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Investigating technological processing supporting the assessment of novel proteins in food and feed risk assessment

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Abstract

The safety of novel proteins is routinely evaluated in various regulated areas of the food and feed chain, including genetically modified (GM) crops and novel foods (NFs). This project aimed to map the food and feed products containing protein from the main GM crops, relevant food categories falling under the NF Regulation, and unconventional feed, together with their production processes and to discuss the effect of the mapped processes on the safety of the corresponding novel proteins. A scoping literature review (1,325 documents included), an open online survey and a stakeholder workshop were the basis to build up the mappings for products and processes, also including operational conditions for each processing step. In the case of crops, the information gathered also helped identify more than 40 products, and the corresponding production processes, not included in the OECD consensus documents for compositional considerations of GM crops. Moreover, a systematic literature review (154 documents included), carried out within the project, assisted in the identification of the available evidence on the impact of processing on protein safety. Overall, certain processes, such as thermal treatments, fermentation, or enzymatic hydrolysis, significantly enhanced protein digestibility across various food/feed matrices. Similarly, fermentation, ensiling, and extraction processes have been shown to improve nutritional properties in various products. The data collected seemed to indicate that heating can effectively reduce the activity of NEPs from GM crops and that heating and enzymatic hydrolysis can reduce IgE reactivity for certain proteins

and operational conditions. However, exceptions to these trends were also reported in the literature, and in certain cases (e.g., impact on gut microbiota), the evidence gathered was insufficient to draw substantiated conclusions. This project also contributed to identify existing knowledge gaps and research needs towards regulatory risk assessment of food and feed products containing protein.

Keywords: Novel foods, unconventional feed, GMs, protein, safety, process, parameters.

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Summary

In the EU, the safety of (novel) proteins is evaluated in regulatory risk assessments for genetically modified organisms (GMOs), novel foods (NFs) and other products. As part of the risk assessment, EFSA evaluates the safety of the production process for the product under assessment. In this context, the objectives of this project were the following:

1. To map food and feed products containing protein, produced from:

(i) the main GM crops

(ii) relevant food categories falling under Regulation (EU) 2015/2283 on NFs

(iii) unconventional feed (UF).

2. To map the production processes and processing steps for the (i), (ii) and (iii) products, including the purpose and range of operational conditions for each individual step.

3. To discuss the differences between the products and processes mapped in Objectives 1 and 2 applied to GM crops, as compared to the ones already proposed in the OECD consensus documents for GM crops.

4. To discuss the impact of processing on protein safety regarding toxicology, immunotoxicity, allergenicity, nutrition, digestibility, gut microbiota of the host for the food and feed products and the corresponding production processes identified in Objectives 1 and 2.

To address Objectives 1 and 2, a scoping literature review, online survey and stakeholder workshop were used to gather information on (i), (ii) and/or (iii) products and their production processes. A total of 1,325 documents, including 725 research and review articles, 313 patents and also book chapters and other document types were included as relevant. The extracted data allowed the creation of a repository of novel protein-containing food and feed products including: (a) the composition of 97 NFs, nine UF categories and food and feed products derived from the eight main crops, (b) a flowchart depicting the corresponding production process(es) and (c) a table including the operational conditions for each processing step, when available.

To achieve Objective 3, the maps constructed in Objectives 1 and 2 were compared with the OECD consensus documents for each crop. As a result, more than 40 new food and feed products (and their corresponding production processes) were identified as relevant to be considered in addition to the OECD consensus documents.

Finally, a systematic literature review (registered in PROSPERO: CRD42024554500) was conducted to discuss the impact of processing on protein safety concerning toxicology, immunotoxicity, allergenicity, nutrition, digestibility, and the gut microbiota of the host for these novel protein sources (Objective 4). 154 scientific articles were included as relevant, out of which 151 passed the risk of bias appraisal.

From this review it can be concluded that:

-Certain processes/technologies, such as thermal treatments, fermentation, enzymatic hydrolysis, and germination, can significantly enhance protein digestibility across various food and feed matrices, although some exceptions have also been reported. Overall, their effectiveness varies based on the food category/formulation, processing method, and treatment parameters/conditions. Non-thermal technologies and drying methods can also improve protein digestibility, but more research is needed to validate their effects, especially regarding innovative methods like supercritical fluid extraction.

-The impact of thermal treatments on the nutritional properties of legume seeds, cereals, and insects varies depending on temperature, time, and processing method. While methods like autoclaving and boiling can enhance digestibility, excessive heat (e.g., frying) may reduce protein quality due to reactions like the Maillard reaction. Fermentation, ensiling, and extraction processes have been shown to improve nutritional quality in various products (e.g., insect flour, forages, brewing by-products).

-Thermal treatment can reduce the activity of the newly expressed proteins (NEPs) from GM crops. However, further research is needed since the effect of other technological processes is hardly reported.

-The data gathered indicate that heating often reduces IgE reactivity in plant-based proteins but has varying effects on insects. Additionally, enzymatic hydrolysis can reduce IgE reactivity in plant-based proteins, insects, and crustaceans, although its effectiveness depends on the specific protein and conditions.

In any case, these general conclusions, and specially making extrapolations from them, should be approached with caution due to various factors: i) the food matrix, which may significantly influence digestibility, nutritional properties, and potential toxicity of proteins, as well as the impact of processing ii) the physiological differences across species, which can influence the effect of processing on protein safety, and iii) the limitations of the studies consulted to predict human allergic responses.

Finally, it should be noted that, in some cases, the information gathered was insufficient to draw solid conclusions, such as on the effect of processing on protein safety regarding the gut microbiota of the host or the effect of treatments others than heating on protein toxicity.

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1 Introduction

1.1 Background and terms of reference as provided by the requestor

This contract was awarded by EFSA to: Universidad de Zaragoza, Zaragoza, Spain

Contractor: Universidad de Zaragoza

Contract title: Investigating technological processing supporting the assessment of novel proteins in food and feed risk assessment scientific report

Contract number: NP/EFSA/NIF/2023/01

1.1.1 Background

In the European Union (EU), the safety of (novel) proteins is routinely evaluated in various regulated areas of the food and feed chain, more particularly in the regulatory risk assessments of products such as Genetically Modified Organisms (GMO), novel foods (NFs), enzymes, food/feed additives or pesticides. As part of the risk assessment, EFSA evaluates the safety of the production process used in the manufacture of the product under assessment, including e.g., information on raw materials and processing aids, processing steps and operational conditions, safety assurance measures and the potential impact on e.g. composition, presence of undesirable substances, nutritional value and metabolism.

The Organisation for Economic Co-operation and Development (OECD) has published a series of documents for the safety assessment of GM foods and feeds of plant origin, which are considered during the risk assessment by EFSA. These documents include processing steps to produce various plant-derived products. However, the OECD documents are limited to certain product categories, and therefore to the associated production processes. For example, the document for rapeseed (OECD, 2011) did not consider rapeseed protein isolate and rapeseed powder production, which both have been assessed as NFs by the EFSA NDA Panel in 2013 and 2020, respectively.

In the framework of the NF Regulation, novel aspects of the production process should also be characterised when that has not been used for food production within the EU before 15 May 1997 and causes a significant impact on the product (considered as a NF).

On this background, EFSA is devoting additional resources to map the types of protein-rich food and feed products and the associated processing technologies and operational conditions, and to assess whether/how processing affects the proteins present in the raw commodity (e.g. degradation, denaturation, aggregation or interactions with the food matrix, e.g. Maillard reactions). This information on the fate of proteins following processing of the raw commodities will support the risk assessment with regards to potential hazards and providing information relevant for the hazard assessment and exposure considerations.

This call is based on EFSA Funding regulation and EFSA's 2023 Work Programme for grants and operational procurements as presented in Annex XII of the Programming Document 2023–2025, available on the EFSA's website[1].

1.1.2 Objectives

The objectives of the contract resulting from this procurement procedure are as follows:

1. To map food and feed products containing protein used either as whole foods or as ingredients, produced from:

(i) the main (GM) crops (including maize, soybean, rapeseed, cotton, sugar beet, camelina, rice, potato)[2].

(ii) relevant food categories falling under the NF Regulation or (iii) recognised as unconventional feed. Particularly the following sources shall be considered: insects, plant-based proteins (e.g. water lentils, pulses), microalgae, protein-rich microbial biomass, and fermentation-derived proteins.

2. To map the processes, the processing steps, the purpose of the process/processing steps, and the range for operational conditions (such as time, temperature, pressure, solvent type/concentration etc.) applied to the above-mentioned products. Known/well-established processing technologies (such as conventional heating, extruding, fermentation, filtering, hydrolysis, etc.), but also innovative ones should be included when having market potential.

3. To discuss the differences between the processing technologies, processing steps and operational conditions from Objective 2 applied to (GM) crops compared to the ones already proposed in the OECD consensus documents for (GM) crops.

4. To discuss the impact of processing on protein safety regarding toxicology, immunotoxicity, allergenicity, nutrition, digestibility, GIT microbiome of the host for the food and feed products identified in Objective 1. The discussion should consider the specific needs of EFSA i.e. risk assessment of food and feed safety and include a list of known manifestations. It is noted that the effect of the intended food/feed matrix (e.g. interactions with particular food components, the effect of pH, ionic strength) should be considered in the discussion.

For Objectives 1 & 2:

Contractors are asked to conduct a **scoping literature review**, to identify and assess the state of evidence for the Objectives 1-2 listed above. The EFSA Guidance on Systematic review (SR) does not prescribe in detail the approaches for scoping reviews, therefore more **details on the requested requirements** are as follows:

[1] https://www.efsa.europa.eu/sites/default/files/2022-01/amp2325.pdf
[2] In the context of GMO area, the interest is for the newly expressed proteins (NEPs) resulting from the genetic modification and also for plant-specific proteins (e.g. in nutritionally enhanced GM plants)

Requirement	Explanation, additional details and/or examples
1. Preparing the review: developing the review protocol and setting the logistics	The key steps to cover in a SR protocol are summarized in Table 6 of the EFSA Guidance on SR. Since the activity foreseen will not be a full SR, selected steps shall be specified to be covered in the protocol. The protocol is expected to include: a) background section; b) the description of the review questions properly formulated; c) the description of the methods that will be applied for searching for studies and selecting the relevant studies, including eligibility criteria and uncertainty analysis; d) the logistics and timelines for doing the review. The proposed protocol will be discussed at the kick-off meeting.
2. Searching for studies	Develop and run specific search strings (at least one per each question listed in Objectives 1 and 2 above) and define the information sources to be used to gather scientific evidence. The keywords should take into consideration Objectives 1-4. Apart from scientific literature databases, grey literature and patents should also be considered.
3. Selecting studies for inclusion or exclusion in the review (e.g., screening for title and abstracts)	Selection of studies for inclusion/exclusion according to eligibility criteria described in the review protocol. The studies will be selected by screening of title and abstract in parallel by two independent reviewers. The study selection shall preferably be performed using the web-based SR software DistillerSR (EFSA will grant the contractor access to the system) or otherwise, a software compatible with the DistillerSR environment

2 Methodologies

2.1 General overview

Different methodologies were applied in order to reach the four objectives defined above. A scoping literature review and mapping methodology was applied to reach Objectives 1, 2 and 3, while a SR was carried out to reach Objective 4.

Scoping reviews are a relatively new approach to evidence synthesis that are particularly useful to provide overviews or maps of evidence Munn et al. (2019). They are also usually applied to identify knowledge gaps, scope a body of literature, clarify concepts, investigate research conduct and, in many cases, as precursors to SR. Scoping reviews aim to be comprehensive, transparent, reproducible, and unbiased, and are the better choice when the review is not intended to answer a *clinically* meaningful question or provide evidence to inform practice, but to help in identifying certain characteristics/concepts in papers or studies, and in the mapping, reporting or discussion of these characteristics/concepts. That is why this strategy was chosen to tackle Objectives 1, 2 and 3.

As described below, this review was conducted by following specific steps outlined in the EFSA guidance on SR (EFSA, 2010). Given the particular characteristics of this type of review, exclusion criteria and assessment of the methodological quality were tackled in a more flexible approach than for SR, in order to minimize the risk of data loss and allow a broader coverage with the resulting maps.

Data extracted from this scoping review were used to map the products and processes (Objectives 1 & 2) that were integrated in a data repository (see section 3.1.1.4). In the particular case of (GM) crops, the results of this mapping were compared and discussed against the maps/flow charts already proposed in the OECD consensus documents for (GM) crops (Objective 3).

Systematic reviews (SR) are a well-established synthesis strategy for published research with a very well-defined methodology. Thus, explicit, systematic methods are followed to minimize the risk of bias in the results, thus providing more robust conclusions for further decision making (Higgins and Green, 2011). The objective of SR is to provide critically appraised and synthesised results/answers to particular questions, which aligns well with the purpose of Objective 4. Therefore, as further described below, this SR was designed to determine whether processing has any effect on safety risks associated to novel proteins present in food and/or feed products.

The SR was designed strictly following the general principles for SR as specified by EFSA (2010) and PRISMA-S (Preferred Reporting Items for SR and Meta-Analyses) methodology - an extension to the Statement for Reporting Literature Searches in SR. The SR has been registered in PROSPERO (International prospective register of SR).

The results of this SR were used to discuss the impact of processing on protein safety regarding toxicology, immunotoxicity, allergenicity, nutrition, digestibility, GIT microbiome of the host for the food and feed products under study [NF, UF and (GM) crops]. The discussion also took into consideration the results of the scoping review and the intended conditions of

use of these novel protein sources, in order to identify the full set of variables affecting protein safety in relation to processing and food matrix composition/properties.

2.1.1 General considerations

The following considerations apply to the protocols/methods implemented to meet Objectives 1-4.

2.1.1.1 Products

It is noteworthy that only protein-containing products were considered for the purpose of Objective 1.

For **NF,** the search was limited to those falling under Regulation (EU) 2015/2283 at the time the searches were carried out (19/03/2024), including: (i) Authorized NF included in the Union list (https://food.ec.europa.eu/safety/novel-food/authorisations/union-list-novel-foods_en); (ii) NF currently under risk assessment by EFSA (summary available on open EFSA); (iii) Products to be considered as NF according to the outcomes of the consultation process on NF status, available on the EC website; and (iv) Products to be considered as NF according to the NF catalogue, available on the EC website (https://ec.europa.eu/food/food-feed-portal/screen/novel-food-catalogue/search).

Since there is no legal definition for the term "**Unconventional Feed**" (UF) in the EU, for the purpose of this procurement the following product categories were considered as UF, based on scientific literature and expert judgement: insects, fungi/mycelial biomass, plant-based (including aquatic plants) proteins, macroalgae, microalgae, krill, protein-rich microbial biomass, fermentation-derived proteins, former food products, biofuel by-products, industrial by-products, and processed animal proteins.

Regarding (**GM) crops**, the review is focused on the following eight crops in relation to food and feed applications: maize, soybean, rapeseed, cotton, sugar beet, camelina, rice and potato. It should be noted that the interest is for newly expressed proteins (NEPs) resulting from the genetic modification and also for plant-specific proteins (e.g. in nutritionally enhanced GM plants). However, since (GM) crops are typically processed in the same way as conventional (non-GM) crops, both GM and non-(GM) crops were considered.

1.1.2.1 Processes

For Objectives 1 and 2 (mapping of products and processes), the processes applied in the manufacture of the relevant NFs (food ingredient or whole food), UFs (feed ingredient or whole feed) and the food and feed (ingredients or grains/seeds and forage) obtained from the crops under consideration were considered/studied. Moreover, additional variables potentially affecting protein safety in relation to the (NF, UF and crop) matrix itself were identified. For Objectives 3 and 4, the addition of NF into particular food categories, UF into relevant feed categories and (GM) crop ingredients into selected food/feed categories were considered, in order to identify additional variables related to the intended use of the NF, UF or crop material as ingredients that could affect protein safety (e.g., related to the processing or composition of the corresponding food categories).

1.2 Logical and structured step-by-step explanation of the methodology

1.2.1 Protocol for the scoping literature review (Objectives 1-3)

1.2.1.1 Definition of the question/s

The scoping review was designed to simultaneously answer the following questions:

Q1. Which are the protein-containing food/feed products produced from the main (GM) crops (maize, soybean, rapeseed, cotton, sugar beet, camelina, rice, potato) or falling under the NF Regulation or recognized as UF?

Q2. Which overall production processes, individual processing steps and operational conditions (e.g., time, temperature, pressure, solvent type/concentration etc.) are applied in the production of the food or feed products resulting from Q1 and for what purpose?

1.2.1.2 Information sources

The following information sources were used:

➲ Scientific literature databases. Journals and books recorded in the following electronic bibliographic databases were used: PubMed, Scopus and Web of Science.

- Reference lists: The reference list from relevant publications including reviews and guidelines was also checked to identify additional studies that had not been retrieved otherwise.

➲ Grey literature. This included:

- Patents. The Espacenet search engine was used.
- Survey: A stakeholder survey was designed and launched through EU survey and was disseminated through EFSA website (https://www.efsa.europa.eu/en/news/novel-protein-production-processes-take-part-our-survey).

  The objective of the survey was identifying: i) novel protein sources potentially relevant under Regulation (EU) 2015/2283 on Novel Foods (excluding traditional foods from third countries), ii) unconventional protein sources potentially relevant as feed and iii) food and feed products containing protein derived from GM* or conventional plant crops, particularly maize, soybean, rapeseed, cotton, sugar beet, camelina, rice and potato, and characterizing the production process of the products under (i), (ii) and (iii).

  The survey was primarily intended for stakeholders involved in the production, use, distribution and/or research & innovation activities on products under the scope of this project and it included a series of multiple-choice and short-answer questions aimed at gathering information about the characteristics of the manufactured/investigated products and their production process, as well as general questions such as stakeholder category and sector of activity.

  The survey is available at: https://ec.europa.eu/eusurvey/runner/ProcessingProteins.

- Workshop: A workshop was held online on 25 June 2024 and was divided into two sessions of two hours each. Appendix A includes the detailed program for each of the sessions.

  A total of 36 experts attended these sessions, including 25 external experts, seven members of the working team, and four from the Nutrition and Food Innovation Unit of EFSA. The external experts were individually invited and selected for their expertise in processes for obtaining and transforming the products under study in this project and/or in protein-safety-related aspects. This group of external experts included both academics and industry professionals.

  The objective of the first session was to review with the invited experts the current knowledge about the production processes of NF, UF, and (GM) crops as well as to evaluate the format of the tables and flowcharts included in Annexes 1.1, 1.2, and 1.3 (main results of Objectives 1 and 2: mapping of products and processes). For this purpose, several case studies extracted from the maps were used that were provided in advance to the participants.

  The information gathered in this session of the workshop was used to refine the maps constructed as well as to improve their format.

  The objective of the second session was to compile and discuss the existing knowledge about the effect of processing on protein safety and to determine if the lessons learned from "standard" foods could be extrapolated to NF/UF/(GM) crops, similar to an expert elicitation, as well as to determine if the project's approach might be missing any risks associated with proteins that can be modified by processing.

  The contributions of the experts participating in this second session were taken into consideration for the discussion of the impact of processing on the protein safety of the products of relevance for the project (see results: Objective 4) and, particularly for assessing the validity of the methodologies used in the documents retrieved in the SR.

In addition, the following documents/information sources were included:

- Outcomes from the consultation process on NF status according to Article 4 of Regulation (EU) 2015/2283 (European Commission) (https://food.ec.europa.eu/safety/novel-food/consultation-process-novel-food-status_en)
- EU NF status Catalogue (https://food.ec.europa.eu/safety/novel-food/novel-food-catalogue_en).
- EFSA scientific opinions on the safety of NF (OpenEFSA) and ongoing dossiers under risk assessment (for the selection of keywords).

EFSA Guidance on the preparation and submission of an application for authorization of a NF in the context of Regulation (EFSA NDA Panel, 2021).

1.2.1.3 Search strategy

2.2.1.3.1. Constraints in the search strategy

- Fields: Title and Abstract
- Dates: No temporal constraint
- Languages: English
- Information sources: see 2.2.1.2

2.2.1.3.2. Keywords and search strings

Search strings were written in PubMed format and then translated to Scopus and Web of Science format using the Polyglot search tool (https://sr-accelerator.com/#/polyglot).

A dual search strategy was developed for the three types of products under study, i.e., NFs, UF and crops. In the case of NFs, a "NF specific" and a "NF category" search strategies/strings were implemented. The "NF category" strategy (e.g., microalgae) was intended to complement the "NF-specific" one (e.g., *Euglena gracilis*), towards a broader coverage of potentially relevant hits from the consulted literature sources, given that the search strategy was implemented at title and abstract level. It is noteworthy that these search strategies were adapted for each particular NF / NF category, as described below. The same applies to the dual search strategies for relevant UF and crops. The list of keywords and the structured search strings are reported in Appendix B.

- **Novel Foods**

a) NF-specific search strategy

The "NF-specific" search strategy (Figure 1) was designed to narrow down the search on the NF under study (please refer to section 2.1.1.1) since the term "novel foods" is commonly used in the literature but not necessarily to describe products falling under Regulation (EU) 2015/2283. Thus, specific keywords defined for each NF under study (246) were included in the first search block.

The second search block included the keyword "food" to introduce a search constraint in relation the respective intended use of NFs as/in food products.

Taking into consideration that NFs may not contain protein, potentially eligible NFs, as described in section 2.1.1.1, were sorted according to their protein content (Yes/No) before the search. In principle, those NFs not containing protein were not excluded from the searches, but instead a third search block including keywords related to the proteinaceous nature of the NF was included in the search string for these particular NFs.

The different search blocks were combined in the search string by using the Boolean operator "AND" (Figure 1).

Independent searches were conducted for each NF. Preliminary searches in Pubmed allowed to identify those NFs for which no records were found. In those cases, the search block "food" was removed from the search string in order to broaden the search results/maps.

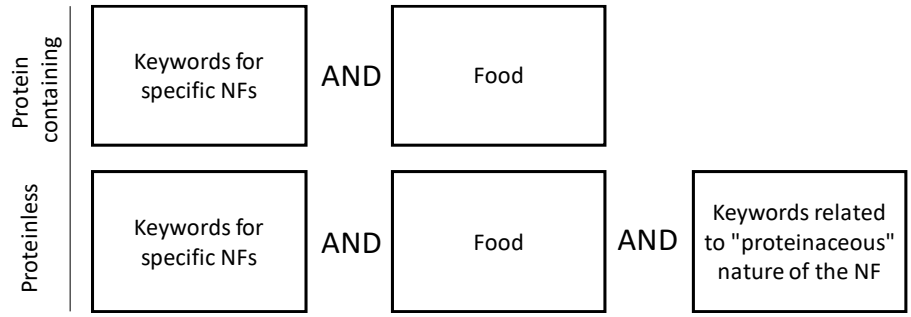

Figure 1: NF-specific search strings

b) NF-category search strategy

The following search blocks were combined in the search string by using the Boolean operator "AND" (Figure 2):

-A list of keywords related to the NF categories of major interest, as indicated in the objectives of the project.

