soya kit. As shown in Table 16, gluten was detected above the scope of the standard curve which was 200 mg/kg gluten. Since the sample : buffer ratio had been increased by a factor of 4 for these samples analysed by the G12 kit, it appears that the level of gluten detected was at least 50 mg/kg but further work would be required to confirm this by re-testing at the ratio for which the kit was designed. Lower levels of gluten (approximately 2-8 mg/kg gluten) were also detected in the homogenised insects raised on the control (chick starter feed) diet using the G12 kit. These levels are below the LOD of the R5 kit and gluten was detected using this kit.

Soya was detected in the crushed insect samples fed on the chick starter diet at a mean level of 5.12 mg/kg soya protein which is equivalent to 13.12 mg/kg soya. Soya was not detected in the buffer used to extract soya from the outside of the insects.

Table 15. Table showing the results of the testing of insects for dietary gluten with the RIDASCREEN® FAST Gliadin Sensitive (R5) ELISA (Article number R7051)

Material sample number	Sample descriptor	Species	Level of gliadin and gluten, mg/kg gliadin and gluten
S24-001591	Black Soldier Fly, raised on regular 'chick starter crumb*' diet (FIRU)	*H. illucens* , (Black Soldier Fly)	<LOD (<0.2 mg/kg gliadin, equivalent to <0.4 mg/kg gluten)
S24-001592	Mealworm, raised on regular 'chick starter crumb*' diet (FIRU)	Yellow Mealworms, *T.molitor*	<LOD (<0.2 mg/kg gliadin, equivalent to <0.4 mg/kg gluten)
S24-001593	Black Soldier Fly, raised on white bread diet	*H. illucens* , (Black Soldier Fly)	>20 mg gliadin /kg) equivalent to >40mg/kg gluten)
S24-002384	White bread sample from diet of S24-001593	Not applicable	>20 mg gliadin /kg) equivalent to >40mg/kg gluten)

*Chick starter crumb was a commercial feed comprising wheat, wheatfeed, dehulled soya meal, full fat soya, calcium carbonate, mono-calcium phosphate, soya oil, sodium chloride, sodium carbonate (no compositional levels provided) plus vitamins.

Table 16. Table showing the results of the testing of insects for dietary gluten with the showing the results of the testing of insects for dietary gluten with the AgraQuant® Gluten G12 ELISA test kit (Article number 10001994)

Material sample number	Sample descriptor	Species	Level of gluten, mg/kg gluten
S24-001591	Black Soldier Fly, raised on regular 'chick starter crumb*' diet (FIRU), homogenised	*H. illucens,* (Black Soldier Fly)	2.0 mg/kg
S24-001592	Mealworm, raised on regular 'chick starter crumb*' diet (FIRU)	Yellow Mealworms, *T.molitor*	7.6 mg/kg
S24-001593	Black Soldier Fly, raised on white bread diet	*H. illucens,* (Black Soldier Fly)	>200 mg/kg
Intact BSF, raised on white bread	Intact BSF, raised on white bread diet	*H. illucens,* (Black Soldier Fly)	>200 mg/kg
S24-002384	White bread sample from diet of S24-001593	Not applicable	>200 mg/kg

Table 17. Table showing the results of the testing of insects for dietary soya with the RIDASCREEN® FAST Soya ELISA test kit (Article Number R7102)

Material Reference	Sample descriptor	Species	Level of soya protein, mg/kg
S24-001591	Black Soldier Fly, raised on regular 'chick starter crumb*' diet (FIRU), homogenised	*H. illucens,* (Black Soldier Fly)	5.12 mg/kg soya protein (equivalent to 13.12 mg/kg soya)
BSF, control diet, intact	Buffer used to extract feed from the outer surfaces of the intact insects used to prepare S24-001591	*H. illucens,* (Black	<LOD (<0.32 mg/kg soya protein)

Material Reference	Sample descriptor	Species	Level of soya protein, mg/kg
		Soldier Fly)	

7.5.4. Discussion for insect diet protein

Gluten was detected in the insect samples raised on bread at a level above the internationally agreed maximum level considered safe to consume for people with coeliac disease (20 mg gluten/kg food). These levels of gluten were detected in both the homogenised samples and intact insects, suggesting that gluten was adhered to the insect bodies, even after blanching. Whether gluten was present in the insect guts requires further investigation. These were small studies and require replicating with larger sample numbers to confirm the findings. Additional further study, involving excising and testing the content of the insect guts and their outer surfaces separately would provide further information regarding the location of the feed.

Soya was detected in insects raised on soya-containing animal feed at a level of 5.12 mg/kg soya. Soya was not detected above the limit of detection of the kit in the buffer used to extract soya from the outside of the insects. While these data suggest that soya was not present on the outside of the insects, it must be noted that it is possible that, while no soya was extracted from the insect surfaces in this small study, it is possible that there may be food substrate adhered to the crevices in the insect bodies. However, irrespective of where the allergens are located on the insects, it has been possible to detect dietary allergens (gluten and soya) originating from the animal feed in insects.