-A list of keywords related to the "NF" status.

-A list of keywords related to the "proteinaceous nature" of the NF

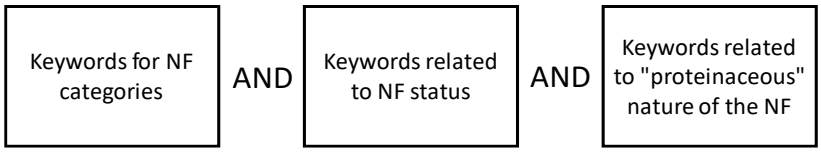

Figure 2: NF-category search strings

- **Unconventional Feed**

a) UF-category specific search strategy

The following search blocks were combined in the search string by using the Boolean operator "AND" (Figure 3):

-A list of specific keywords for each of the 12 UF categories initially under study: insects, plant-based proteins, macroalgae, microalgae, protein-rich microbial biomass, fungi/mycelial biomass, food by-products, fermentation by-products, industrial by-products, krill, nanoparticles/nanomaterials and Processed Animal Proteins.

-A list of keywords related to the term "Unconventional Feed"

-A list of keywords related to the "proteinaceous" nature of UF

Searches were conducted separately for each UF product category.

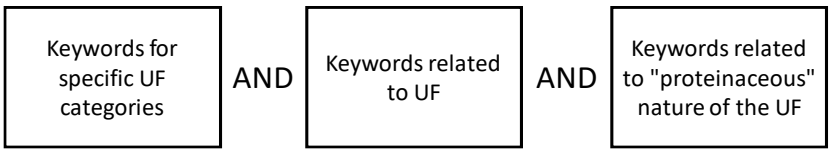

Figure 3: UF-category specific search strings

b) UF-general category search strategy

The following search blocks were combined in the search string by using the Boolean operator "AND" (Figure 4):

-A list of keywords related to the "Novel" nature of UF.

-A list of keywords related to the term "Feed".

-A list of keywords related to the "proteinaceous" nature of UF

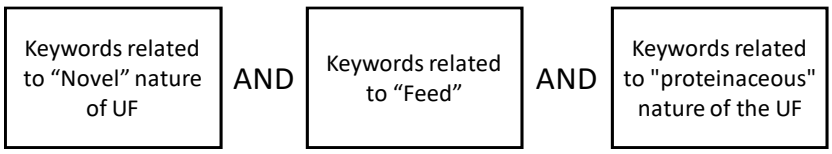

Figure 4: UF-general category search strings

- **(GM) crops**

a) (GM) crops-specific search strategy

The following search blocks were combined in the search string by using the Boolean operator "AND" (Figure 5):

-A list of specific keywords for each of the 8 (GM) crops under study.

-A list of keywords related to the terms "NEP" and "GM".

-A list of keywords related to the "proteinaceous" nature of (GM) crops

Searches were conducted separately for each (GM) crop.

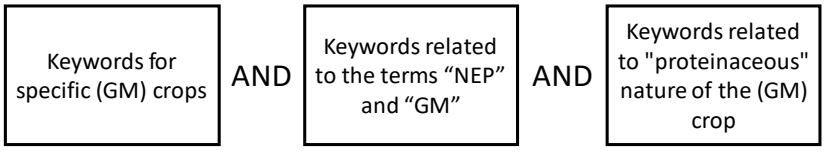

Figure 5: (GM) crops-specific search strings

b) (GM) crops general category search strategy

The following search blocks were combined in the search string by using the Boolean operator "AND" (Figure 6):

-A list of specific keywords for each of the 8 (GM) crops under study.

-A list of keywords related to the terms "Food" and "Feed".

-A third block including a list of keywords related to terms "NEP" and "GM" and to the "proteinaceous" nature of (GM) crops.

Searches were conducted separately for "food" and "feed" related products.

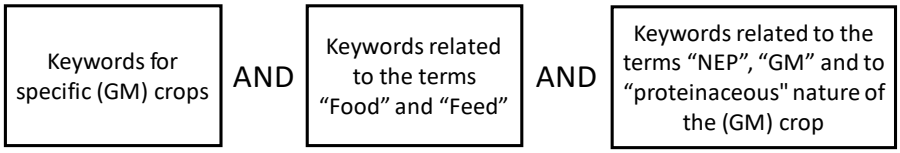

Figure 6: (GM) crops-general category search strings

2.1.1.2 Documenting and reporting the search process

In the case of PubMed, searches were directly performed using DistillerSR. For Scopus and Web of Science, the respective searches were carried out on the respective websites/search engines and then the search results/references were exported to EndNote to create the RIS files that were eventually transferred to DistillerSR. The full search strings were recorded exactly as run, together with the number of records retrieved.

References were labelled using DistillerSR according to the search strategy through which they were obtained: product category (NF, UF or Crops), search strategy (specific or category/general) and source (PubMed, WoS or Scopus).

DistillerSR automatically removed the duplicates from PubMed searches during the upload process. Once all the references from Scopus and Web of Science were uploaded, the DistillerSR Duplicate Detection tool was used to find and quarantine duplicates. Detection options were applied sequentially (title, authors, abstract) and the process was monitored to guarantee no loss of information/references.

1.2.1.4 Selecting studies for inclusion and exclusion in the review

2.2.1.5.1. Exclusion criteria

Tables 1 and 2 report the exclusion criteria applied for title and abstract screening, and full text screening, respectively.

Table 1: Title and Abstract screening exclusion criteria

Description of the exclusion criteria	**Exclusion code**
Do not have access to the abstract or the abstract is not in English	EC 1
Documents not related to Food/Feed Science and Technology or that do not have interest for the topic of the work because speculate, are too general or describe historical or irrelevant data	EC 2
Documents describing/studying a product that does not contain protein (e.g. oils or sugars)	EC 3
Documents describing/studying a product that cannot be considered as NF or UF according to the definitions indicated above or it is not produced from the eight crops listed	EC 4

Table 2: Full text screening exclusion criteria

Description of the exclusion criteria	**Exclusion code**
Do not have access to the full text or the full text is not in English	EC 1
Documents not related to Food/Feed Science and Technology or that are not related to the topic of the work because speculate, are too general or describe historical or irrelevant data	EC 2
Documents describing/studying a product that does not contain protein (e.g. oils or sugars)	EC 3
Documents describing/studying a product that cannot be considered as NF or UF according to the definitions indicated above or it is not produced from the eight crops listed	EC 4
Documents describing/studying a process/technology that cannot be applied to produce any NF or UF according to the definitions indicated above or to produce a protein product from the eight crops listed	EC 5
Documents describing/studying a process/technology that cannot be applied to proteins or has a low TRL (Technology Readiness Level)	EC 6

2.2.1.5.2. Study selection

Title and abstract screening were carried out in parallel by two independent reviewers. Articles marked as irrelevant by the two reviewers were excluded without proceeding to the full-text analysis. In case of a lack of consensus among the reviewers or if the two experts had doubts about a specific document, a conservative approach was followed to ensure completeness and the document was considered for full text screening.

Full text screening was carried out by a single reviewer. Relevant data from the selected articles were extracted according to section 2.2.1.7.

The resulting hits from each screening step is reported in the results section, which includes a flow chart following the provisions of the PRISMA statement (Moher et al., 2009), a structured framework ensuring the transparent and systematic reporting of reviews.

Each step of the article screening/selection process was documented within the DistillerSR environment to assure transparency and reproducibility.

The shortlisted included and excluded studies and the reasons of selection are provided in Appendixes C.1 and C.2.

1.2.1.5 Protocol for grey literature search and screening

A specific methodology for searching, documenting, reporting and selecting the grey literature was applied according to Godin et al., 2015. Nevertheless, similarly to other information sources, grey literature followed SR reporting standards as established in the PRISMA statement (Moher et al., 2009).

- Patents

The search strings used for the standard literature review were manually translated into Spacenet syntax. Eligibility criteria (inclusion/exclusion, Tables 1 and 2) were the same as those for other information sources.

Title and abstract screening were carried out in parallel by two independent reviewers. Articles marked as irrelevant by the two reviewers were excluded without proceeding to the full-text analysis. In case of a lack of consensus among the reviewers or if the two experts had doubts about a specific document, a conservative approach was followed to ensure completeness and the document was considered for full text screening. Full text screening was carried out by a single reviewer. Relevant data from the selected articles were extracted according to 2.2.1.7.

The resulting hits from each screening step are reported in Figure 9 which includes a flow chart following the provisions of the PRISMA statement (Moher et al., 2009).

Each step of the article screening/selection process was documented to assure transparency and reproducibility. Information about the expertise of the reviewers was also recorded.

The shortlisted included and excluded studies and the reasons of selection are provided in Annexes C.1 and C.2.

- Surveys

Survey responses were collected and revised by a single reviewer. Data extraction was carried out simultaneously.

-Workshop

The information gathered from the two workshop sessions was used to refine the maps and develop/discuss the SR results.

1.2.1.6 Data extraction into evidence tables

Excel spreadsheets were used for data extraction from the eligible studies after full-text screening, including the following fields:

- Bibliographic information: Authors, year, journal, title, type of document
- Search Category: NF, UF, Crops.

- Source of proteins: major category (NF, UF, crops), product category (crops, pulses, water plants, other plant-based proteins, insects, microalgae, macroalgae, fungi, microbial protein/single cell protein, fermentation-derived proteins, krill, others)
- Product description: species, variety and composition, if available
- Use: general category (food or feed), type of use (whole, dietary supplement or ingredient). If used as a food ingredient also the food category (bakery, dairy, meat, fish, fresh vegetable food, dry food e.g. pasta, canned food, others)
- Processing technology: History of use (known/well-established or innovative technologies), specific technology and operational conditions under which the corresponding results were obtained.
- Operational conditions (time, temperature, pressure, solvent type and concentration…). As a conservative approach, all the parameters or conditions described in the study were extracted, even if it was unknown whether the specific parameter could affect proteins.

Depending on the particular field, the collected information was numerical (for example operational conditions), fixed text (such as food/feed) or free text.

1.2.2 Protocol for the mappings

Data extracted from the selected studies into evidence tables were synthesized in both tabular and narrative formats. In addition, Visio software was used to build flowcharts from the collected data. Based on this, a data repository of products and processes was constructed (see section 3.1.1.4).

1.2.2.1 Mapping of the Novel Food, Unconventional Feed and GM crop protein products

The outcome of this mapping exercise was a list of relevant protein products (see section 3.1.1.4).

1.2.2.2 Mapping of the process and processing conditions

Based on the data extracted, flowcharts of the production process for each of the protein-product combinations were constructed (see section 3.1.1.4).

1.2.3 Processing conditions applied to (GM) crops: comparison with OECD consensus documents (Objective 3)

Comparative flowcharts were constructed to illustrate the differences found between the production processes and processing conditions mapped (Objectives 1 and 2) for (GM) crops and those reported in OECD consensus documents (OECD 2002a, 2002b, 2004, 2011, 2012, 2016, 2020). For this purpose, the flowcharts from the OECD consensus documents were used as a basis, and new processes/processing lines and/or products were incorporated into them. Section 3.2 provides more detailed information on the new products and processes incorporated into the flowcharts, as well as the sources. Such differences were discussed in detail based on expert judgement. Data were also presented in narrative and tabular formats to ensure their readiness for food and feed risk assessments.

1.2.4 Protocol for the Systematic (literature) Review (Objective 4)

1.2.4.1 Definition of the question/s

The key elements of the SR question were identified in order to verify that the question was suitable for a SR. the standard PICO/PECO approach was used, in which the key elements are the population of interest (P), the intervention (I) or exposure of interest (E), a comparator (a control – C) and the outcomes of interest (O).

The PICO/PECO elements identified were the following:

-Population: the food/feed product containing novel proteins [NF, UF and (GM) crops]

-Intervention/Exposure: the process applied to the product (e.g. thermal treatment)

-Comparator: control, non-exposure (food/feed product not exposed to the processing technology or exposed to a technology with known/quantifiable effect)

-Outcome: the safety related effect (e.g. toxicity)

Thus, the SR was designed to answer the following question: Which is the impact of processing on the safety of novel food/feed proteins?

1.2.4.2 Information sources

The following information sources were used:

➲ Scientific literature databases: The following databases were used: PubMed/MEDLINE, (https://www.ncbi.nlm.nih.gov/PubMed), Scopus (https://www.Scopus.com/home.uri) and Web of Science (https://www.webofknowledge.com).

➲ Reference lists: The reference list at the end of relevant publications including reviews and guidelines was checked to identify studies that had not been otherwise retrieved.

1.2.4.3 Search strategy

2.2.4.3.1 Keywords and Search Strings

The following search string (used to capture the key elements of the research questions) was used:

(Processing OR manufacture OR production OR fabrication OR Washing OR Cleaning OR blending OR cutting OR crushing OR chopping OR grinding OR milling OR disruption OR lysis OR "pressure homogenization" OR "bead beating" OR "innovative technologies" OR pressing OR "pulsed light" OR "ultrasounds" OR cavitation OR "cold plasma" OR "hydrostatic pressure" OR ultraviolet OR UV OR irradiation OR "electric fields" OR "magnetic fields" OR Extraction OR distillation OR solvent OR Separation OR centrifugation OR decantation OR defatting OR dewatering OR dialysis OR filtration OR Sieving OR filtering OR flocculation OR Precipitation OR Crystallisation OR Crystallization OR Steeping OR Infusion OR Mixing OR homogeneisation OR homogenization OR "Chemical reaction" OR "Chemical hydrolysis" OR "Enzymatic reaction" OR "Enzymatic hydrolysis" OR Enzyme OR hydrolysis OR Fermentation OR cultivation OR

Refining OR purification OR "cation exchange" OR "anion exchange" OR "affinity chromatography" OR heating OR Pasteurization OR Sterilization OR "heat treatment" OR "thermal treatment" OR "heat process" OR "thermal process" OR Thermalized OR Blanching OR Boiling OR Baking OR Frying OR steaming OR ensiling OR delinting OR dehulling OR bleaching OR drying OR Evaporating OR evaporation OR Dehydrating OR Desiccating OR Desiccation OR Freeze-Drying OR "Vacuum Drying" OR "spray drying" OR "spray dry" OR atomization OR "Atomized drying" OR de-watering OR "radiofrequency drying" OR "Microwave Drying" OR "Supercritical Fluid Drying" OR "Infrared Drying" OR "Pulse Combustion Drying" OR "Ultrasound-Assisted Drying" OR Extrusion OR extruding OR "pH adjustment" OR acidification OR "acid treatment" OR alkalinisation OR alkalization OR "alkaline treatment" OR decolourisation) AND (Toxicology OR immunotoxicity OR allergenicity OR nutrition OR nutritive OR Antinutritive OR digestibility OR "GIT microbiome" OR "gastrointestinal microbiome" OR Disbiosis OR toxicology OR toxicity OR toxic OR poisonous OR lethal OR noxious OR venomous OR deadly OR harmful OR unsafe OR detrimental OR allergic OR sensitizing OR allergens OR allergen OR allergenic OR Hypersensitivity [Title/Abstract]OR Anti-absorptive OR Bioavailability OR Absorption OR Assimilation OR "gut flora" OR "gut microbiota" OR "intestinal microbiota" OR "chemical risk" OR "chemical hazard" OR "cancer" OR "carcinogenic" OR "mutagenic" OR "genotoxic" OR "mutagenic" OR "teratogenic") AND ("Novel feed" OR "Innovative feed" OR "alternative feed" OR "new feed" OR "Recent feed" OR "Emerging feed" OR "Modern feed" OR "Revolutionary feed" OR "Pioneering feed" OR "Advanced feed" OR "unconventional feed" OR "Novel ingredient" OR "Innovative ingredient" OR "alternative ingredient" OR "new ingredient" OR "Recent ingredient" OR "Emerging ingredient" OR "Modern ingredient" OR "Revolutionary ingredient" OR "Pioneering ingredient" OR "Advanced ingredient" OR "unconventional ingredient" OR "Novel food" OR "Innovative food" OR "New food" OR "Recent food" OR "Emerging food" OR "Modern food" OR "Revolutionary food" OR "Pioneering food" OR "Advanced food" OR "Emerging Culinary Trends" OR "Modern Gastronomy" OR "unconventional food" OR ((NEPs OR "newly expressed proteins" OR Cry OR IPD OR Mpp OR Vip3OR Vpb OR ATHB17 OR HAHB4 OR EPSPS OR AAD OR APH4 OR AHAS OR FT_T OR PAT OR GAT OR GOX OR ASL OR HPPD OR PMI OR TDO OR DMO OR NPTII) AND (maize OR "Zea mays" OR Corn OR Soybean OR "glycine max" OR Soy OR rapeseed OR "brassica napus" OR Canola OR Cotton OR "Gossypium hirsutum" OR "sugar beet" OR "Beta vulgaris" OR Cameline OR "Camelina" OR Rice OR "Oryza sativa" OR Potato OR "Solanum tuberosum"))) AND (Protein OR "Protein-rich" OR "Amino Acids-rich" OR "Peptide-rich" OR "High-protein" OR "Protein-packed" OR "Protein-dense" OR "Protein-laden" OR "Protein-heavy" OR "Protein-filled" OR "Protein-abundant" OR "Protein-loaded" OR "Protein-intensive" OR "Protein-nutrient" OR "Protein-concentrated" OR "Protein-adequate" OR "Protein-saturated" OR "Proteinaceous" OR "Aminoacid-rich" OR "Amino-protein-rich" OR "Peptide-based" OR "Protein-containing" OR "Peptidic-rich")

2.2.4.3.2. Constraints in the search strategy

- Fields: Title and abstract
- Dates: No temporal constraint.
- Languages: English

Information sources: see 2.2.4.2

1.2.4.4 Documenting and reporting the search process

In the case of PubMed, searches were directly performed using DistillerSR. For Scopus and Web of Science, searches were carried out in their respective websites/search engines and then the search results/references were imported to EndNote, which was used to create the RIS files that were then exported to DistillerSR. The full search strings were recorded exactly as run, together with the number of records retrieved.

DistillerSR automatically removed the duplicates from PubMed searches during the upload process. Once all the references had been uploaded, the DistillerSR Duplicate Detection tool was used to find and quarantine duplicates. Detection options were applied sequentially (Title, authors, abstract) and the process was monitored to guarantee no loss of information/references.

1.2.4.5 Selecting studies for inclusion and exclusion in the review

2.2.4.5.1. Exclusion criteria

Table 3 reports the exclusion criteria applied for title-abstract screening and full text screening.

Table 3: Exclusion criteria used in the Systematic Review

Description of the exclusion criteria	**Exclusion code**
Full text not available	EC1
Studies/experiments not associated to novel foods, unconventional feed or (GM) crops or to food/feed products that do not contain protein	EC2
Studies/experiments not involving food/feed processing	EC3
Articles that do not evaluate protein safety (protein toxicity, immunotoxicity, allergenicity, nutritional value, digestibility and impact on GIT microbiome)	EC4
Studies that do not include a comparator/control (or a reference to it) as per the definition above	EC5
Documents with general speculation, general description, or historical description of food/feed processing, novel protein sources or protein safety or any other document that cannot be categorized in inclusion criteria and cannot be excluded according to the above-mentioned criteria	EC6

2.2.4.5.2. Study selection

Two independent researchers performed the initial screening of the articles based on study titles and abstracts using an assessment form and keeping into account inclusion and exclusion criteria indicated above. At this stage, if one of the reviewers considered the study to be eligible for inclusion, the reference was included for the full text screening.

After retrieving the complete articles, full text review was carried out to confirm the eligibility of the studies. As for Title and Abstract screening each reviewer separately applied the inclusion/exclusion criteria and completed an assessment form. If at least one of the reviewers considered the study eligible for inclusion, the reference was included for data extraction and

risk of bias assessment. The selected articles underwent the data extraction process (see 2.2.4.6) and risk of bias assessment (2.2.4.7).

The whole process of the selection of articles was documented and reported in a PRISMA flow chart (Figure 44).

The shortlist of included and excluded studies and the reasons of selection is provided in Appendixes D.1 (included) and D.2 (excluded) in an openness way.

1.2.4.6 Collecting the data from the included studies and creating evidence tables

Specifically designed Excel spreadsheets (Appendix E) were used for data extraction.

The information collected from included studies was the following:

- Bibliographic information:
  - o Title
  - o Abstract
  - o Author
  - o DOI
  - o URL
  - o Item type
  - o Journal
  - o Language
  - o Volume
  - o Pages
  - o Year
- PICO/PECO definition:
  - o Study design
    - -Type of study, number of intervention/exposure groups and controls, inclusion and exclusion criteria.
  - o Population: Food/Feed product
    - -Food product (including species and variety) and population category (Novel food, Unconventional feed or GM crop).
    - -Intended use: Ingredient vs whole and category.
    - -Protein studied and concentration (if available)
    - -Composition and physico-chemical characteristics of the food product (if available)

o Intervention of interest/processing technology

-Processing technologies applied (known/well-established and innovative technologies) and magnitude and definition of each one of the operational conditions (time, temperature, pressure, solvent type and concentration…).

o Outcome(s)

-Methodology/technique used to measure the outcome

-Changes in toxicity, immunotoxicity, allergenicity, nutritional value, digestibility and impact on host GIT microbiome

Number of reviewers: two, one for data extraction and one for checking the extracted data.

Disagreements: In case of borderline data to extract, a conservative approach was used to ensure that no information was lost. Data were checked by a second member of the team for confirmation.

For each quantitative data a point estimate (mean) was extracted together with an estimate of variability (standard deviation, standard error or confidence interval). When required WebPlotDigitalizer was used to extract quantitative data from figures.

1.2.4.1 Appraisal of the risk of bias

The methodological quality of each included study was critically appraised. Aspects of the design, execution, analysis and reporting of each study that may lead it to give a biased result were assessed. Each study underwent a standardized assessment, checking whether or not it meets a predefined list of methodological characteristics.

Two independent reviewers scored each one of the studies according to risk of bias. The following points were analysed: Selection bias, Performance bias, Detection bias, Attrition bias and Reporting bias. The studies were judged on three levels of bias: high, low and unclear. This step was done independently by the reviewers. Disagreements regarding scores were resolved by discussion and consultation with a third reviewer where needed.

This information was embedded in the same file for data extraction recording indicated in the previous point (2.2.4.6).

This assessment was used to interpret the results of primary studies and reviews when synthesized in this review and in the formulation of conclusions. As a criterion, a global risk of bias score was not used; instead, articles for which a high risk was determined in any of the five evaluated aspects were not included in the evidence table and were excluded from the discussion and conclusions. Those, mostly reviews, for which the outcome of this evaluation was unclear for all or some of the parameters were still considered, but the original data source was consulted for extraction (this source was also evaluated following the methodology).

1.2.5 Synthesis and discussion of the data obtained from the studies included in the SR

The information was synthesized in a tabular format and discussed in a narrative way. When required figures were included.

In order to discuss the impact of processing on protein safety regarding toxicology, immunotoxicity, allergenicity, nutrition, digestibility, GIT microbiome of the host for the food and feed products studied, the discussion was enriched, when required, with the data/results of the scoping review (including workshop minutes and stakeholder survey) and mapping (Objectives 1 and 2). In addition, the following documents/information sources were also considered:

- Consultation process on NF status (European Commission) (https://food.ec.europa.eu/safety/novel-food/consultation-process-novel-food-status_en)
- EU NF Catalogue (https://food.ec.europa.eu/safety/novel-food/novel-food-catalogue_en)
- Scientific opinions on NF published in EFSA journal (open EFSA) and ongoing dossiers under risk assessment.
- EFSA Guidance on the preparation and presentation of an application for authorisation of a NF in the context of Regulation (EU) 2015/2283 (EFSA NDA Panel, 2016)
- Scientific opinions on GMs published in in EFSA journal (open EFSA) and ongoing dossiers under risk assessment
- EU GMO register (https://food.ec.europa.eu/plants/genetically-modified-organisms/gmo-register_en)

2 Assessment/Results

2.1 Objectives 1 & 2

2.1.1 Search strategy

The search strategy was developed as described in section 2.2.1.

2.1.1.1 Results from the scoping literature search

Table 4 shows the results obtained from the searches in scientific databases and Spacenet. It should be noted that since searches in PubMed were carried out directly with DistillerSR, this software automatically removed some duplicated documents (same ref ID) during the upload process.

Table 4: Search results

Query date	Number of hits
1 – PubMed_19/03/2024	*3890*
2 – WoS_19/03/2024	*3740*
3.- Scopus_19/03/2024	*3636*
4. –Spacenet_14-16/03/2024	*3021*
Total with duplicates	*14287*
Total without duplicates	***9636***

2.1.1.2 Screening of relevance

After the title and abstract screening 2,269 documents were found relevant, whereas 7,367 were excluded. Another 1,016 documents were excluded after full text screening. Therefore, a total of 1,325 documents were found as relevant (Table 5), including 34 out of the 69 survey responses and 224 documents from the category other document types (mainly consultations, opinions…).

Table 5 and Figure 7 display the number of documents, per type, finally included in the data extraction process. Most of the documents fell into the "research articles + reviews" category. Both subcategories of documents are presented together as no distinctions were made between them during the data extraction process. It can also be observed that very few book chapters were included in the data extraction process, since in most cases their approach/focus was considered very general by the reviewers. Finally, the number of "other document types" is also quite high since it includes all EFSA scientific opinions on the safety of NFs, consultations on NF status and NF authorisations.

Table 5: Relevant documents after full text examination

Type of document identification	Number of documents
Relevant	***1325***
Research articles + reviews	*750*
Books/book chapters	*4*
Patents	*313*
Survey responses	*34*
Other document types	***224***

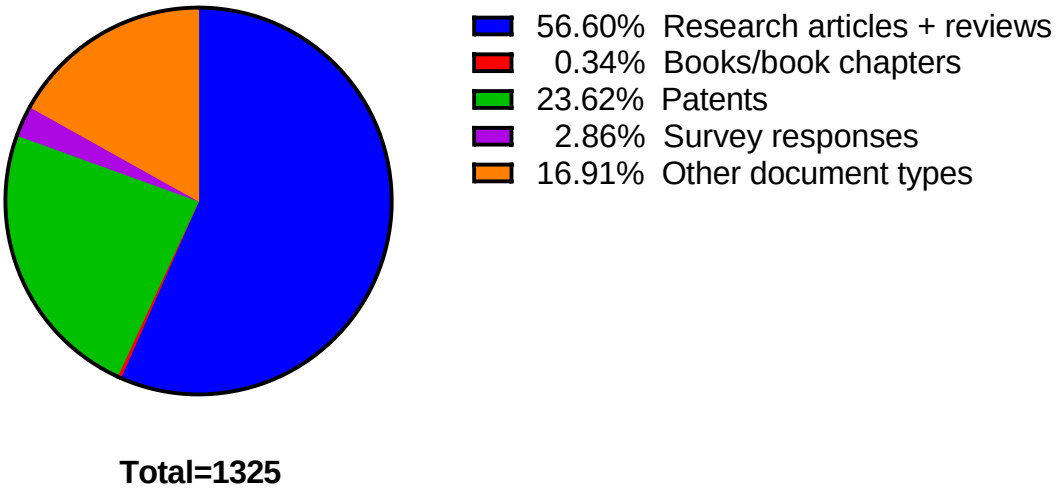

Figure 7: Distribution of relevant documents by type.

Regarding the relevant documents retrieved by search category the number of documents was very similar for the three of them as can be observed in Figure 8. It should be noted that the figure does not include the surveys or the "other document types".

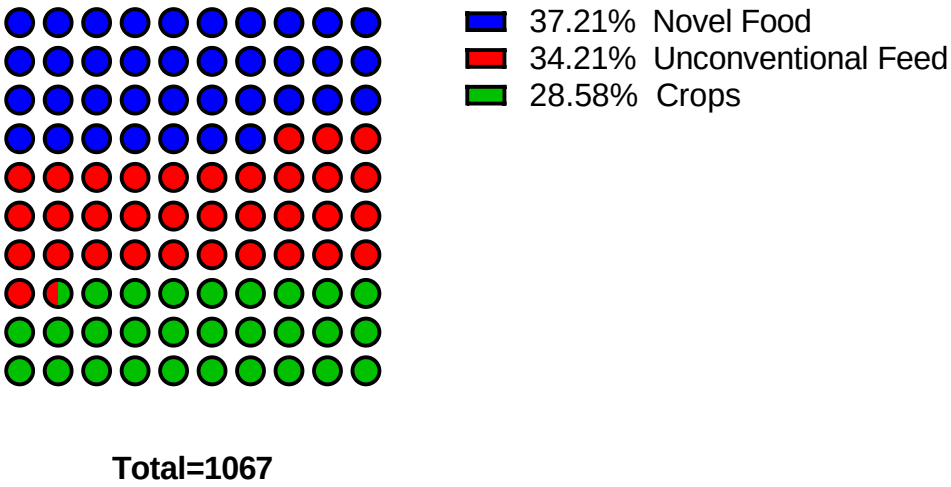

Figure 8: Distribution of relevant documents by search string category.

2.1.1.1 PRISMA statement

This section outlines the adherence to the PRISMA guidelines (Moher et al., 2009). The flowchart (Figure 9) exemplifies the methodical approach taken for the identification, screening, and inclusion of studies, reviews, books and other type of articles, highlighting the integrity and robustness of the research process adopted for this call.

The major cause of exclusion at the title and abstract screening step was the Exclusion Criteria 2 (EC 2) "documents not related to Food/Feed Science and Technology or that do not have interest for the topic of the work because speculate, are too general or describe historical or irrelevant data", which was the cause for article exclusion in almost 79% of the cases (Figure 9). It was followed by EC 3 (documents describing/studying a product that does not contain protein), accounting for 8% of the exclusions, EC 4 (documents describing/studying a product that cannot be considered as NF or UF according to the definitions indicated above or it is not produced from the eight crops listed), for 4%, and EC 1 (record for which we did not had access to the abstract or it was not in English) for 3%. It should also be noted that up to 451 documents (6%) were excluded due to several concurrent criteria.

Regarding the full text screening step, the EC 2 was again the major cause for article exclusion (61%), although EC 4 substantially increased (27%) since in many cases the studies/patents dealt with food products that did not fall with the category of NF as defined above (products authorized in the EU or under risk assessment by EFSA at the time of the searches).

Finally, 35 of the responses to the survey were excluded because they did not provide data on the product/production process, or they were incomplete.

The full list of included and excluded documents is indicated in Figure 9 and provided in Appendixes C1 (included) and C2 (excluded).

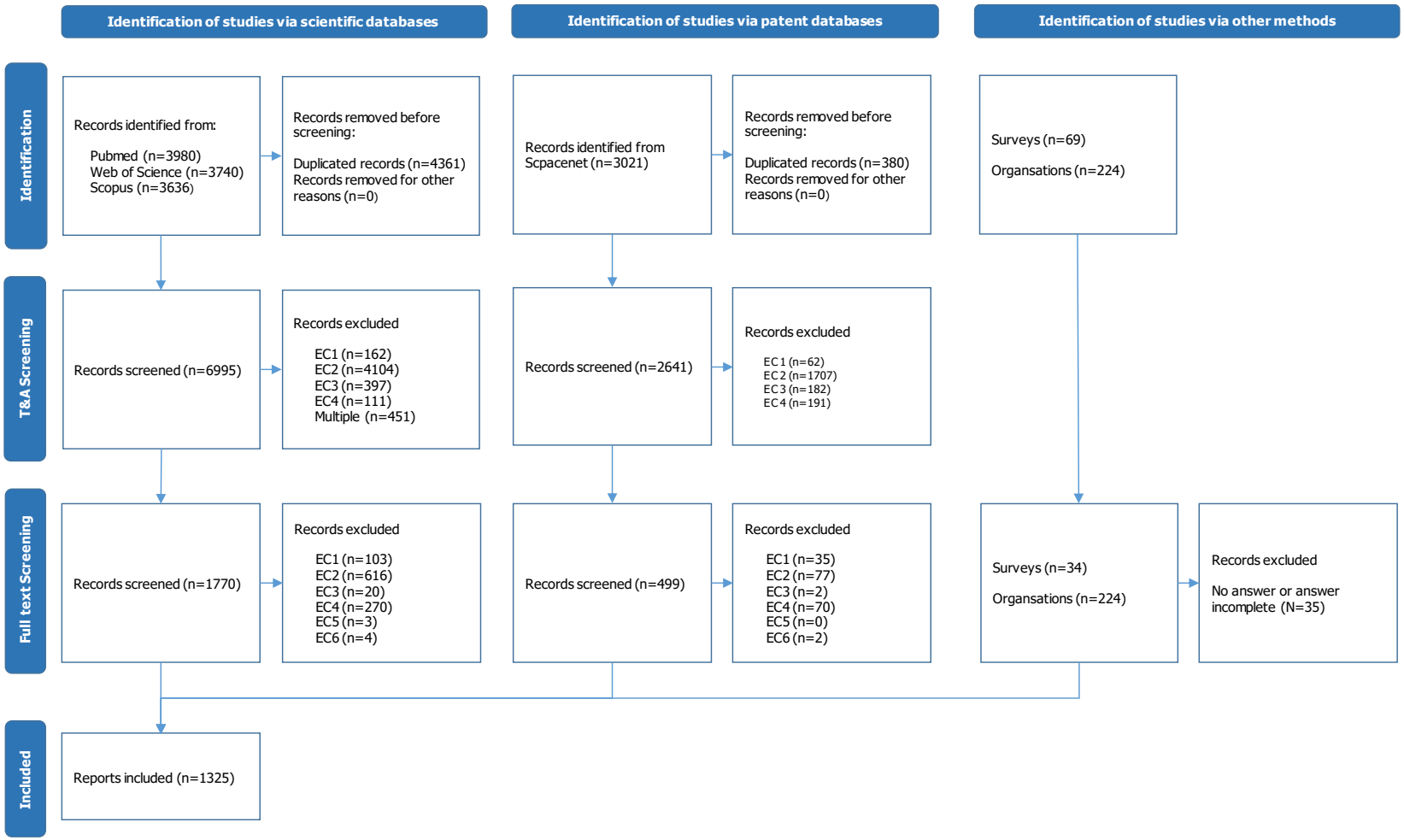

Figure 9: Scoping review PRISMA flowchart

2.1.1.1 Data extraction and repository/map construction

Data extraction was performed as described in section 2.2.1.7. The data were used to build the repository of novel protein containing food and feed products.

The repository has been divided in Annexes A.1 (Crops), A.2 (NFs) and A.3 (UFs), each one containing several excel sheets (e.g., Figure 10) including the information obtained for each of eight crops under study and for the different food/feed products for which enough information was found to build the flowcharts (see below). Thus, each sheet contains the following data:

-The basic composition of the NFs, UFs and food and feed products derived from the 8 main crops

-A flowchart depicting the corresponding production process(es)

-A table including the operational conditions for each processing step (when it was available).

-A list of the references used to build the map/flowchart.

In addition, the repository also contains an index/table of contents, a description and, in a separated document, a list of terms found/glossary (Annex A.4).

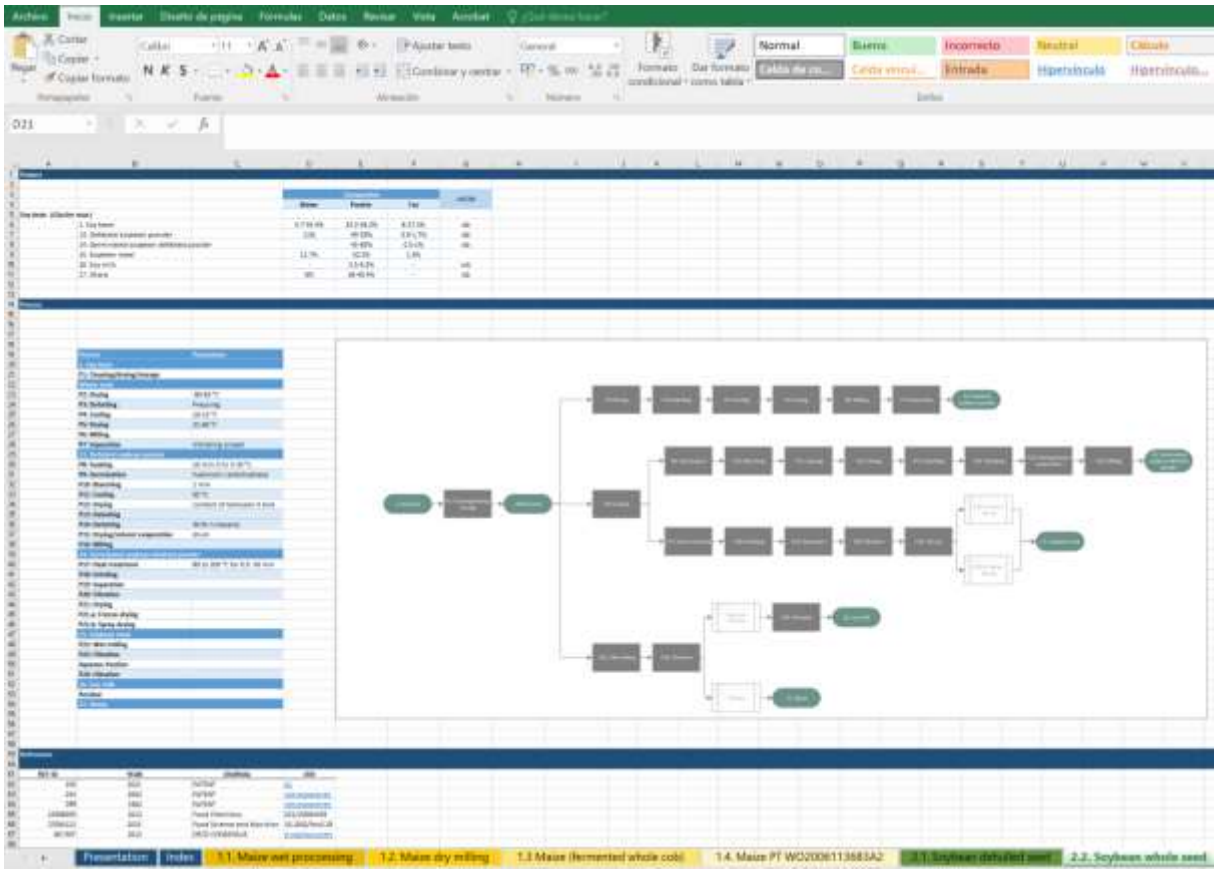

Figure 10: Structure of the Excel sheets from the repository

The flowcharts have been constructed using exclusively the data extracted from the documents gathered from the scoping review. Therefore, some processing steps might be missing in some flowcharts. The same happens for many processing parameters and product characteristics. In fact, it should be noted that there are various cases in which only a single document describing the product/process was retrieved. In most of these cases the document provided sufficient information for drawing a basic flowchart and, therefore, it was included. In any case, this should be interpreted with caution. On the other hand, in some cases, e.g. for krill, the selected papers and patents did not include information about the production process and, therefore, the corresponding product and production process were not included in the repository.

It is also noteworthy that slight differences can be found in the structure/content of Annexes A.1, A.2 and A.3. For instance, for NFs (Annex A.2), due to their particular characteristics, a separate Excel sheet (with the corresponding flowchart) was created for each individual NF, even if originating from the same source (e.g., different NFs from *Olea europaea*). However, the sheets/flowcharts in Annex A.3 for UFs were sorted by category (e.g., insects, algae…) and therefore are more generic. For some of the eight crops under study (Annex A.1), the different transformation processes (e.g. dry and wet milling) were included in different Excel sheets. This annex includes mainly information about grain/seed processing but also forage is included when products obtained from it were found (e.g. from sugar beet leaves or rice straw). Finally, it should be indicated that, in any case, and particularly in the case of crops and unconventional food, the flow diagrams have been designed to be coherent and as simple to interpret as possible, without losing information. That is why, in some cases (such as crops), the transformation processes have been divided into several diagrams, while in the case of UF, the included diagrams do not exactly correspond to the product groups initially proposed and used for the search.

2.2 Objective 3

The Objective 3 of the project was to compare the results of the product and process mappings for the eight crops studied (particularly the flow charts constructed) with those reported in the OECD consensus documents, with the aim of identifying new processes and/or products that could be obtained from these crops.

In this regard, it is important to point out that:

- Since the focus of the project, and therefore the product and process mappings in Annexes A.1, A.2 and A.3, was on novel protein sources, the comparison with the OECD consensus documents was limited to protein-containing products and the corresponding processes.

- There is no OECD consensus document for camelina and therefore only the other seven crops have been discussed.

- The new processes/products identified in relation to those reported in the OECD consensus documents, were retrieved from patents and/or scientific articles, so very often they were only tested on a laboratory and/or pilot plant scales.

-Patents are subject to examination by patent examiners, not to scientific peer review. In several cases, the new processes/products were retrieved from single literature sources.

Based on the above, the representativeness and scalability of the identified processes and products should be considered with caution. Furthermore, Annex A.1 (i.e., product and process mappings for the eight crops) should not be perceived as an "enriched" or "consolidated" version of the processes/products reported in the OECD consensus documents, but as a preliminary effort to document the possible alternatives for the eight crops investigated as retrieved under a certain methodology from sources selected under this project. Further work would be required towards completeness/validation of the new processes/products identified.

As a result of this comparison 47 New Products (NP) with their production processes and 13 Alternative processes (AP) not specifically included or described in the OECD consensus documents were identified. In the context of this project/objective, these "New Products" are not necessarily new/recent or involve innovative processes/technologies. It only means that they were not specifically included in the OECD consensus documents. On the other hand, the term Alternative Processes has been used to designate those processes/processing lines not found (or not fully described) in OECD consensus documents but leading to the production of products already outlined in them.

Table 7 includes all the NP and AP classified by crop. It also includes: a) the name of the product and its number as recorded/designated in Annex A.1., b) the reference source and c) a rough estimate of its technology maturity level (high/established vs low/unclear). This latter classification has been established based on the expertise of the project team members. To determine this, both the number/quality of the bibliographic sources and the technological maturity of each of the technologies/processes involved have been considered. On the other hand, other aspects, such as the commercial viability of the process/product, have not been taken into account. The use of the Technology Readiness Level (TRL) scale as proposed in Horizon 2020 and Horizon Europe (European Commission, 2013) has been deliberately avoided due to the significant challenges in determining this point based on the limited amount of available data in most cases. Nevertheless, the level of technological maturity classified as 'high' or 'established' could correspond to a TRL above 6 (established), although it has also been assigned to processes which, while only described at the laboratory level (TRL <5), do not present, at first glance, any apparent obstacles to reaching a high level of maturity. On the other hand, in cases where the technology/process in question was considered immature or where it was difficult or impossible to determine its technological maturity based on the available data, it has been labelled as 'low/unclear,' which tentatively could be equivalent to a TRL below 5 or simply unknown. For instance, NP that were gathered from a single source, particularly if it was a patent, were initially classified as 'low/unclear' unless the proposed/additional process was well-established (for example, extrusion). On the other hand, there are also cases where, whilst there was a single source, they were classified as 'high/established' (e.g., nixtamalization) since it was a robust and a known/already implemented (established) process or its potential to reach this maturity level was considered high.

Table 6: Summary of all the New Products and Processes identified. NP: new products; AP: alternative processes. Numbering in ID and Product columns are detailed in Sections 3.2.1-3.2.7 and in Annex A.1.