It must be noted that these results have been obtained from crushed BSF insects which were produced for research into animal feed, and not from a final commercial product ready for human consumption. The kits therefore are capable of detecting allergens in a background of insect protein. No matter the location of the dietary allergens in insect protein, these observations warrant further investigation to assess the risks associated with allergen carry over from the rearing substrates, especially to inform regarding risk assessments for preparing commercial insect protein destined for human consumption. Further investigation of whether the allergens from the rearing substrate accumulate in the insect gut or on their surface will be important to inform future industry washing procedures that minimise allergen carry over from the substrate to the final insect product. From a very brief investigation of the labels on insect protein for human consumption, feed allergens are not declared as standard.

7.6. Final conclusions and discussion to the testing section

Given the information declared on the product labels, ELISA test kits sensitive to milk and crustacea can be applied to detect PF milk protein and insect protein, respectively. Gluten- and soya-sensitive kits could be applied to detect dietary allergens in a background of insect protein. The studies also showed evidence that allergens (at least gluten) may be detected on the bodies of the insects, even following blanching in water during final stages of the insect preparation process. These data warrant further investigation to determine whether dietary proteins are present in commercially available insect protein for human consumption.

None of the kits for which false positive investigations were conducted showed false positive data in these small studies, providing evidence that the ELISA kits tested are specific to the analytes in question and that cross-reactivity has not been detected among the challenge samples included in these small studies.

The crustacean sensitive kits did not respond in a quantitative manner when detecting levels of insect protein in cookies incurred with insect protein. This may be due to the levels of the target proteins in the insect material being below or near the limit of detection of the kits and this needs to be investigated further in the future. The LOD for insects appeared much higher than is standard for allergen testing kits. It is likely that, since the levels of tropomyosin are much lower in insects than in crustacea, new kits will need to be developed in the future which are specific to insects rather than applying crustacean-sensitive kits, to meet sufficiently sensitive limits of detection for insect allergens. Clinical data will also be required to understand the eliciting level of insect allergens in insect protein products.

While milk protein was detected in the PF milk products, it must be noted that, due to the nature of PF products, all of which are individual and may therefore differ regarding the particular milk protein expressed during production, the applicability of these milk testing kits would need to be tested for every PF milk product of interest. There will always be the possibility that a PF milk protein produced by the fermentation process may not contain the epitope detected by the antibodies used in some or all dairy milk-sensitive ELISA kits. Similarly, the conformation of the PF milk protein may result in the antibodies not detecting the protein.

It was interesting that Laboratory 4 reported casein in all three PF milk samples which did not declare casein. An explanation for this relates to the possibility that the antibody upon which this kit is based was raised to a casein extract which contained trace amounts of β-LG and this β-

LG is being detected. All four laboratories reported casein in sample S24-000588. Again, it would be interesting to understand the reason for this. A mass spectrometry study to identify the proteins and their sequences in this sample may provide some information to explain why this kit is reporting positive data for this sample as it appears that the proteins are somehow interfering with the test.

Following these small studies, while further work is required to determine the performance of conventional ELISA kits to react to alternative protein products, it is promising that the kits appeared to be sensitive to detecting the presence of alternative proteins.

8. Estimate of costs to perform ELISA studies

In order that FSA are in a position to react to risks relating to allergens in alternative proteins, costs are detailed below, first to purchase the required consumables and equipment to set up a laboratory for allergen testing, and secondly providing current charges (February 2024) for commercial allergen testing by accredited laboratories.

8.1. Estimated costs to perform testing

The estimated costs to perform testing, in terms of consumables required test kits and reference materials, equipment costs and annual expenses necessary to develop and maintain accredited testing status, are detailed in Table 18 (Appendix 3) along with an estimate of staff time requirements.

8.2. Charges by UKAS-accredited testing laboratories

Currently, UKAS accredited laboratories are charging approximately £70 per sample per allergen test (e.g. β-LG, casein, 'entire' milk or crustacea) to test foods for allergens by ELISA.

9. Overall Final Conclusions and Future Direction

The safety of our food is paramount. When considering the introduction of novel foods to our diets, allergenicity risk assessments comprise a critical part of determining the safety of these alternative forms of protein.

There is no simple or rapid guaranteed solution to determine the allergenicity of novel foods within a short timeframe. A scenario is foreseen whereby consumption occurs by Western consumers (as is permitted at

present under regulation in Israel and USA for PF protein and across Europe for four species of insect) which will provide a growing dataset against which to assess allergy risks to inform future regulation and current weight-of-evidence procedures. As consumption grows, clinical data will be gathered and clinical studies of human subjects can occur. In the meantime, data can be gathered using allergen prediction tools and digestibility studies to provide risk assessments to regulators. While there is evidence that certain types of food processing reduce the allergenicity of certain foods, no suitable food processing method or methods are available to eradicate allergenicity across a wide range of food types. More definitive data are required regarding the effect of processing on allergenicity with total protein hydrolysis showing potential to reduce and even to remove allergenicity but this is at the expense of destroying the functional properties of the proteins.