ID	Product (as in Annex A.1)	Reference	Technology maturity level[(a)]
Maize			
NP1	4. Fermented high protein corn liquor and straw feed	Xu et al., 2013	Established/High
NP2	5. Fermented high protein corn starch residue	Kou et al., 2016	Established/High
NP3	8. Fermented corn gluten	Seo et al., 2014	Established/High
NP4	14. Fermented corn straw	Wang et al., 2015; Yodou et al., 2011; Zhu et al., 2018; Zhang et al., 2018	Established/High
NP5	15. Maize meal	Kalumbi et al., 2019	Established/High
AP1	27. Fermentation residue/mash	Gibbons et al., 1983	Established/High
NP6	28. Nixtamalised whole corn flour	Ayala Rodríguez et al., 2009	Established/High
NP7	29. Single-cell protein and a high-protein product from corn fermented mash	Lin et al., 2020	Low/Unclear
NP8	30. Nixtamalized extruded maize flour	Milan-Carrillo et al., 2007	Established/High
NP9	31. Fermented high-protein corn cob	Ke et al., 2016	Low/Unclear
NP10	32. Germ residue by-products	Abbas et al., 2006	Low/Unclear
NP11	33. Starch slurry fine fiber protein		
NP12	34. Protein concentrate		
NP13	35. Starch and fine fiber		
NP14	36. Fermentation solids and fine fiber		
NP15	37. Corn based animal feed		
NP16	38. Non-fiber fractions by-products		
NP17	39. Fiber fraction by-products		

(a) See body text for definitions
(b) Depends on the solvent used

Table 6 *(cont.)*

ID	Product (as in Annex A.1)	Reference	Technology maturity level[(a)]
Soy			
NP18	5. Fried soybean meal	Wu et al., 2019	*Low/Unclear*
AP2	6. Soybean powder	Han et al., 2021; Ferree et al., 1943	*Established/High*
NP19	7. Soybean foam	Ferree et al., 1943	*Low/Unclear*
AP3	9. Defatted-toasted soybean flakes	Kunimoto et al., 2024; Toomer et al., 2023; Lee et al., 2019	*Established/High*
AP4	10. Defatted soybeans		
AP5	11. Defatted soy flakes		
NP20	14. Germinated soybean defatted powder	Marais et al., 2002	*Low/Unclear*
NP21	15. Soybean meal	Baba et al., 1982; Stanojevic et al., 2012	*Established/High*
AP6	16. Soy milk	Yokotsuka et al., 1977; Wakana et al., 1980; Xin et al., 2021; Jiang et al., 2016; Han et al., 2021; Kang et al., 2018; Van den Berg et al., 2022	*Established/High*
AP7	17. Solid soy residue (Okara)	Nti et al., 2015	*Established/High*
NP22	18. Soy milk powder	Xin et al., 2021; Jiang et al., 2016; Han et al., 2021	*Established/High*
AP8	19. Tofu	Kang et al., 2018; Van den Berg et al., 2022	*Established/High*
AP9	20. Extruded soybean meal	Singh et al., 2021; Kumar et al., 2019; Guo et al., 2018; Karunanithy et al., 2012; Jansons et al., 2021; de Moura et al., 2011; Cruz-Ortiz et al., 2020	*Established/High*
AP10	21. Soybean hydrolysate	Okajima et al., 2003; de Regil et al., 2004; Al Loman et al., 2016; Rayaprolu et al., 2017; Zhang et al., 2018; Sitanggang et al., 2021	*Established/High*

(a) See body text for definitions
(b) Depends on the solvent used

Table 6 *(cont.)*

ID	Product (as in Annex A.1)	Reference	Technology maturity level[(a)]
AP11	22. Soybean protein concentrate	Yokotsuka et al., 1977; Alakov et al., 2004; de Moura et al., 2011; Allocco et al., 2014; Li et al., 2019; Lee et al., 2019; Wanga et al., 2021; Singh et al., 2022; Zheng et al., 2023	*Established/High*
AP12	23. Soybean protein isolate	Han et al., 2021; Ferree et al., 1943	*Established/High*
NP23	24. Soybean fractionated protein	Hirotsuka et al., 1995	*Established/High*
NP24	25. Feed from fermented soybean hulls	Han et al., 2019	*Low/Unclear*
NP25	26. Fermented soybean meal (Natto)	Ishii, 1991; Hong, 2019	*Established/High*
NP26	27. Fermented soybean-based meal	Ellegard et al., 2021	*Low/Unclear*
NP27	28. Fermented soybean powder	Ju, 2021	*Low/Unclear*
Rapeseed			
NP28	7. Rapeseed protein concentrate	Tan et al., 2011; Rodrigues et al., 2016; Ivanova et al., 2018; Zhanga et al., 2020; Lia et al., 2020; Zahari et al., 2021; Thi Le et al., 2021; Chairez-Jimenez et al., 2023; Di Lena et al., 2023	*Established/High*
NP29	8. Rapeseed protein isolate	Turck et al., 2020; Tian et al., 2022	*Established/High*
NP30	3. Defatted and fermented rapeseed meal	Croat et al. 2016; Alhomodi et al., 2022; Tian et al., 2023	*Established/High*
NP31	4. Extruded rapeseed meal	Zhanga et al., 2017	*Established/High*
NP32	6. Irradiated rapeseed meal	Xiong et al., 2024	*Established/High*
AP13	1. Defatted rapeseed meal	Navarro et al., 2018; Di Lena et al.; 2021	*Variable*[(b)]
Sugar Beet			
NP33	8. Sugar beet leaves protein concentrate	Akyüz et al., 2021; Brouwer et al., 2023; Goktayoglu et al., 2023; Akyüzs et al., 2023; Akyüz et al., 2024	*Established/High*

(a) See body text for definitions
(b) Depends on the solvent used

Table 6 *(cont.)*

ID	Product (as in Annex A.1)	Reference	Technology maturity level[(a)]
Rice			
NP34	7. Defatted and fermented rice bran	Debi et al., 2021; Ugyen et al., 2023; Shih, 2003	*Established/High*
NP35	8. Extruded rice grits	Zaczuk et al., 2015	*Established/High*
NP36	9. Rice bran protein hydrolysate	Lei et al., 2015; Shih, 2003	*Established/High*
NP37	10. Fermented rice bran	Debi et al., 2021; Ugyen et al., 2023; Shih, 2003	*Established/High*
NP38	17. Rice starch protein hydrolysate	Babini et al., 2020	*Established/High*
NP39	18. Rice protein concentrate	Shih et al., 2003	*Established/High*
NP40	19. Rice protein hydrolysate	Chang et al., 1986; Shih et al., 2004; Chang et al., 2010; Lei, 2015	*Established/High*
NP41	20. Fermented whole rice	Nnam and Obiakor, 2003; Totakul et al., 2020	*Low/Unclear*
NP42	21. Protein from rice straw	Li et al., 2023	*Low/Unclear*
Potato			
NP43	7. Potato powder	Shepherd et al., 2005; Broothaerts et al., 2007	*Established/High*
NP44	8. Potato fruit juice	Akbari et al., 2019	*Established/High*
NP45	9. Fermented potato powder	Liu, 2014	*Low/Unclear*
NP46	10. Potato protein concentrate	Akbari et al., 2019	*Established/High*
NP47	11. Potato protein hydrolysate	Miedzianka et al., 2014; Akbari et al., 2019; Gao et al., 2023	*Established/High*

(a) See body text for definitions
(b) Depends on the solvent used

These products and processes will be discussed in more detail below, each one within the section corresponding to the crop they originate from. In order to facilitate comparisons and easily identify where these new products/processes would fit within the charts of the OECD consensus documents, the latter have been redrawn/adapted, indicating where the processing line leading to the obtention of these new products would start or where the alternative processes would be located. In these charts the codes (numbers) used for the products in Annex A.1 have been included to also facilitate comparisons with it. The processing lines for each of these new products/alternative processes are also included below, starting from the stage or product (whether intermediate or final) from the OECD consensus documents charts from they would begin. As for the redrawn/adapted OECD consensus charts, the product and process numbers from Annex A.1 have been retained in the figures so the processing parameters can be easily checked in that Annex.

Lastly, in order to enable/facilitate this comparison in a reverse yet straightforward manner, Appendix G includes the flow diagrams from Annex A.1, in which the new products and alternative processes have been indicated in red.

2.2.1 Maize

As per the OECD consensus document for maize (OECD, 2002a), this section has been divided in wet processing (3.2.1.1.), dry milling (3.2.1.2.) and Feed Processing (3.2.1.3.) of Maize.

2.1.1.2 Wet processing

In the case of maize wet processing, the searches allowed the identification of five products not described in the OECD consensus document for maize (OECD, 2002a) (Figure 11), together with their production processes (Figure 12). These include four products (NP1-NP4) obtained through fermentation of products already described in the OECD consensus document: corn syrup/liquor (NP1), corn starch residue (NP2), corn gluten (NP3), and corn straw from cleanings (NP4) (Liang et al., 2003; Yudou et al., 2011; Xu et al., 2013, Zhu et al., 2018; Zhang et al., 2018; Kou et al., 2016; Wang et al., 2015; Seo et al., 2014). In addition, the process for obtention of what Kalumbi et al. (2019) denominate "Maize meal" (NP5), a product that is proposed to be part of a maize-based stiff porridge mixed with flour made from hydrothermally treated soybeans to combat protein-energy malnutrition in sub-Saharan Africa, has also been documented (NP5).

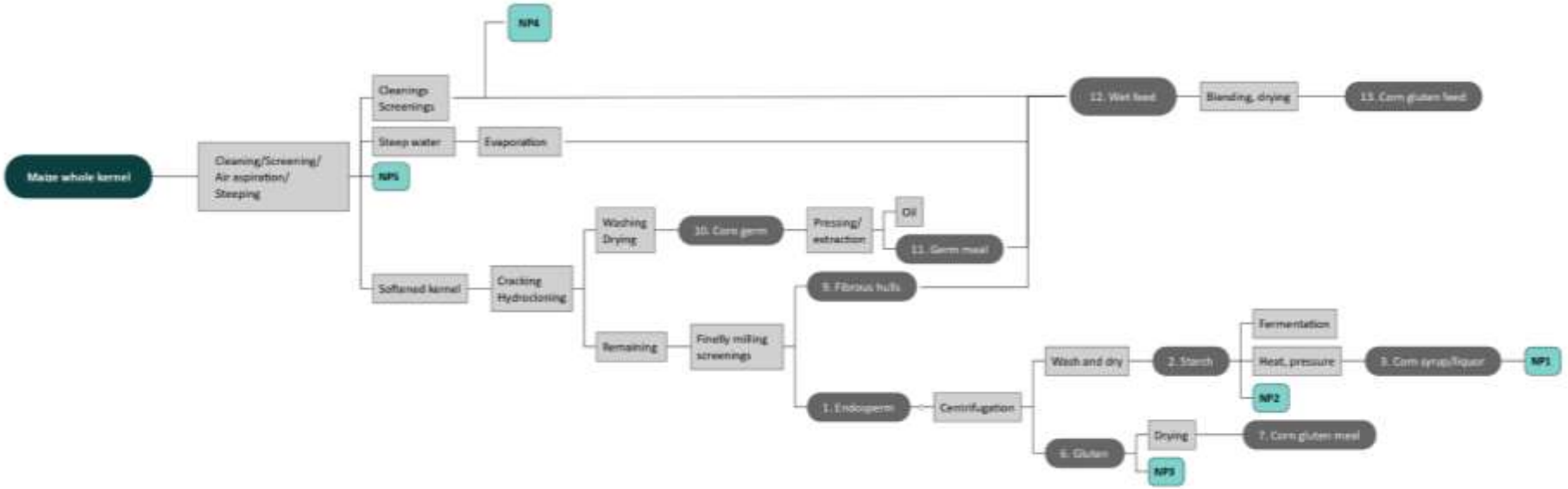

Figure 11: Maize wet processing. Adapted from OECD (2002). New products (NP) are indicated in green.

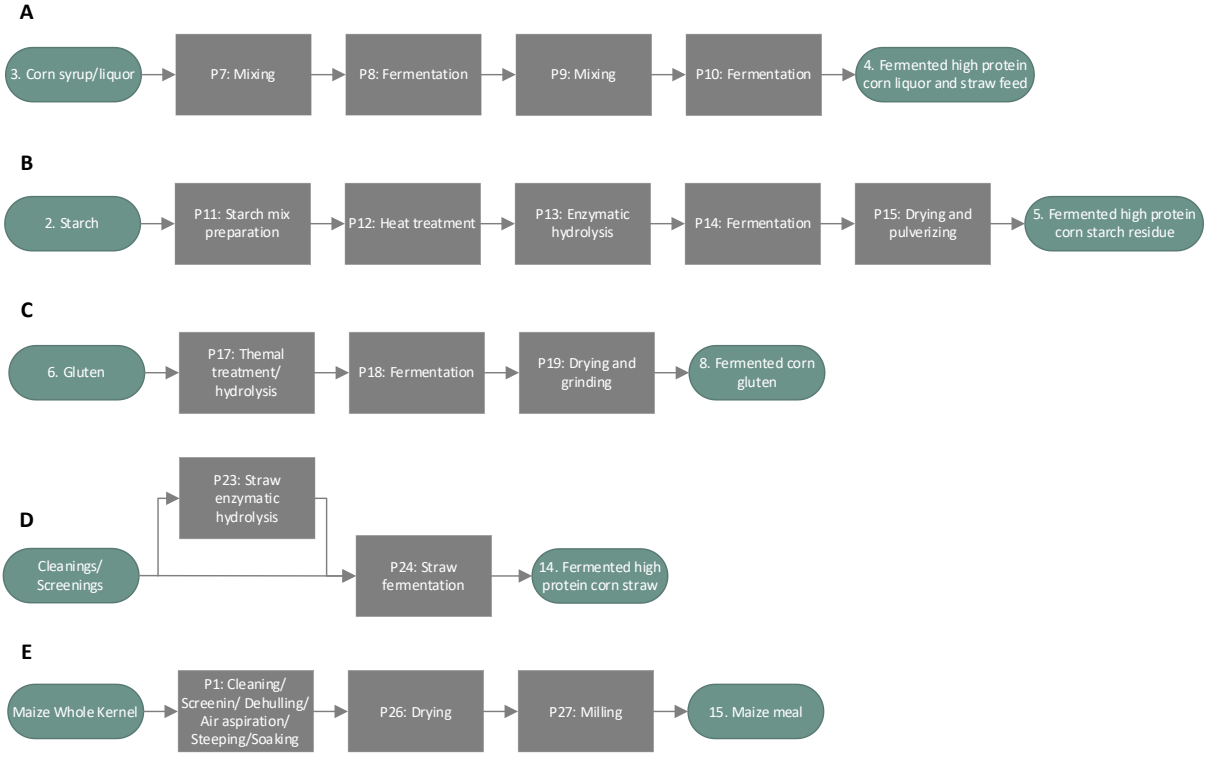

Figure 12: Process for obtaining NP1 (fermented high protein corn liquor and straw feed, A), NP2 (fermented high protein corn starch residue, B), NP3 (fermented corn gluten, C), NP 4 (fermented high protein corn straw, D) and NP 5 (maize meal, E) from maize. Adapted from the flowchart included in Annex A.1: Tab 1.1 Maize wet processing, where treatment parameters are indicated.

2.1.1.3 Dry milling

Regarding maize dry processing, the searches have allowed the identification of three products not described in the OECD consensus document for maize (OECD, 2002a) (Figure 13), as well as their production processes (Figure 14). They include a new product (NP6: Nixtamalised whole corn flour) whose production process starts directly from corn clear grain and based on the nixtamalisation process (basically consisting on soaking and cooking maize in an alkaline solution) (Ayala Rodríguez et al., 2009), another one (NP8: Nixtamalised extruded maize flour) starting from corn fine or coarse grits based on the use of the same technology/process (Milan-Carrillo et al, 2007) and a third one (NP7: Single-cell protein and a high-protein product from corn) obtained from the enzymatic hydrolysis of the fermentation residue/mash (Lin et al., 2020) have been documented. Regarding this latter one, the retrieved information also allowed for the characterization of an alternative process (AP1) for obtaining this fermentation residue/mash from corn clear grain.

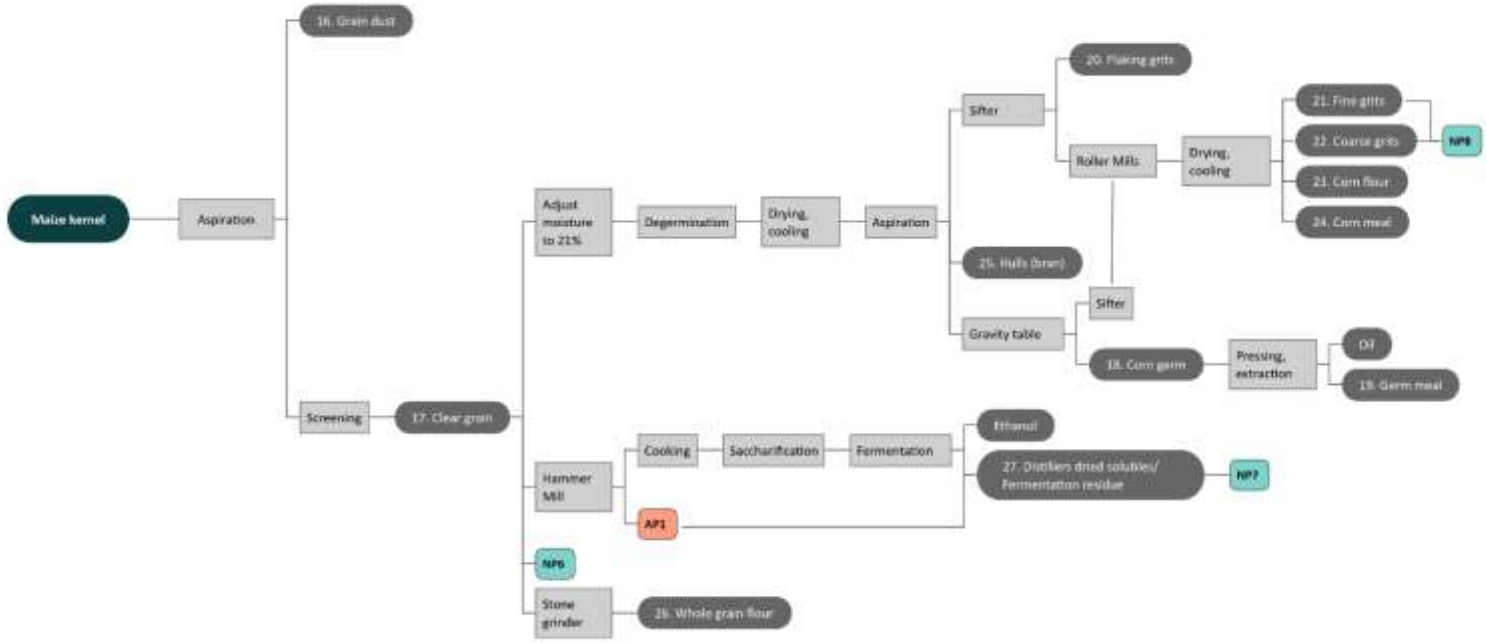

Figure 13: Maize dry milling. Adapted from OECD (2002). New products (NP) are indicated in green and alternatives processes (AP) in orange.

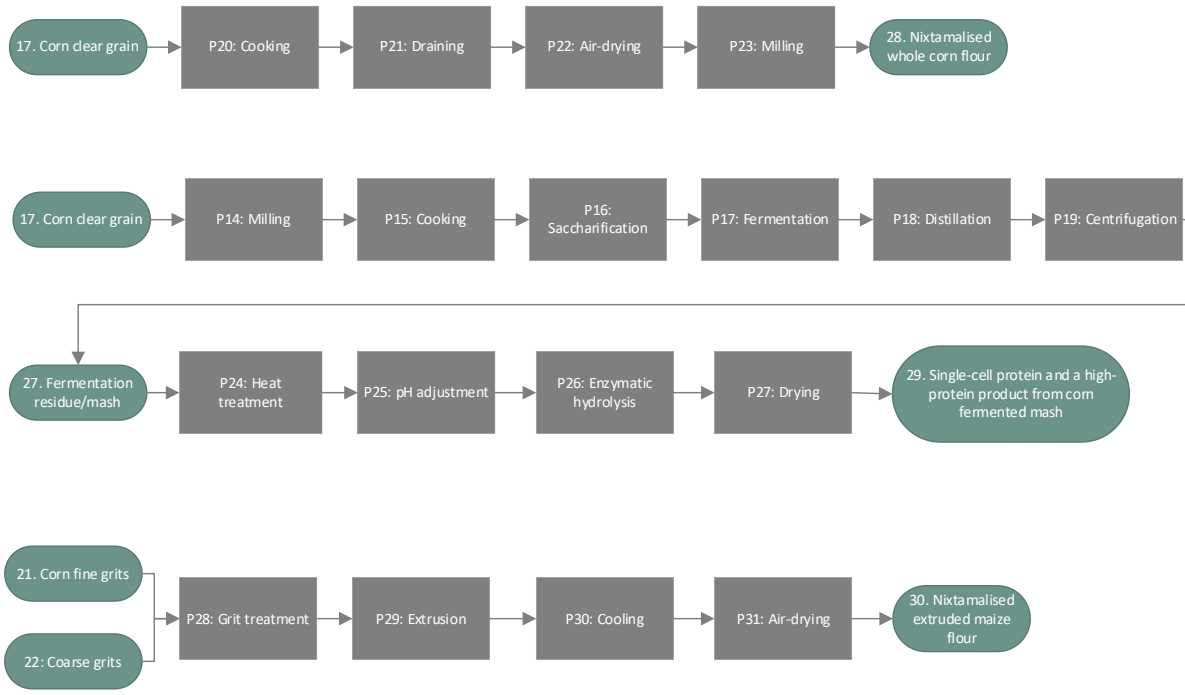

Figure 14: Process for obtaining NP6 (Nixtamalised whole corn flour, A), NP7 (Single-cell protein and a high-protein product from corn, B), NP8 (Nixtamalised extruded maize flour, C) and alternative process (AP1) for the obtention of fermentation residue/mash (B) from maize. Adapted from the flowchart included in Annex A.1: Tab 1.2. Maize dry milling, where treatment parameters are indicated.

2.1.1.4 Whole Plant/ Feed Processing

Finally, two new production processes leading to protein containing products from maize are depicted in Figures 15 (comparison with OECD, 2002a consensus diagrams) and 16 (flowcharts).

For obtaining NP9 (Fermented whole cob) the whole corn cob is subject to a two-stage fermentation process with *Pleurotus ostreatus* and *Saccharomyces cerevisiae*, leading to a fermented feed product with an increased protein content (approx. two-fold) (Ke et al., 2016) (Figure 16A).

On the other hand, in Figure 16B (Abbas et al, 2006), a method to produce a protein concentrate, ethanol and a modified animal feed is depicted. The process includes pericarp and germ removal from the corn kernel, which are further processed into by-products. The protein is extracted into a protein concentrate and the remaining starch is then fermented and distilled to ethanol and stillage. The modified animal feed comprises the pericarp and germ removed from corn kernels and, optionally, by-products of the pericarp and germ processing, and lignocellulosic materials. Therefore, and according to the inventors of this patent, through this method the starch in corn-based animal feed is replaced with biomass fiber treated to make it more digestible by animals.

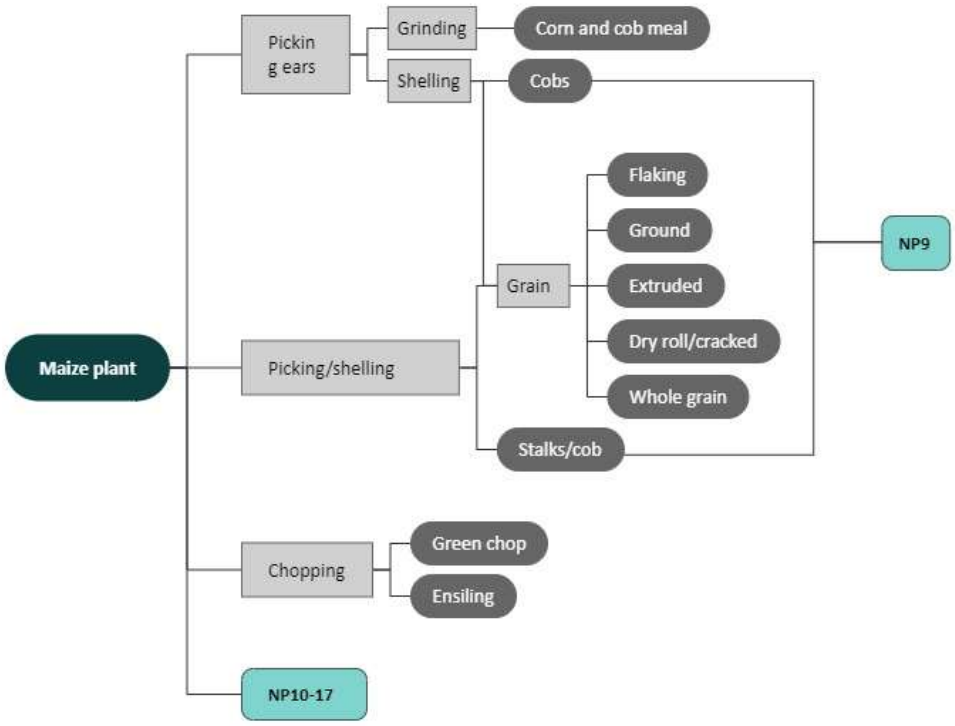

Figure 15: Maize Feed Processing. Adapted from OECD (2002a). New products (NP) are indicated in green.

2.2.2 Soybean

Soybean is the crop under study for which the greatest variety of products and transformation processes has been described as per the conducted scoping literature review. Moreover, different products (final, intermediate, by-products) reported in the OECD consensus document (OECD, 2012) are used as starting materials for several new processes (such as the production of hydrolysates/concentrates/isolates). Therefore, and to make the use and interpretation of the transformation flowchart for this crop easier, seven independent flowcharts were created and included in Annex A.1. However, in order to make comparisons with the OECD consensus document again this section has been divided in the same sections of the OECD consensus document: whole soybean processing (3.2.2.1) and Defatted soybean flakes processing (3.2.2.2)

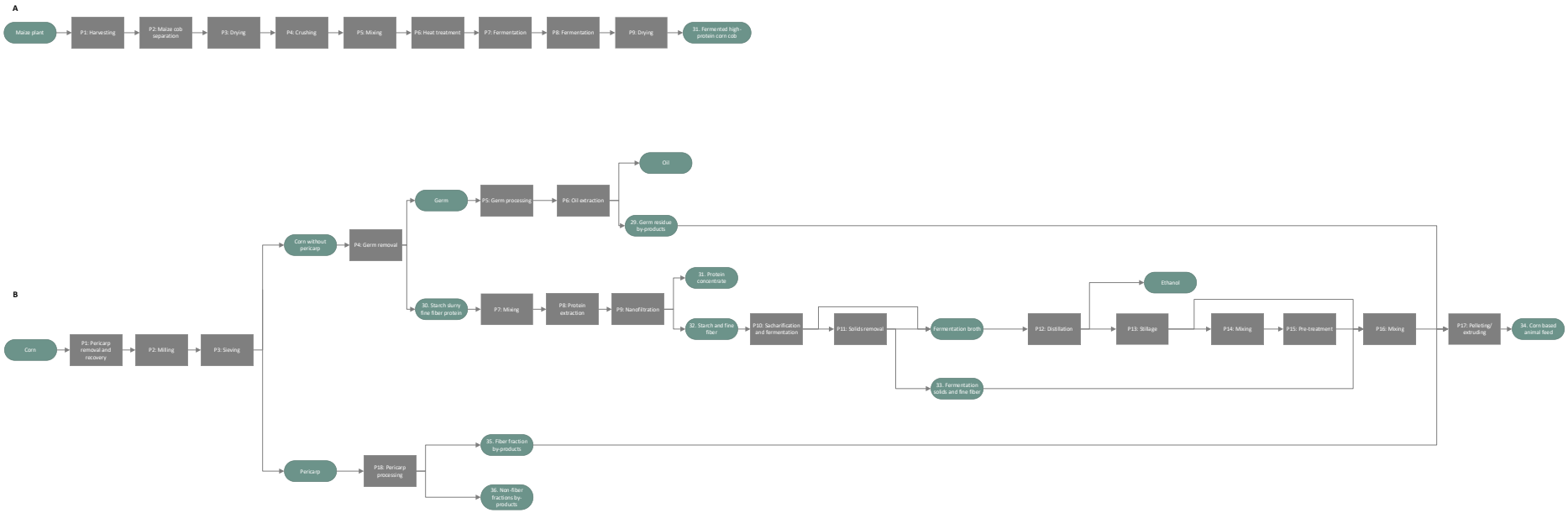

Figure 16: Process for obtaining NP9 (Fermented whole cob, A) and NP 10-17 (B) from maize. Adapted from the flowcharts included in Annex A.1: Tab 1.3. Maize (Fermented whole cob) (A) and Annex A.1: Tab 1.4. Maize PT WO2006113683A2 (B), where treatment parameters are indicated.

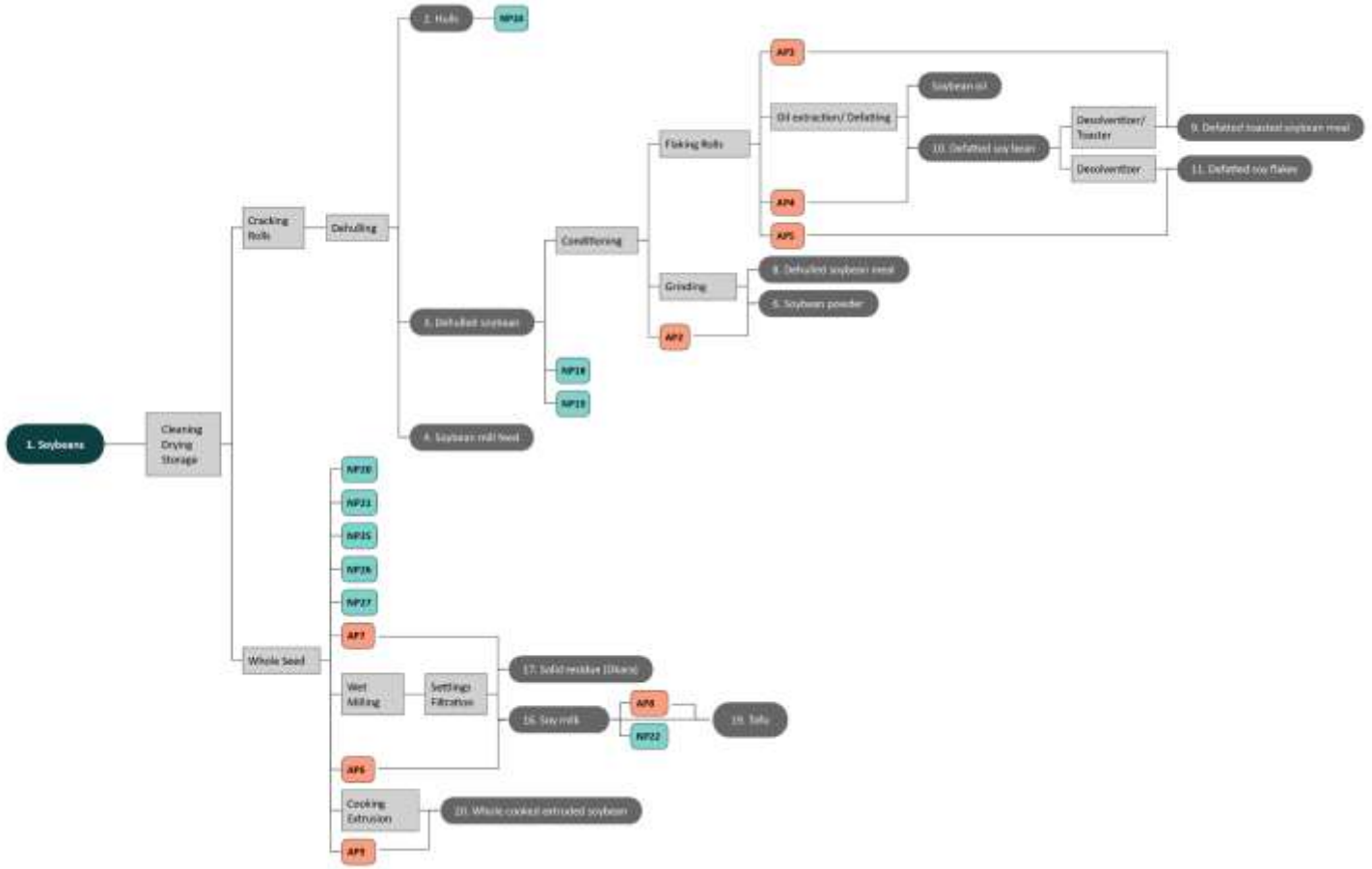

Figure 17: Whole soybean processing. Adapted from OECD (2012). New products (NP) are indicated in green and alternatives processes (AP) in orange.

2.1.1.1 Whole soybean processing

Several products (nine) and (alternative) processes (eight) not included in the whole soybean processing flowchart/diagram from the soybean OECD consensus document OECD, 2012) were found in the scoping review (Figure 17).

As can be observed in Figure 18, NP18 and NP19 correspond to fried soybean meal (Figure 18A) which involves a combined heat + enzymatic treatment and then a frying process as reported in the patent of Wu et al. (2019), and NP 19 (soybean foam), that according to Ferree et al. (1943) might be used as a substitute for egg white. The figure also depicts that different processing lines might be used for obtaining soybean powder (AP2).

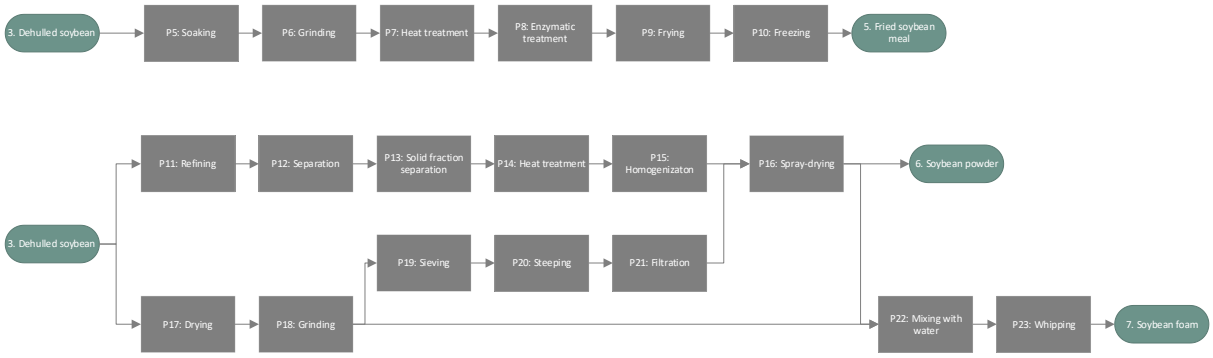

Figure 18: Process for obtaining NP18 (Fried soybean meal, A) and NP19 (Soybean foam, B) and Alternative process for obtaining soybean powder (AP2, B) from soybean. Adapted from the flowcharts included in Annex A.1: Tab 2.1. Soybean dehulled seed, where treatment parameters are indicated.

On the other hand, Figure 19 summarizes the different alternative processing lines (AP3-AP5) for obtaining three products already described in the OECD consensus (2012) (Kunimoto et al, 2024; Toomer et al, 2023; Lee et al, 2019).

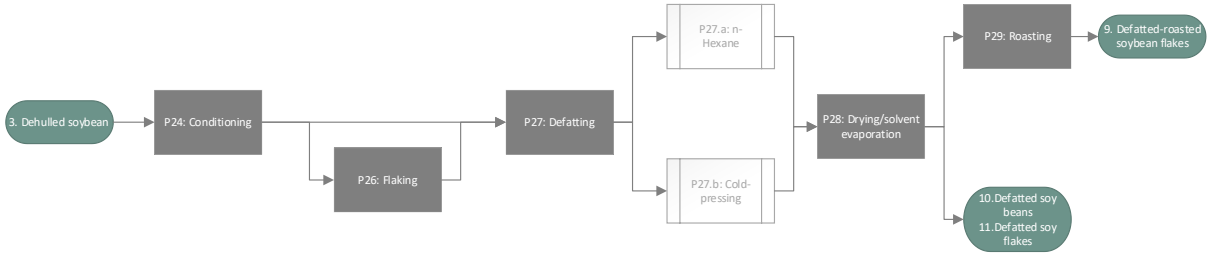

Figure 19: Alternative processes for obtaining defatted toasted soybean flakes (AP3), defatted soybeans (AP4) and defatted soy flakes (AP5) from soybean. Adapted from the flowcharts included in Annex A.1: Tab 2.1. Soybean dehulled seed, where treatment parameters are indicated

Finally, starting from the whole soybean another two products not specifically mentioned in the OECD consensus document can be obtained as described in Figure 20. The first one is NP20 (Germinated soybean defatted powder) which results from the application of standard

defatting and drying process to germinated soya been seeds (Marais et al, 2002) whereas the second was denominated soyabean meal (NP21) by the authors (Baba et al, 1982) and has the particularity of not being defatted.

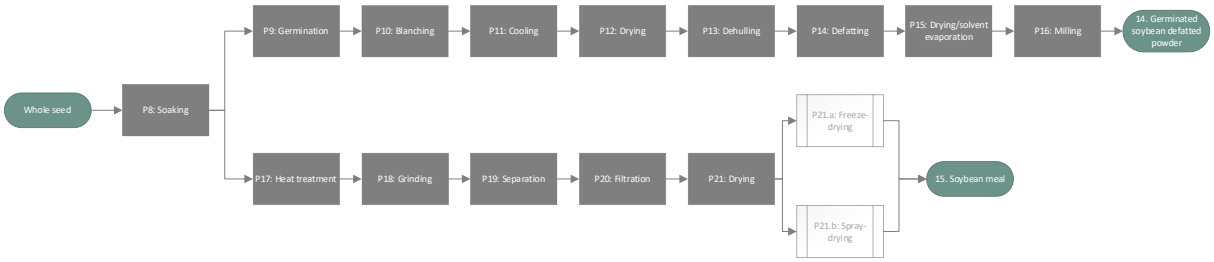

Figure 20: Process for obtaining NP20 (Germinated soybean defatted powder) and NP21 (Soybean meal) from soybean. Adapted from the flowchart included in Annex A.1: Tab 2.2. Soybean whole seed, where treatment parameters are indicated.

Figure 21 includes the processing steps for the obtention of Soy milk powder (NP22; Xin et al, 2021; Jiang et al, 2016; Han et al, 2021) and expands the information available in the OECD consensus document (OECD, 2012) about the production of soy milk, its derivatives and co-products not only by providing a detailed description of the steps required but also the different/alternative processes for its production (AP 6-8) as described by which is not reported in the OECD consensus document (OECD, 2012), is described elsewhere (Yokotsuka et al, 1977; Wakana et al, 1980; Nti et al, 2015; Jiang et al, 2016; Kang et al, 2018; Han et al, 2021; Xin et al, 2021; Van den Berg et al, 2022 in Figure 17 (Annex A.1 – Tab 2.3 Soybean milk and tofu), including different patented alternatives for the production of soy milk (Jiang et al, 2016; Xin et al, 2021).

On the other hand, the diagram in Figure 22 includes, in a simplified and "aggregated" form, the production process for extruded soy products (AP9), including the alternative processes and/or/optional steps, and also considering that these extruded products can be produced from different intermediate products (Singh et al., 2021; Kumar et al., 2019; Guo et al., 2018; Karunanithy et al., 2012; Jansons et al., 2021; de Moura et al., 2011; Cruz-Ortiz et al., 2020). At this point it should be noted that extrusion processes are extremely common, and that Figure 22 is not exhaustive, as extrusion processing also depends on the characteristics of the particular starting product. However, it has been included here since it does not specifically appear in the OECD consensus document (OECD, 2012).

Finally, the production process of four fermented soy products NP24 (Feed from fermented soybean hulls), NP25 (Fermented soybean meal), NP26 (Fermented soybean-based meal) and NP 27 (Fermented soybean powder) is included in figure 23 (Ishii, 1991; Han et al, 2019; Hong, 2019; Ellegard et al, 2021; Ju, 2021).

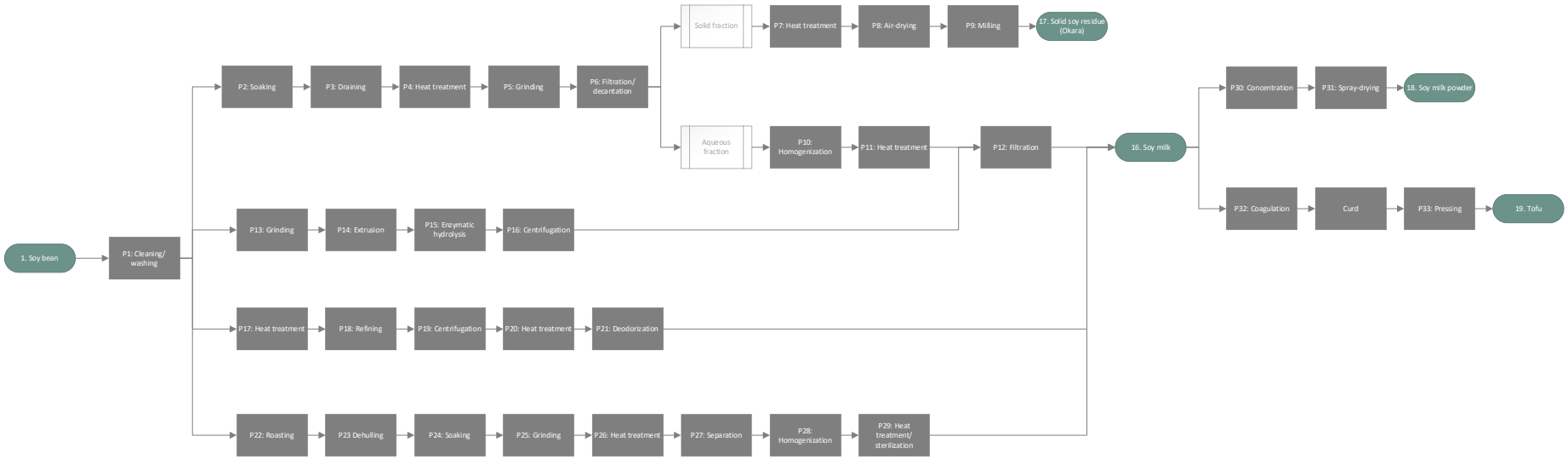

Figure 21: Process for obtaining NP22 (Soy milk powder) and alternative process for obtaining soy milk (AP6), solid soy residue (AP7) and tofu (AP8) from soybean. Adapted from the flowchart included in Annex A.1: Tab 2.3. Soybean milk and Tofu, where treatment parameters are indicated.

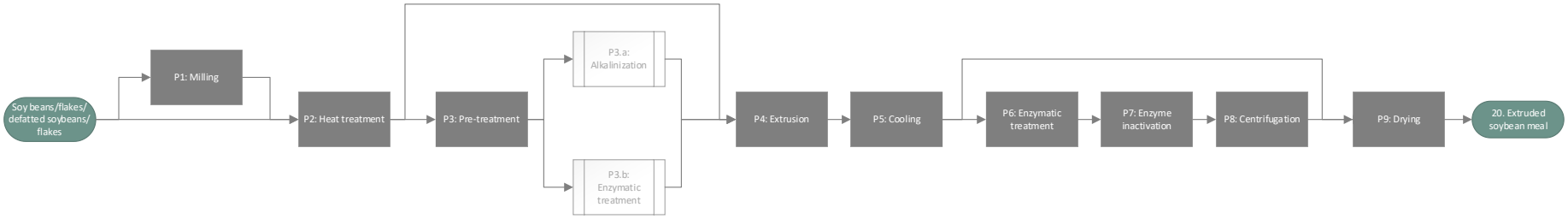

Figure 22: Processes for obtaining extruded soybean meal, including AP9. Adapted from the flowchart included in Annex A.1: Tab 2.4. Soybean extruded, where treatment parameters are indicated.

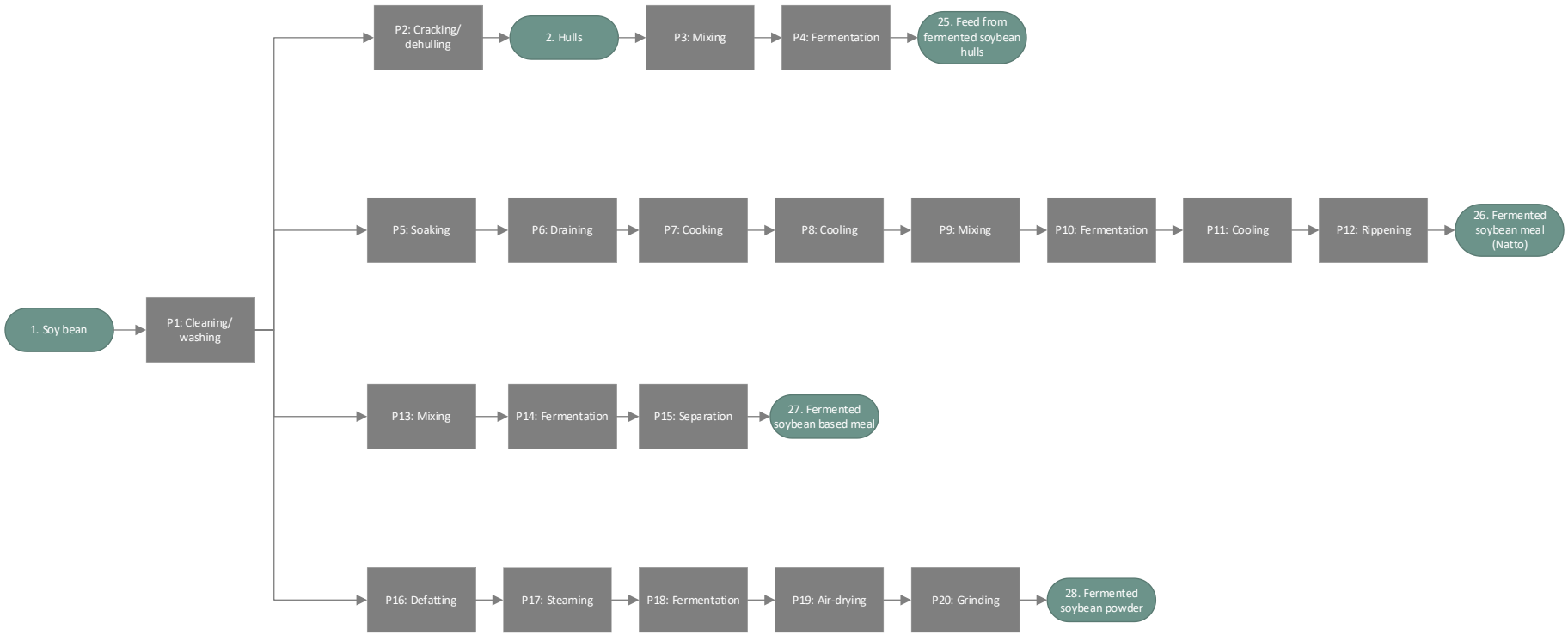

Figure 23: Process for obtaining NP24 (Feed from fermented soybean hulls), NP25 (Fermented soybean meal), NP26 (Fermented soybean-based meal) and NP 27 (Fermented soybean powder). This flowchart can be found in Annex A.1: Tab 2.7. Soybean fermented products where treatment parameters are indicated

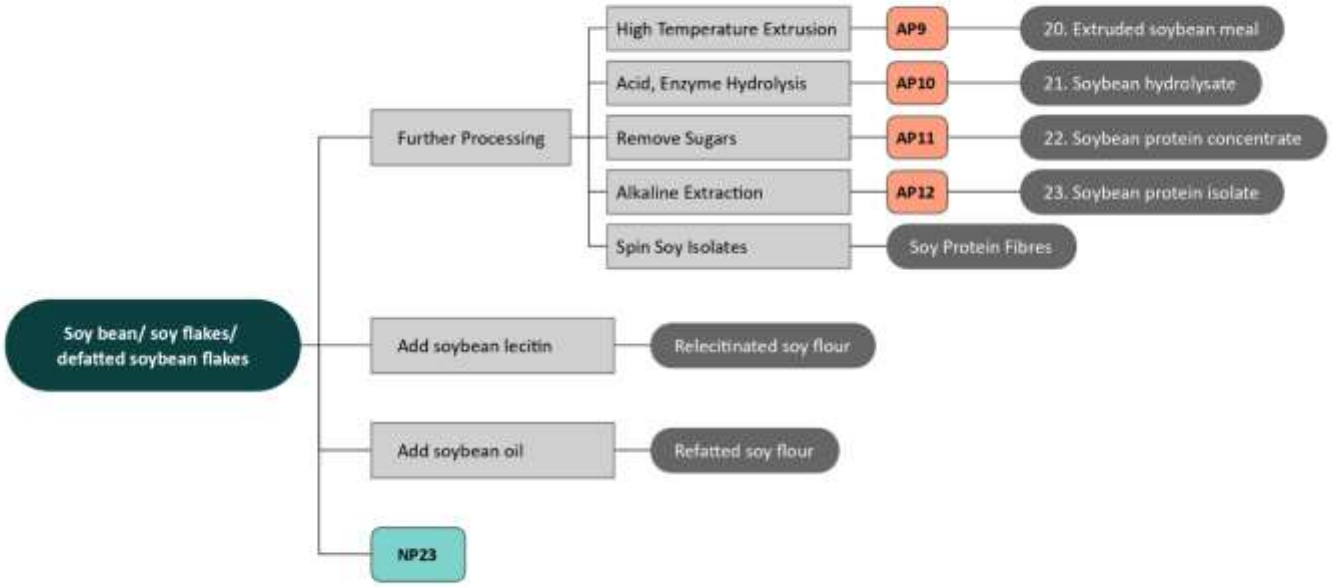

Figure 24: Defatted soybean flakes processing. Adapted from OECD (2012). New products (NP) are indicated in green and alternatives processes (AP) in orange.