Considering precision fermentation, PF is under development as a novel form of milk and egg protein. It is clear that the allergenicity of PF egg and milk proteins is not being considered separately by innovators to that of their conventional (dairy) equivalents. The potential effect of PF technology on the allergenicity of the protein is not considered in the literature. Future focus should include the fact that PF protein products will differ depending on factors including the specific gene sequence used, microorganism species, culture media and processing conditions, any of which could contribute to post-translational modifications of the proteins. This may impact the allergenicity of each product and different PF products must be considered on a case-by-case basis.

Regarding the allergenicity of insect protein, there are a great many studies in this area and there are benefits from considerations of cross-reactivity from pan-allergens shared with crustacea. The vast majority of studies focus on predictive analysis of allergenicity, and the potential for *de novo* sensitisation from insect protein must be understood. Perhaps, particularly with reference to the new consumption of insect protein by Western populations, more data regarding allergenicity are required. As discussed throughout this review, much more data are needed relating to human (and not other animal) oral exposure to all novel proteins in Western diets, either by clinical trial or case studies for consumers exhibiting symptoms of allergy to novel foods to understand their allergenicity.

Further knowledge is required relating to the carbohydrate chitin in insect-based foods. While not an allergen, its role in immunoregulation must be better understood to protect consumers of insect protein since, unlike in crustacea, chitin is more intrinsic to the edible part of insects and so its consumption when eating insects cannot be avoided unless it is extracted out of the insect protein.

Innovators developing PF and insect protein products are aware of risks relating to allergenicity of these products and intend to label their products as containing milk or egg allergens or containing insect allergens which are unsuitable for consumption by crustacea-sensitive consumers. For this reason, innovators are therefore not conducting allergen testing during the development stage. As with conventional food, fit-for-purpose testing methods to detect allergens in novel foods must be available to protect customers from potential cross-contamination and authenticity concerns in the supply chain. Stakeholders have called for faster approval processes for novel foods and more regulatory advice and direction, for example regarding validated methods for testing and allergen eliciting levels of alternative proteins.

The applicability of current testing methods on currently available novel foods must be determined in order to inform regulators according to our testing capabilities in this area. It should be noted that, as novel proteins are further developed, testing capabilities must develop in line.

Data from small preliminary studies including an ELISA kit comparison exercise in this report suggest a strong potential that the capability to detect PF milk proteins in the three PF products tests is available using currently available ELISA kits which are sensitive to allergens in conventional milk. It must be noted that, due to the unique nature of each PF product based on the microbe used, protein sequence expressed and processing conditions, the success of such detectability will be product-specific and testing of each PF product is required. Each of the kits reported positive data for casein in one of the three PF samples for which casein wasn't declared. Further study involving mass spectrometry to identify the proteins present in this sample and the protein sequences may provide information which may explain why this kit reports positive data for these samples.

While crustacea allergen detection kits reacted to insect protein, these kits did not show the sensitivity of ELISA kits for other allergens. It is not ideal to use the cross-reactivity of insect allergens with those of crustacean allergens to form the basis of a testing method for insect allergens. Data are needed to understand the elicitation levels of insect protein and it may be that further development of testing kits is required to achieve the required sensitivity. Given the large differences in the concentration of the muscle proteins to which the ELISA methods are sensitive in crustacea compared to insects, we conjecture that it is likely that kits will require to be developed using antibodies raised specifically to insect proteins rather than crustacea proteins in order to attain sensitive LOD and quantitation capabilities which are typical of ELISA methods for allergens in conventional foods. Clinical data relating to the allergen elicitation levels of insect protein are also required.

Previous research has raised concerns that dietary allergens can be carried over to insect protein from the insect feed. Small studies in the current project, based on insects reared for research purposes but replicating certain industry methods demonstrated the detection of allergens from insect feed to the final product, either from undigested allergens present in the insect gut or from adherence to the insect body. Further work to excise and extract the alimentary canal of insects is required to determine whether the source of the allergens which were detected was the insect gut, adhesion to the outer surfaces of the insects or both locations. The data would inform in order to optimise insect washing practices in industry. These data must be urgently considered for commercial insect protein to manage risk to consumers.

Future work would also include more detailed studies to determine the suitability of current test kits and to develop insect-specific test kits. Studies and surveys to determine whether allergens are present in commercially available insect protein are required. The collation of clinical data relating to consumption of novel foods, including the generation of human oral exposure clinical trial data will provide improved knowledge regarding the allergenicity risk of novel foods.

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Supplementary Materials

FS900409 - Appendices (Supplementary)

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