2.1.1.2 Defatted soybean flakes processing

The OECD consensus document for soybean (OECD, 2012) includes soybean protein hydrolysates, concentrates and isolates as products that can be obtained from defatted soybean flakes but does not provide detail in the processing steps, which thanks to the bibliography search carried out have been described in more detail in Annex A.1 Tab 2.5. Soybean protein extracts, and in Figure 25, together with the multiple alternatives for producing them, labelled as AP10-AP12. At this point it should also be noted that, while the starting raw material reported in OECD (2012) is exclusively defatted soybean flakes, other intermediate products are frequently used as starting material in the production of soy protein isolates, concentrates and isolates, although the manufacturing lines are the same as those for defatted soybean flakes (Yokotsuka et al., 1977; Okajima et al., 2003; Alakov et al., 2004; de Regil et al., 2004; de Moura et al., 2011; Allocco et al., 2014; Al Loman et al., 2016; Rayaprolu et al., 2017; Zhang et al., 2018; Lee et al., 2019; Li et al., 2019; Sitanggang et al., 2021; Wanga et al., 2021; Singh et al., 2022; Zheng et al., 2023). In addition, another product not reported in the OECD consensus document and found in this scoping review is that patented by Hirotsuka et al. (1995). This product is a soybean protein isolate/fraction (NP23) obtained by applying an electrolytic reductive aqueous system (Figure 26).

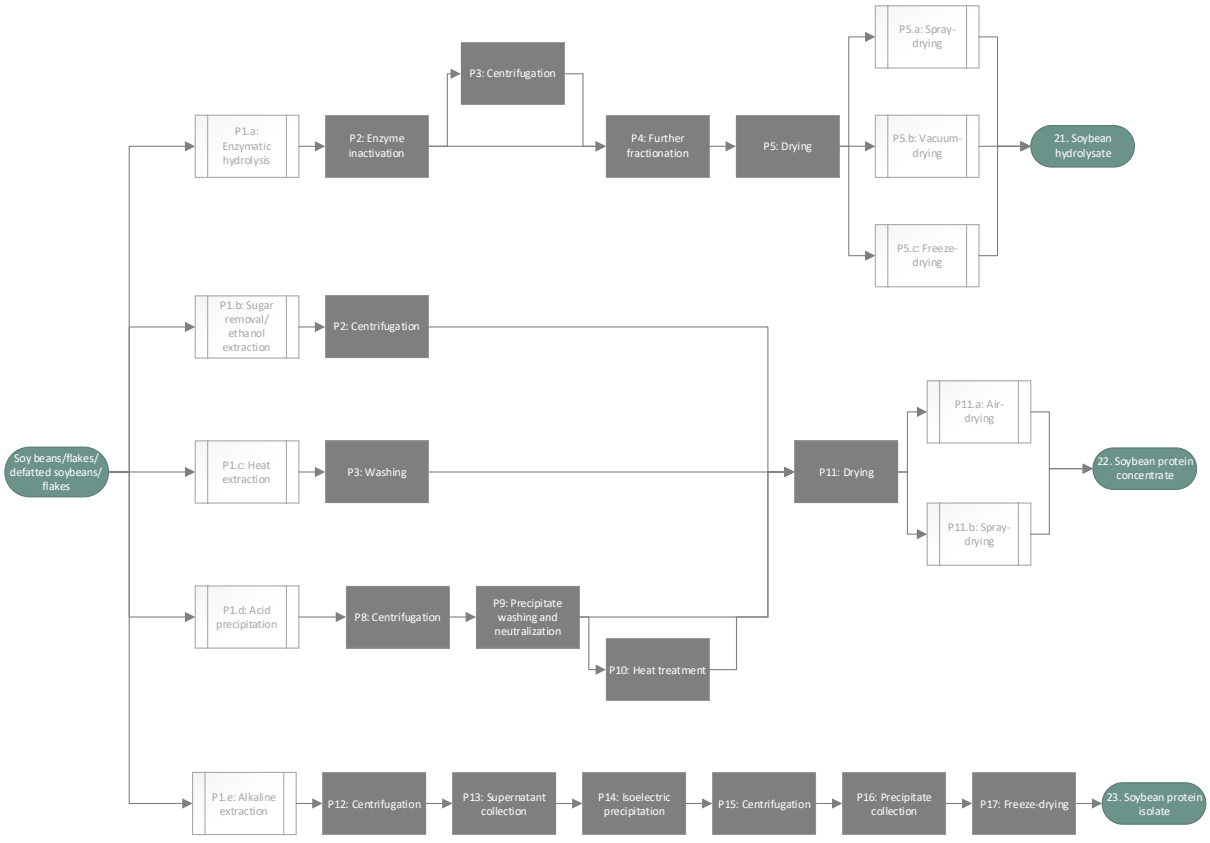

Figure 25: Processes for obtaining soybean hydrolysate, concentrate and isolate, identified as AP10, AP11 and AP12, respectively. This flowchart can be found in Annex A.1: Tab 2.5. Soybean protein extracts, where treatment parameters are indicated.

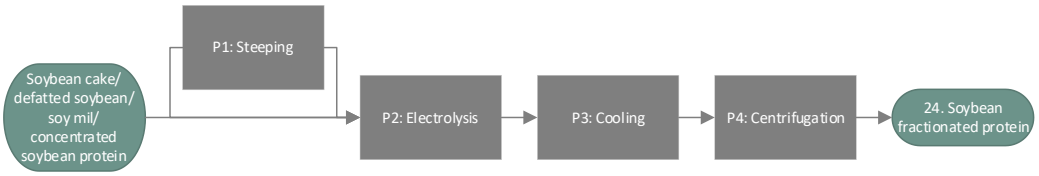

Figure 26: Process for obtaining NP23, Soybean fractionated protein. This flowchart can be found in Annex A.1: Tab 2.6. Soybean fractionated protein where treatment parameters are indicated.

2.2.3 Canola

The OECD consensus document (OECD, 2012) for low erucic acid rapeseed (canola) extensively describes the processing of canola oil, but not of the subsequent processing of canola meal/cake through, e.g. fermentation or protein extraction into concentrates, isolates or hydrolysates. Regarding canola oil production, it reports mechanical extraction/cold pressing. Based on the literature search, the basic flowchart for canola meal has been expanded (Figure 27), including alternative methods for the obtention of oil/cake (AP13) and the Process for obtaining various products from canola meal/cake (NP28-32). Regarding these alternative methods (AP13) they include oil extraction via solvents, such as hexane or petroleum (Figure 28) as described in Navarro et al. (2018) and Di Lena et al. (2021). However, it should be noted that according to Directive 2009/32/EC the use of the latter is not authorized for the production of foodstuffs or food ingredients either in the EU or imported into the EU. On the other hand, products obtained from canola meal can be classified in two groups.

Figure 29 depicts the processing steps for the obtention of canola protein extracts, i.e.: canola protein concentrates (NP28) and isolates (NP29) obtained from rapeseed meal (Tan et al., 2010; Rodrigues et al., 2017; Zhang et al., 2020; Li et al., 2020; Le et al., 2021; Zahari et al., 2021: Tian et al., 2022; Lena et al., 2023; Jiménez et al., 2023). According to these sources, different processes might be applied in order to obtain these concentrates/isolates, including alkaline extraction (either alone or combined with ultrasound), acid extraction or enzymatic hydrolysis procedures, which would/might be followed by standard water removal (filtration, centrifugation) steps. In the case of canola protein hydrolysates (NP32) the use of different enzymes is proposed, including, phytases, alcalases, endo-glucanases followed (or not) by an alkaline extraction + isoelectric precipitation procedure (Turck et al., 2020; Tian et al., 2022).

The second group (Figure 30) comprises defatted and fermented canola meal (NP30; Croat el at., 2016; Alhomodi et al., 2022; Tian et al., 2023), extruded canola meal (NP31; Zhanga et al., 2017) irradiated canola meal (NP32; Xiong et al., 2024).

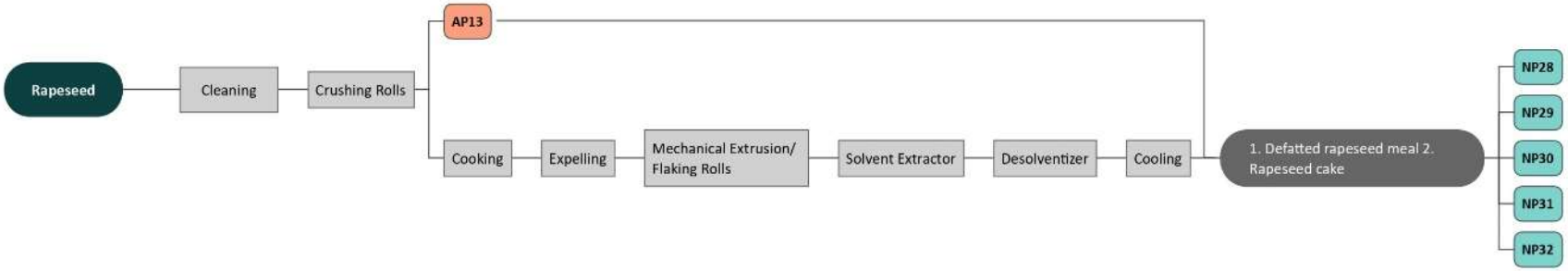

Figure 27: Canola/Rapeseed processing. Adapted from OECD (2012). New products (NP) are indicated in green and alternatives processes (AP) in orange.

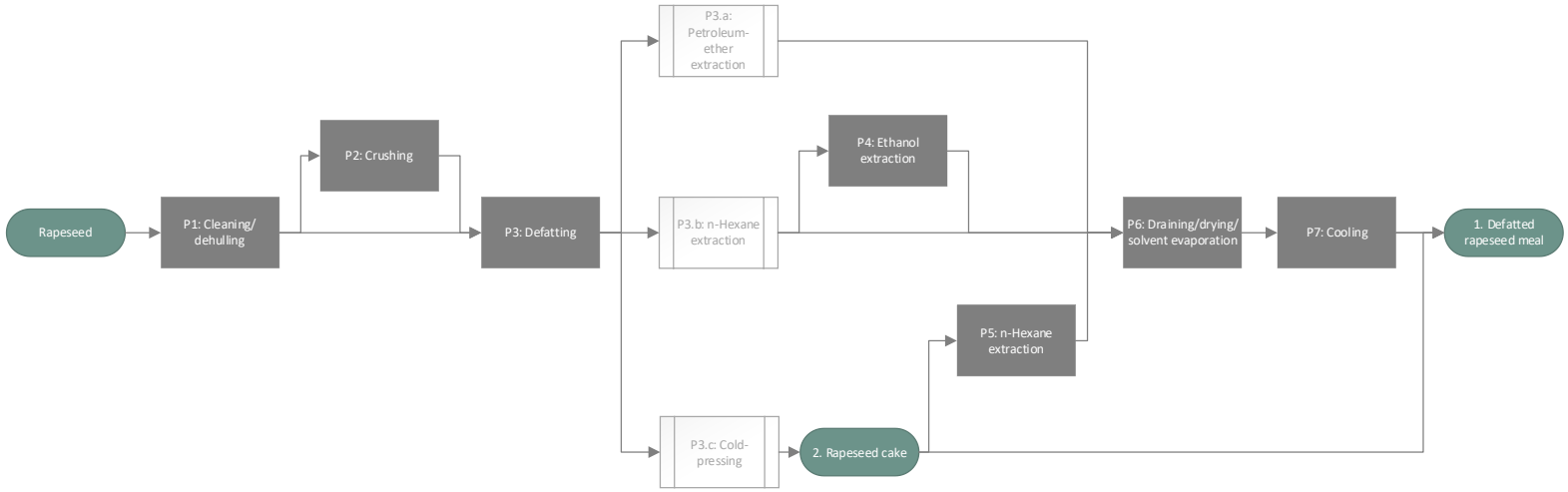

Figure 28: Alternative Process for obtaining canola meal/cake (AP3). Adapted from Annex A.1: Tab 3.1. Canola meal (inc. fermentation) where treatment parameters are indicated.

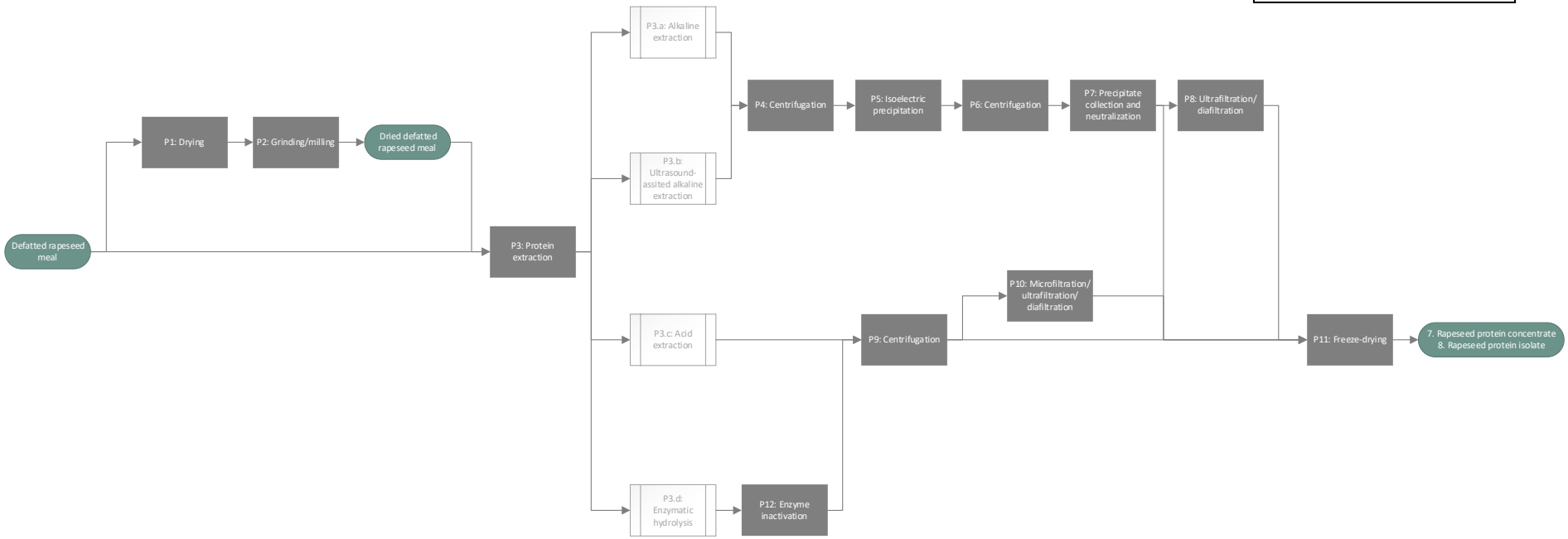

Figure 29: Process for obtaining NP28 (Rapeseed protein concentrate) and NP29 (Rapeseed protein isolate) from rapeseed. Taken from Annex A.1: Tab 3.2. Canola protein extracts, where treatment parameters are indicated.

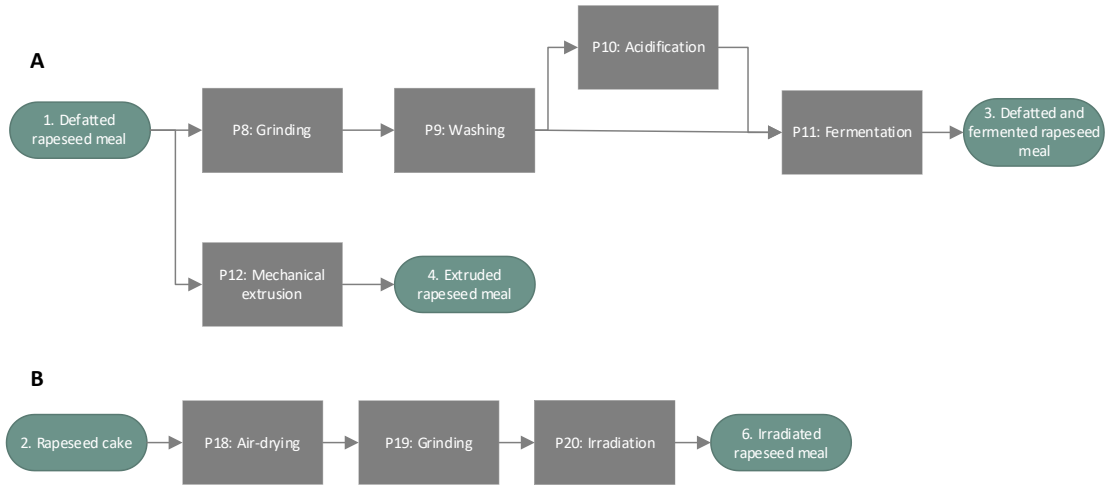

Figure 30: Process for obtaining NP30 (defatted and fermented canola meal) and NP31 (extruded canola meal (A) and NP 32 (irradiated canola meal, B) from rapeseed. Adapted from Annex A.1: Tab 3.1. Canola meal (inc. fermentation) where treatment parameters are indicated.

2.2.4 Cottonseed

Figure 31 shows the process of obtaining protein products derived from cottonseed (adapted from OECD, 2004). In this case, the bibliographic search for 'cottonseed' did not allow the identification of any new or alternative process to those indicated in the OECD consensus documents, and it was only possible to extract the process parameters from several of the different processes/steps and to identify in more detail two of the possible techniques/technologies to reduce the amount of gossypol in cottonseed meal: enzymatic hydrolysis (Zhang et al., 2024) and solid-state fermentation (Zhang et al., 2022) (Figure 32).

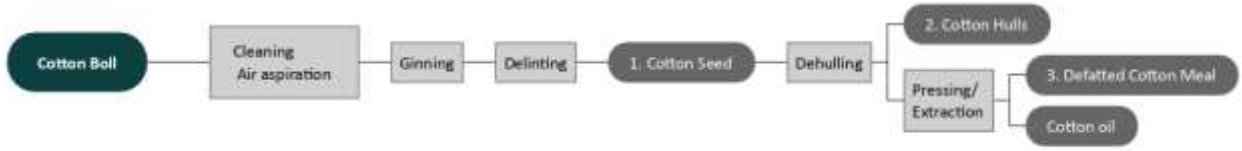

Figure 31: Cottonseed processing. Adapted from OECD (2004).

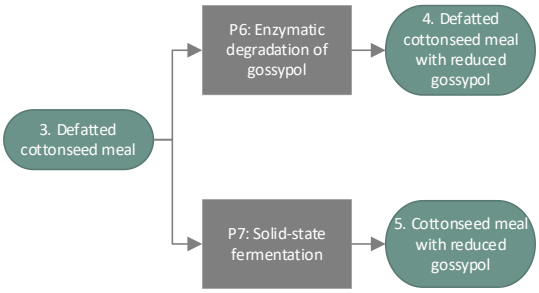

Figure 32: Process for obtaining reduced gossypol cottonseed meal. Adapted from Annex A.1: Tab 4. Cottonseed, where treatment parameters are indicated.

2.2.5 Sugar Beet

In the case of sugar beet, the scoping review led to the identification of a product (Sugar beet leaves protein concentrates, NP33) and the characterization of its production process, which was not mentioned in the OECD consensus document (OECD, 2002b) for sugar beet (in this case, from its leaves) (Figures 33 and 34)

The different processes indicated in Figure 34 (Annex A.1: Tab 5 Sugar Beet) essentially differ in the technique/methodology used for protein extraction, which in turn determines the downstream processing, the resulting protein yield and purity of the protein concentrates (28.9 - 86.4% w/w on a dry matter basis), which also depends on the concentration and purification steps.

2.2.6 Rice

Given the diversity of processes reported in the literature for rice, four flow diagrams were built (Annex A.1) for rice. They include processes for obtaining nine products not reported/included in the OECD consensus document (OECD, 2016) as indicated in figure 35.

The first four new products identified are obtained from rice bran. Two of them result from the fermentation of rice bran, whether defatted (NP34) or not (NP37) (Debi et al., 2021; Ugyen et al., 2023; Shih, 2003). The third one is produced by simply extruding the pre-conditioned rice brans (NP 35), Zaczuk et al., 2015) which is a common step in rice by-products processing, and the last one is a protein hydrolysate from rice bran (NP38), which according to Lei et al., (2015) and Shih, (2003) is produced by enzymatic hydrolysis followed by alkaline extraction and isoelectric precipitation.

NP38-NP40 correspond to protein hydrolysates/concentrates obtained from broken rice. In the case of NP38: "Rice starch protein hydrolysate" this product is obtained from starch residues through fermentation and subsequent fractionation (Babini et al., 2020). In the other two cases the starting material is broken/milled rice/flour and the protein products obtained are a concentrate obtained after alkaline extraction and acid precipitation -an standard method for concentrating proteins- (Shih et al., 2003) and a rice protein hydrolysate that is obtained after enzymatic hydrolysis but for which slightly different pathways (centrifugation vs filtering; one vs two centrifugations) have been described in the bibliography (Chang et al., 1986; Shih et al., 2004; Chang et al., 2010; Lei, 2015).

Finally, Figure 38 includes the two processes starting from whole (brown) rice (A) and rice straw (B), respectively, that were retrieved from the literature search (Nnam and Obiakor, 2003, Totakul et al., 2020, Li et al., 2023). The first process leads to the production of NP 41 (Fermented whole rice, A) using yeasts for fermentation. On the other hand, the second involves the ammonification and further solid-state fermentation of rice straws in order to obtain a protein rich product from rice straw (NP 42).

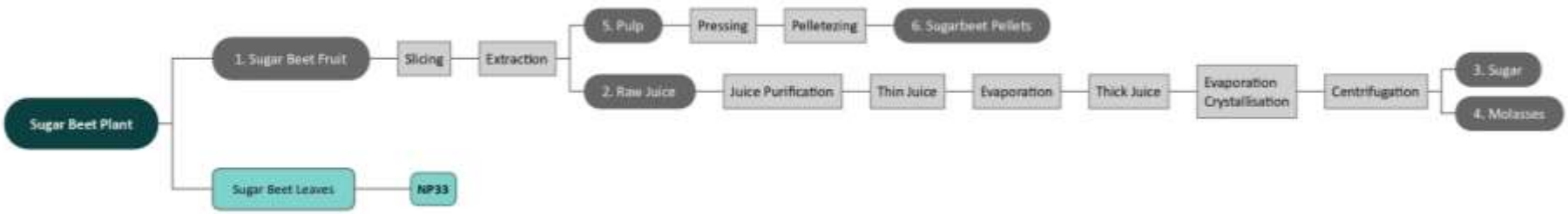

Figure 33: Sugar beet processing. Adapted from OECD (2002b). New products (NP) are indicated in green.

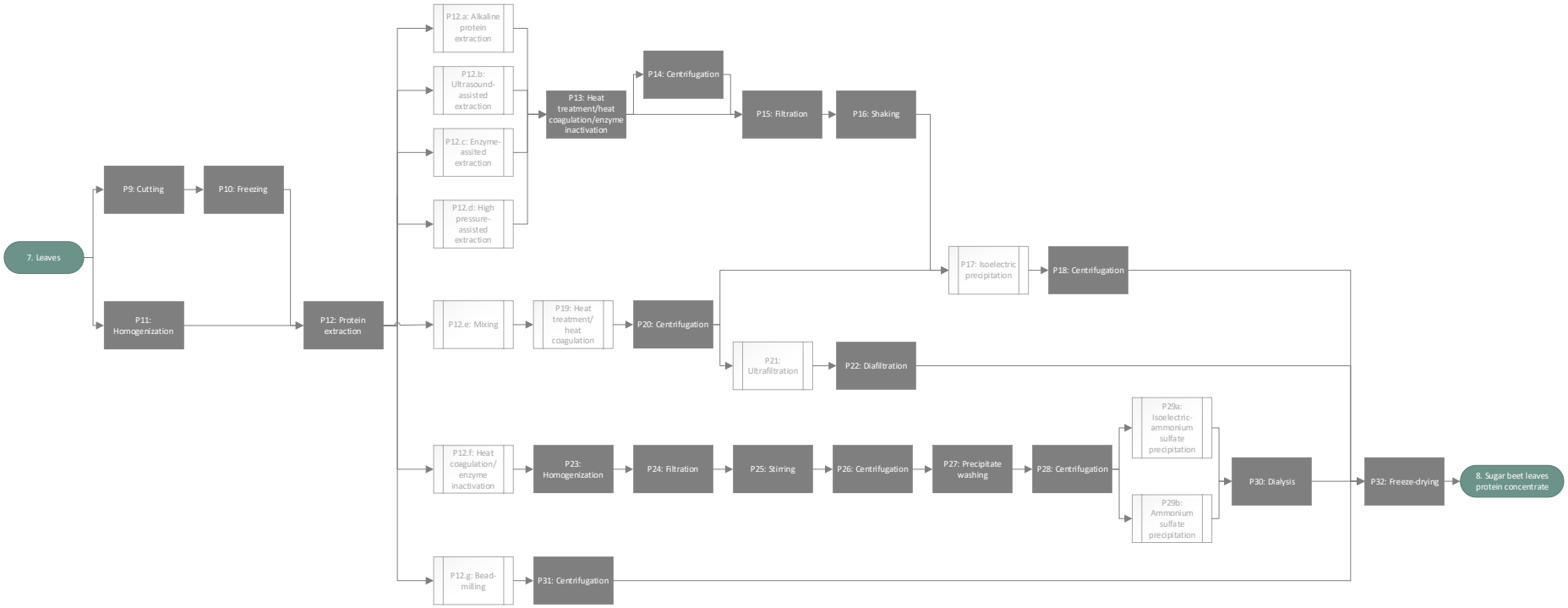

Figure 34: Process for obtaining NP33 (Sugar beet leaves protein concentrates) from sugar beet products. Adapted from Annex A.1: Tab 5. Sugar beet, where treatment parameters are indicated.

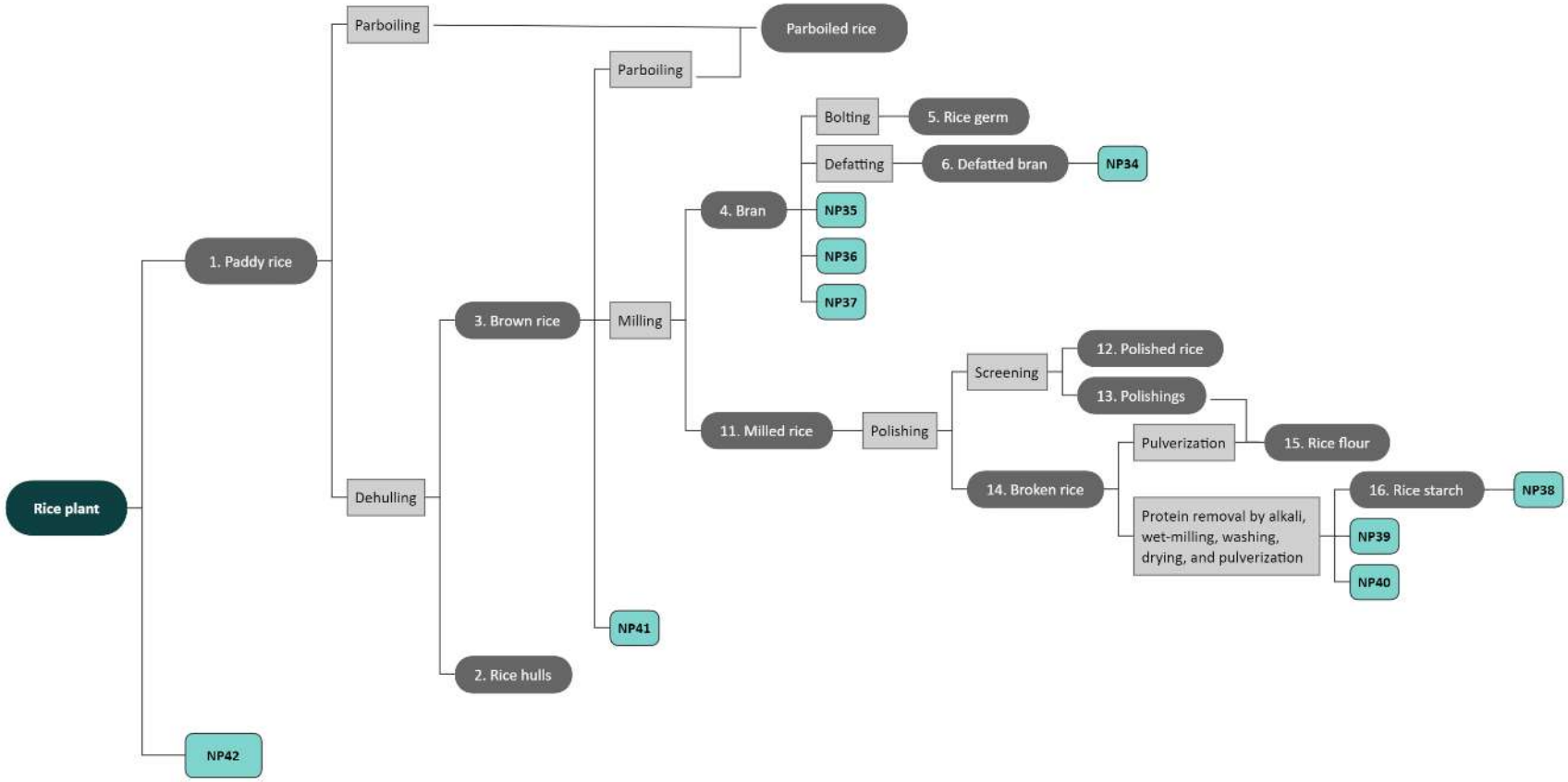

Figure 35: Rice processing. Adapted from OECD (2016). New products (NP) are indicated in green.

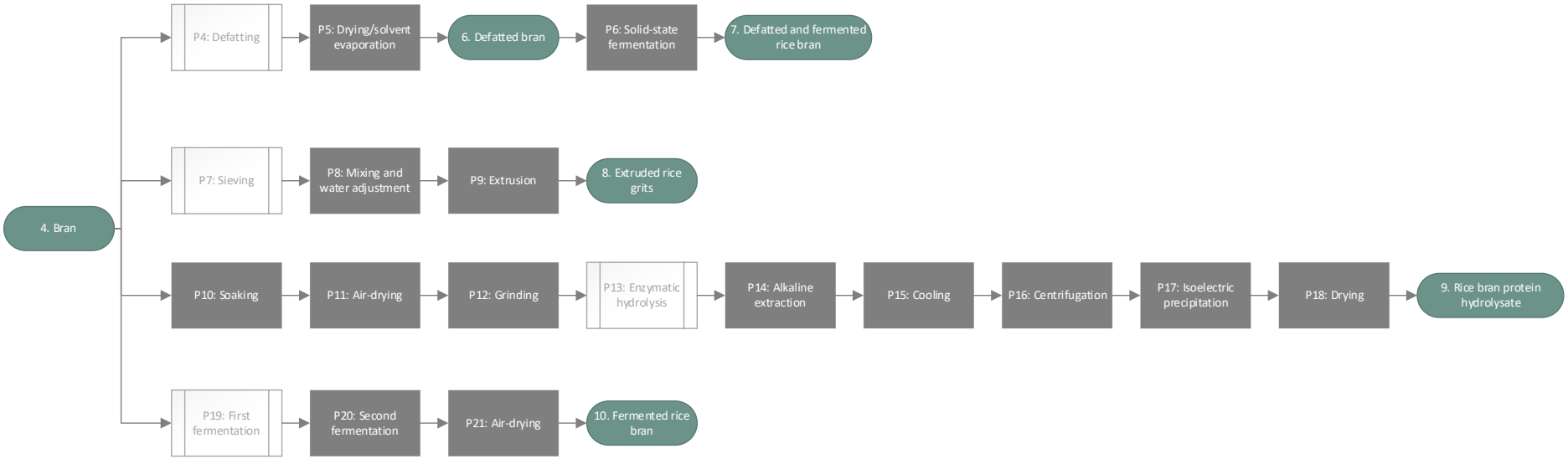

Figure 36: Process for obtaining NP34 (Defatted and fermented rice bran, A), NP35 (Extruded rice grits, B), NP36 (Rice bran protein hydrolysate, C) and NP37 (Fermented rice bran, D) from rice bran. Adapted from Annex A.1: Tab 7.1. Rice bran, where treatment parameters are indicated.

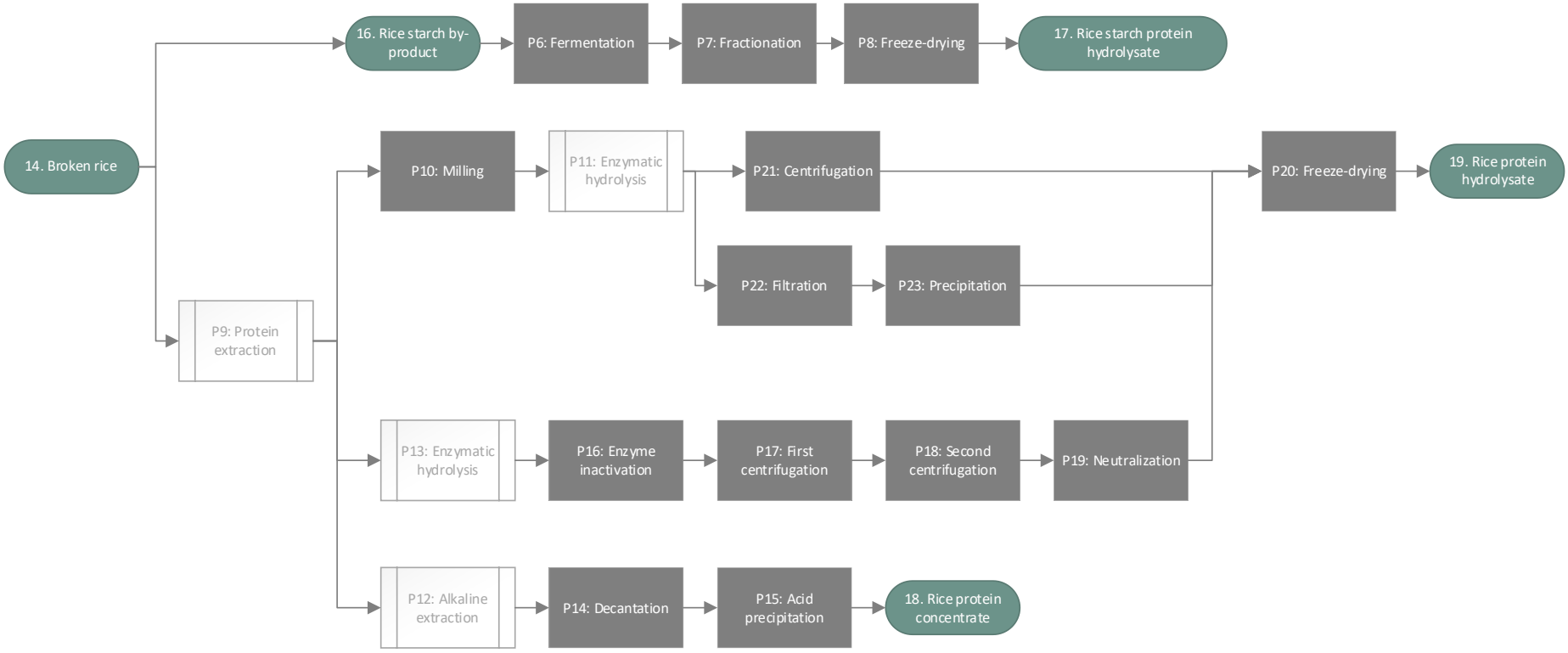

Figure 37: Process for obtaining NP38 (Rice starch protein hydrolysate), NP39 (Rice protein concentrate) and NP40 (Rice protein hydrolysate) from broken rice. Adapted from Annex A.1: Tab 7.2. Milled rice, where treatment parameters are indicated.

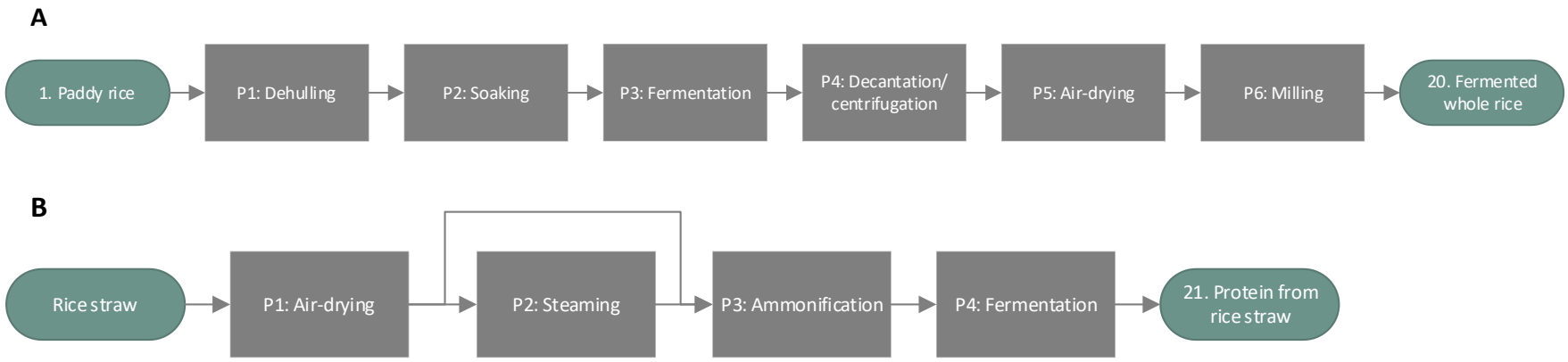

Figure 38: Process for obtaining NP41 (Fermented whole rice, A) and NP42 (Protein from rice straw, B) from rice. Taken from Annex A.1: Tab 7.3. Fermented whole rice and Annex A.1: Tab 7.4 Protein from rice straw, where treatment parameters are indicated.

2.2.7 Potato

Based on the literature review, the process diagram in the OECD consensus document for potato (OECD, 2020) has been expanded as per Figure 42.

Five products (NP43-NP47) not reported in the OECD consensus document for potato were found though the literature search. These products include simple two products obtained through simple/standard processes. This is the case of potato powder (NP43), which in this case was reported to be dried by freeze-drying (Shepherd et al., 2005; Broothaerts et al., 2007) and potato fruit juice (NP44) obtained by simple mechanical extraction (Akbari et al., 2019) (Figure 39 A and B, respectively).

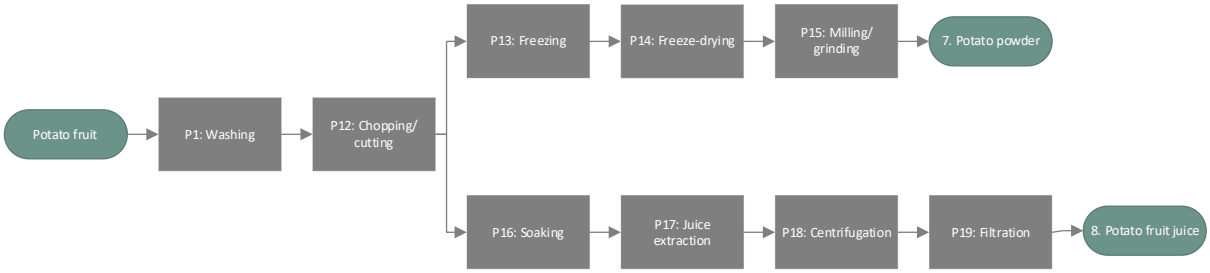

Figure 39: Process for obtaining NP43 (Potato powder, A) and NP44 (Potato fruit juice, B) from potato. Adapted from Annex A.1: Tab 8. Potato, where treatment parameters are indicated.

Figure 40 includes the processing steps for the obtention of fermented potato powder from potato starch (NP45) though solid-state fermentation and subsequent drying (Liu, 2014) (Figure 40A) and Figure 41 those for the obtention of potato concentrate (NP46) and hydrolysate (NP47).

Regarding the concentrate, the process described by Akbari et al. (2019) is based on ethanol protein precipitation, whereas the hydrolysate involved the addition of alcase or alcase + ficin (Gao et al., 2023), although it is possible that other processes/enzymes could be used for the same purposes (Akbari et al., 2019; Gao et al., 2023; Miedzianka et al., 2014).

Figure 40: Process for obtaining NP45 (Fermented potato powder) from potato. Adapted from Annex A.1: Tab 8. Potato, where treatment parameters are indicated.

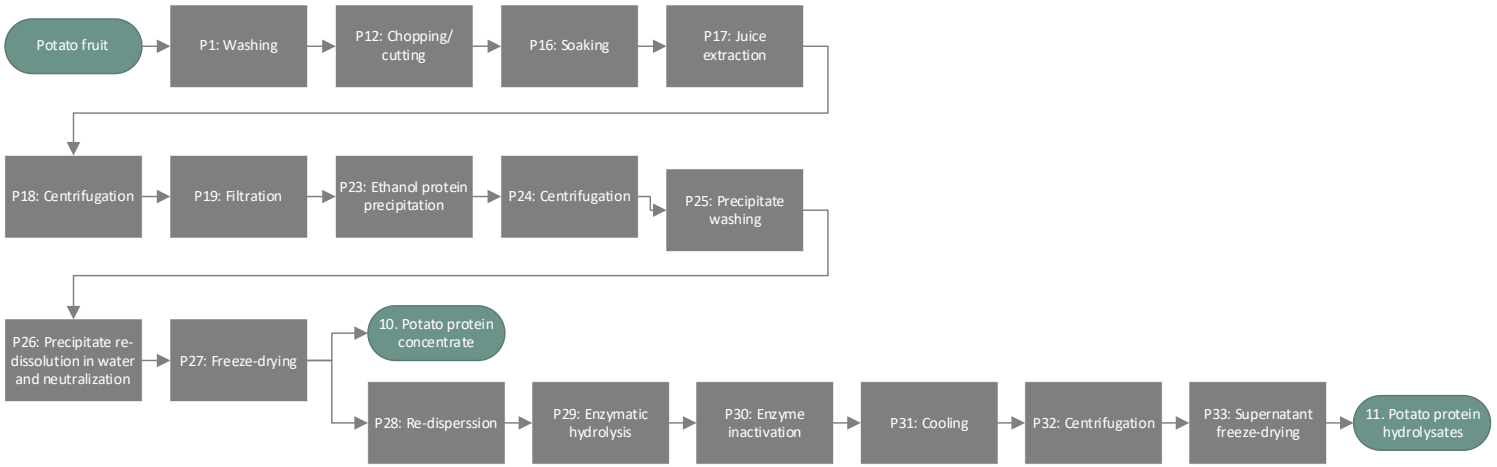

Figure 41: Process for obtaining NP46 (A, Potato protein concentrate) and NP47 (B, Potato protein hydrolysate) from potato. Adapted from Annex A.1: Tab 8. Potato, where treatment parameters are indicated.

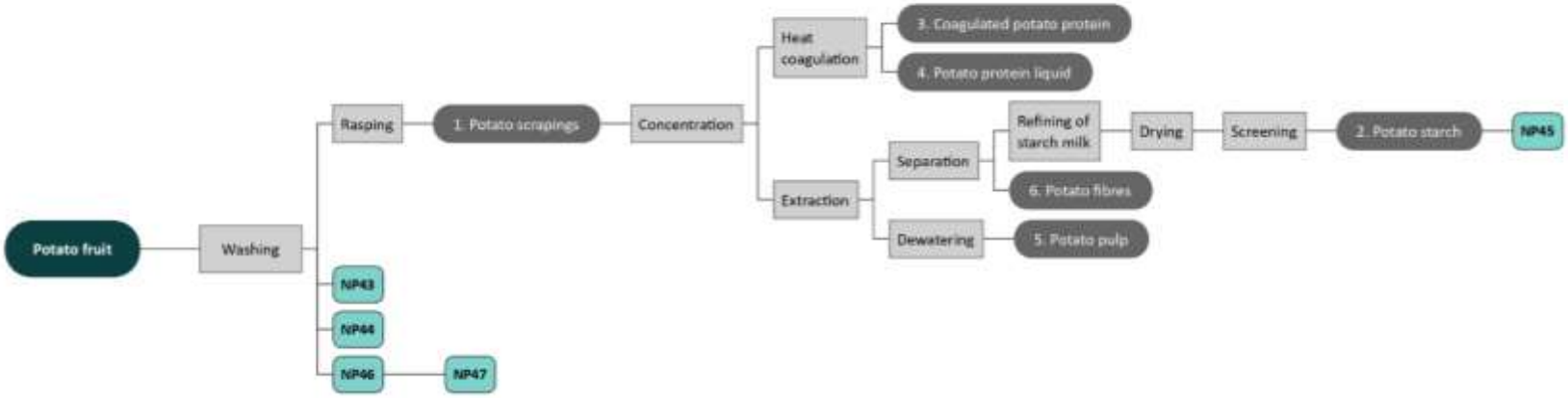

Figure 42: Potato processing. Adapted from OECD (2020). New products (NP) are indicated in green.

2.2 Objective 4

2.2.8 Search strategy

The search strategy was developed as described in section 2.2.4.

This systematic review was registered at Prospero, the International Prospective Register of Systematic Reviews of the National Institute for Health and Care Research of the United Kingdom (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=554500).

2.2.1.1 Results from the systematic literature search

Table 7 shows the results obtained from the searches in scientific databases.

Table 7: SR Search results

Query date	***Number of hits***
1 – PubMed_29/04/2024	*319*
2 – WoS_29/04/2024	*678*
3 – Scopus_29/04/2024	*796*
Total with duplicates	*1,793*
Total without duplicates	*1,076*

2.2.1.2 Screening of relevance

After the title and abstract screening 1,076 documents were found relevant, whereas 817 were excluded. Another 160 documents were excluded after full text screening. Therefore, a total of 154 documents were found as relevant (Table 8, and Figure 43), including 55 documents retrieved from the reference list of the documents identified as relevant from the literature search.

Table 8 displays the number of documents identified as relevant per type. Most of the documents fell into the "research articles" category. Next in descending order of the number of documents are the categories of reviews, other types of documents (including EFSA scientific opinions on the safety of NFs and GMOs and a commentary article) and book chapters. It should be noted that neither reviews nor book chapters were used as a primary source for data extraction; instead, the data were extracted from the articles listed in the references of the corresponding documents.

Regarding the relevant documents retrieved by category, most of them were classified within the "Novel Foods" category although a more precise classification would be as "new products dedicated to human consumption" since not all of them could be classified as Novel Foods according to Regulation (EU) 2015/2283. Another 31 documents were focused on UF and 25 were on GM crops. Similarly, most of the documents studied the effect of processing on NF/UF/GM crops digestibility and nutrition properties, whereas only one (1/154) was dedicated to the effect of food processing on the gut microbiota (Figure 43).

Table 8: Relevant documents after full text examination (total number and by type)

Type of document identification	*Number of documents*
Relevant	***154***
Research articles	*94*
Reviews	*28*
Book chapters	*8*
Other document types	*24*

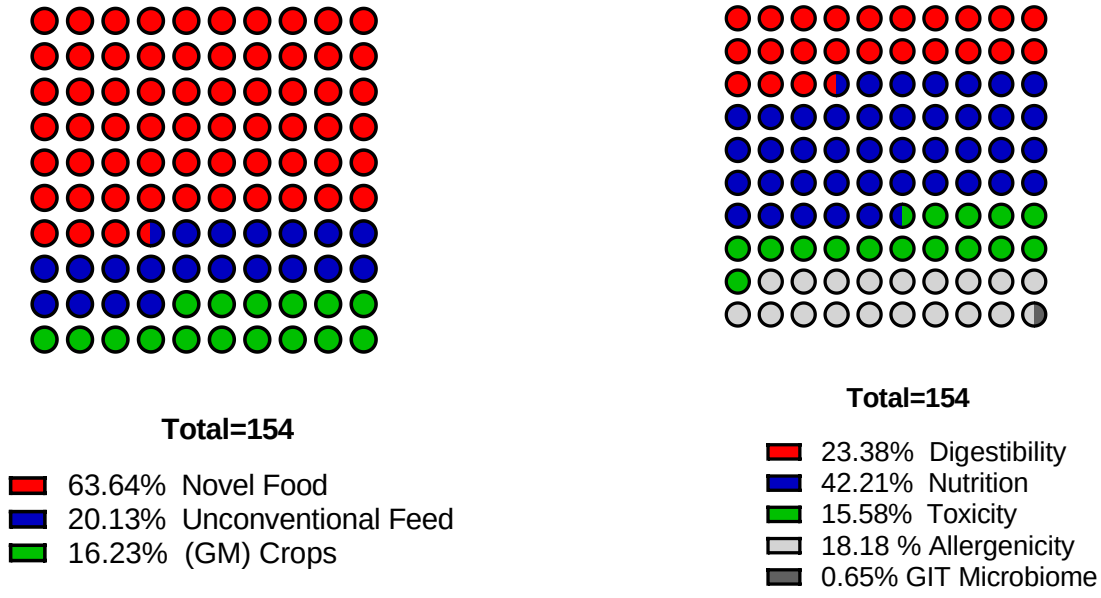

Figure 43: Distribution of relevant documents by search string category.

2.2.1.3 PRISMA statement

This section outlines the adherence to the PRISMA guidelines (Moher et al., 2009). The flowchart (Figure 44) exemplifies the methodical approach taken for the identification, screening, and inclusion of studies, reviews, books and other type of articles, highlighting the integrity and robustness of the research process adopted for this objective.

The major cause of exclusion at the title and abstract screening step was the Exclusion Criterium 3 (EC 3) "Studies/experiments not involving food/feed processing", which was the cause for article exclusion in almost 61% of the cases (Figure 44). It was followed by EC 2 ("Studies/experiments not associated to novel foods, unconventional feed or (GM) crops or to food/feed products that do not contain protein"), accounting for 25% of the exclusions, EC 6 ("Documents with general speculation, general description, or historical description of food/feed processing, novel protein sources or protein safety or any other document that cannot be categorized in inclusion criteria and cannot be excluded according to the above-

mentioned criteria"), for 8%, and EC 4 ("Articles that do not evaluate protein safety (protein toxicity, immunotoxicity, allergenicity, nutritional value, digestibility and impact on GIT microbiome") 6%.

Regarding the full text screening step, EC 3 was again the major cause for article exclusion (36%), followed by EC 4 (21%). It should be noted that at this level several documents (up to 18%) were excluded for different reasons by the two reviewers.

The full list of included and excluded documents is indicated in Figure 44 and provided in Appendixes D.1 and D.2.

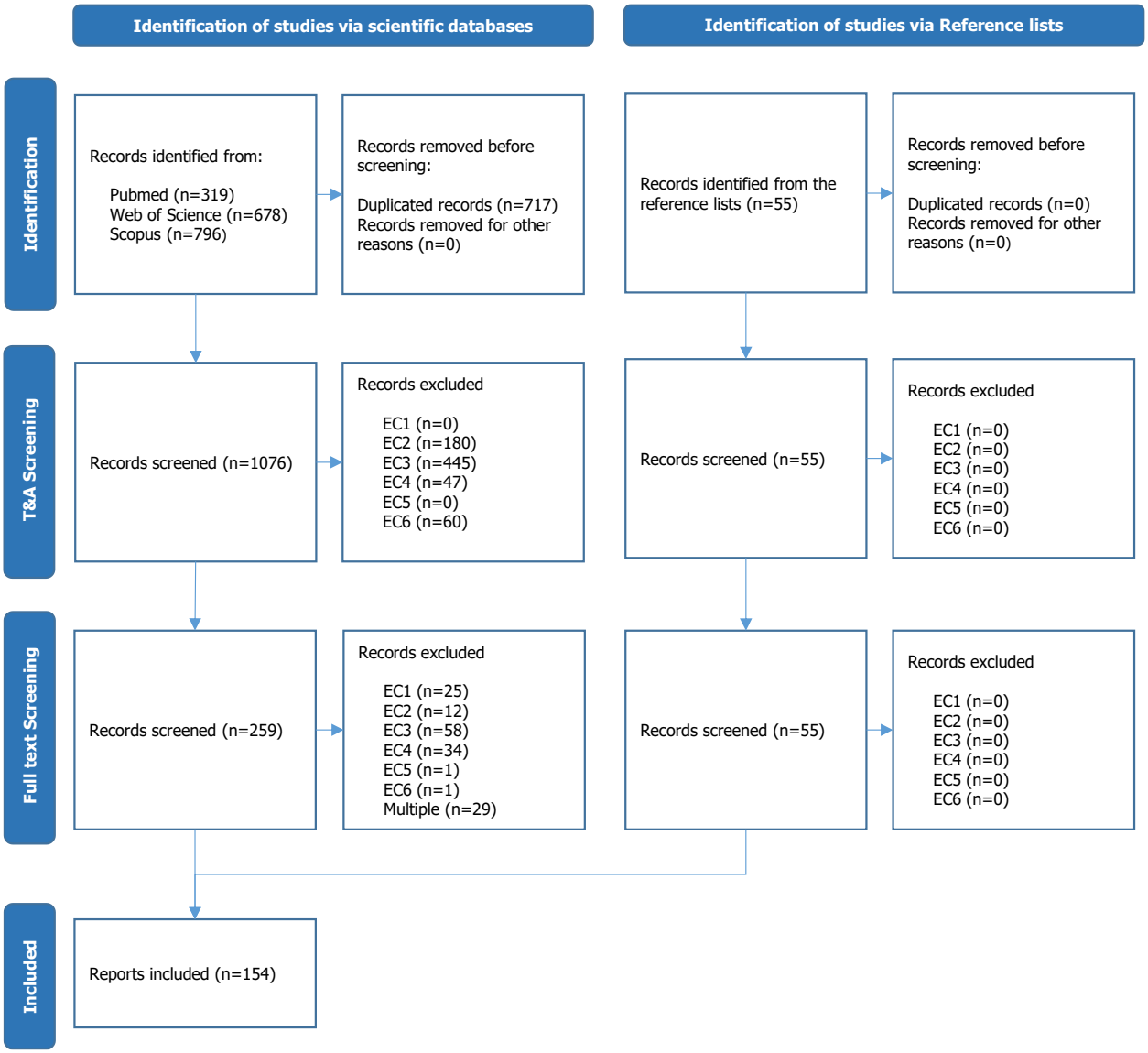

Figure 44: Systematic review PRISMA flowchart

2.2.1.4 Data extraction and evidence table construction

Data extraction was performed as described in section 2.2.4.6. The data were used to build the evidence tables included and discussed in section 3.3.2.

2.2.1.5 Risk of bias assessment

Appendix F includes the results of the risk of bias assessment for the 154 documents from which data extraction was conducted. As can be observed therein, there was a significant number, at least 32 documents, for which the risk of bias assessment was 'unclear' across the five evaluated aspects. These 32 documents include all the reviews and some documents categorized as 'others.' As mentioned previously, these documents were not excluded, but the original sources were used for data extraction, obtained from the reference list. Only three articles were found to have a high risk of bias. In two cases, it was due to reporting bias (dissemination influenced by the nature and direction of the results) and in one case due to performance bias (unequal treatment between study groups). Therefore, the results reported in these three articles were excluded from the evidence tables.

2.2.9 Impact of Processing on protein safety

This section discusses the impact of processing on the safety of food and feed products containing protein, including several foods or food ingredients authorised as NFs (e.g., derived from insects), different UF products and also those obtained from GM crops. Regarding the latter, it should be noted that the evidence gathered largely consisted of Scientific Opinions of the EFSA GMO Panel and only eight articles dealing with the impact of processing on the safety of GM crops were identified as relevant to be included in the SR.

2.2.1.6 Impact of processing of food/feed digestibility

The data extracted from the documents found through the systematic review and regarding the effect of the process on the digestibility of new protein sources are presented and discussed below. Table 9 summarizes the collected data/results along with the bibliographic sources.

3.3.2.1.1. Heat/Thermal Treatments

Thermal processing is one of the oldest and most widely used techniques for preserving food. It consists in the application of a combination of temperature and time to achieve a desired reduction in the number of microorganisms in a food product but is also the art, science and craft of using heat to make food edible and more palatable. The different heat treatment processes (i.e., of different intensity) that are used to make food suitable for human or animal consumption may exert an effect on protein content and availability. In general, it is known that heat treatments affect proteins by causing its denaturation, which might lead, among others, to increased water-holding capacity and/or lower functionality. The aim of this part of the discussion was to address the effect of heat treatments on proteins in NF, UF and crops. Extrusion will be discussed together with heat/thermal treatments although it should be reminded that extrusion involves not only the application of heat but also of pressure to force the material through a specifically designed opening—a die shape—with the desired cross-sectional profile.

Plant based products

Some of the protein-rich NFs/UFs are new species or varieties of legumes. Due to the scarcity of specific studies on the effect of processing on these new legumes, some articles on the thermal processing of traditional legumes will be discussed due to their similarity. Ohanenye et al. (2022) reviewed the effect of physical processing technologies, including thermal treatment, on the protein digestibility of legumes. Heat has been reported to improve protein digestibility, with variations depending on the type of legume, temperature and cooking time. Thermal treatment was found to denature native protein structure and modify the structure of protease inhibitors along with legume storage proteins, while also causing protein aggregation, thus making the proteins more susceptible to digestive proteases during unfolding of the protein. Thermal treatment enhances structural changes, which are further improved by wet heating, as gelatinization and cross-linkages occur between proteins and starch. Articles specifically dealing with the effect of thermal treatment on protein digestibility of legumes are summarized and discussed below.

Heat treatment (80°C up to 9 min) in a water bath enhanced the degree of hydrolysis (DH) of legume *Phaseolus vulgaris L.* protein from 62.34 to 73.64%. It was confirmed that heat treatment changed the structural properties of these proteins and improved its foamability, emulsification, and *in vitro* digestibility (Li et al., 2023). Regarding cooking under pressure (autoclaving at 121°C for 10 min) protein digestibility was improved by 96−105% when compared to raw legumes, for black grams, chickpea, lentil, red and white kidney bean (Avezum et al., 2023). In case of *Vigna unguiculata* (L.) Walp subsp. *unguiculata*, an underutilized legume, the cooking improved the *in vitro* protein digestibility (IVPD) from 71.3 (raw) to 78.7% after 30 min at 100°C - and from 81.6 (raw) to 89.7% after autoclaving (at 121°C for 20 min), which means 7% and 8% increments, respectively (Kalpanadevi and Mohan, 2013; Boye et al., 2012). Similar results were reported years ago for chickpea (*Cicer arietinum*) in which *in-vitro* protein digestibility (IVPD) improved from 71.8 (raw) to 83.5% after autoclaving (120°C for 50 min) (Clemente et al., 1998). Faba bean (*Vicia faba L.*) IVPD improved from 64.6 (raw) to 71.2% after 45 min cooking, or to 73.7% after autoclaving (121°C for 30 min) (Khalil et al., 1995). Velvet bean (*Mucuna pruriens*), a tropical legume native to Africa and tropical Asia, was reported to improve in true ileal digestibility from 48.5 (raw) to 81.6% after autoclaving (120°C for 30 min) (Siddhuraju et al., 1996). In summary, the impact of the thermal treatments on legume protein digestibility seems to be influenced by the legume type and treatment but in general the IVPD tends to increase after processing.

In case of other plant-based NFs such as herbaceous plant finger millet (*Eleusine coracana*), IVPD improved from 79 (raw) to 84.7–86.3% after cooking, although temperature and time were not reported (Annor et al.,2017). True digestibility of sun-dried and unsliced seeds of flowering plant amaranth (*Amaranthus hybridus*) increased from 84.4 to 92.0% after cooking (100°C - 10 min) (Suffo Kamela et al., 2016). On the contrary, heat-treated quinoa albumin (80, 100, and 121°C) showed lower degrees of hydrolysis than the control. The content of AAs in the in vitro digestion product of unheated quinoa albumin was higher than that in heat-treated samples. Among them, the AA content after heat treatment at 121°C was the lowest at the end of digestion, 45.1% lower than the control. It was speculated that high-temperature thermal treatment may cause severe destruction to AAs and induce irreversible

decomposition, leading to a loss of contents and finally a reduced hydrolysis ratio (Yang et al., 2022).

The effect of thermal processing on the proteins of the novel cereal Glabrous canary seed was studied by Rajamohamed et al. (2013). Roasting was performed by dry-heat in an oven at 176°C for 12 min whereas boiling was done in water at 98°C for 12 min. Roasting markedly altered the protein electrophoretic profile with the appearance of large molecular weight aggregates and both thermal processes generally improved Canary seed protein digestibility. On the other hand, Llopart et al. (2014) studied the effects of extrusion conditions on physical and nutritional properties of extruded whole grain red sorghum (*Sorghum spp*). The treatment (182°C, 14% moisture) improved IVPD from 53.2 (raw) to 70.0% (extruded). Similarly, extrusion (120°C, 30% moisture) significantly increased the IVPD of distillers dried grains with solubles (DDGS) ($p < 0.05$). According to existing studies, the shift in protein secondary structure from ⊎-sheet to ⊎-turn usually leads to an increase in IVPD. In the present study, however, the opposite shift was observed, but there was still an increase in IVPD, which was attributed to the disappearance of the lignocellulosic fraction in DDGS, providing a greater surface area for digestive enzymes (Liu et al., 2024).

Insects

In the study of Megido et al., (2018) mealworms (*T. molitor*) were subjected to different thermal treatments. For vacuum cooking, mealworms were vacuum-packed in a plastic bag and immersed in a water bath at 74.0°C for 60 min. For frying, mealworms were pan-fried for 1 min in 15.0 mL of olive oil (preheated for 1 min) and dried on a paper towel. For boiling, mealworms were immersed in a water bath at 100.0 ± 0.5°C for 1 min. For oven cooking, mealworms were cooked in a 70.0°C preheated oven for 15 or 30 min. The higher lipid levels after frying likely induced a proportional dilution of the protein content. The negligible differences in protein content between treatments could be due to the loss of the small fraction of soluble proteins in the insect exudate. Although insect protein content seemed to be similar between treatments (except for frying), insect protein digestibility was altered by cooking: thus, proteins from raw and fried mealworms were less digestible than proteins from all other treatments.

Among all the references studied, the majority were *in vitro* studies. Just Siddhuraju et al. (1996) studied digestibility of Velvet bean (*Mucuna pruriens*) using rats as animal models and Megido et al. (2018), which investigated the digestibility of proteins from *T. molitor*, involved human participants who consumed mealworms prepared using different cooking methods.

3.3.2.1.2. Drying/a_w reduction

Drying or "dehydrating" is a method of food preservation that removes enough moisture from the food so bacteria, yeast and moulds cannot grow. But apart from enhancing shelf life, drying serves multiple purposes beyond microbial preservation such as concentrating nutrients and flavours, reducing weight and volume, improving texture and taste, facilitating easy rehydration or preserving nutrients. However, it is known that dehydration could affect the digestibility of proteins especially if high temperatures are involved or if drying causes structural changes like cross-linking or oxidation, though the extent varies depending on the dehydration method and conditions applied.

Although *Chlorella vulgaris* is not a NF, it can be classified as an UF and there are other microalgae with similar cell morphology and properties that are considered NFs. Accordingly, the study of the effect of dehydration on *Chlorella* digestibility could be relevant to anticipate the effects of drying on other microalgae proteins. Agitated thin film drying (ATFD) of this microalga at temperatures up to 98°C with a residence time of 10 min was studied to keep the digestibility of microalgae protein. N-solubility was significantly higher after ATFD (80.3%) compared to the other drying methods such as solar drying, spray drying or freeze-drying (64.7–69.8%) (Van De Walle et al., 2024).

Different drying methods: freeze-drying (FD), vacuum oven (VOD) and oven drying (OD) were studied on Hempseed (*Cannabis sativa L.*) meal, a product that is relevant for several NFs. FD meal had the highest IVPD (88.2%), followed by OD meal (84.5%) and VOD meal (84.1%). FD product was more digestible and had a conformation more accessible to enzymes when compared to VOD and OD. The lower IVPD in VOD and OD meals was associated with the reactions of carbohydrates with free amino groups under mild heating (Duijsens et al., 2023).

Apart from those addressing NFs, a couple of articles dealing with the effect of drying on UF digestibility were found via the SR. Kisworo et al. (2017) studied different drying methods applied to a solid herbal waste as an alternative feed for ruminants. Gas production, methane, NH_3, microbial protein, *in vitro* degradability of dry matter (IVDMD) and organic matter (IVDOM) in silage and dried samples were lower ($p < 0.05$) compared to sun dry and freeze dry. These results were apparently due to the high content of secondary metabolites especially tannin. Regarding insect processing, Rawski et al. (2020) did not find changes on digestibility after drying (130°C for 1 h or 80°C for 23 h) of black soldier fly larvae, but feed acceptance was also improved in fish.

3.3.2.1.3. Fermentation

Fermentation is the conversion of carbohydrates to alcohol or organic acids using microorganisms—yeasts or bacteria—under anaerobic conditions. It is generally acknowledged that fermentation improves the digestibility of proteins in food through various biochemical processes: protein breakdown (proteolysis), formation of bioactive peptides, reduction of anti-nutritional factors and reduction of protein cross-linking. Proteins could be cross-linked with other food components like carbohydrates or fats, which reduces their digestibility. Fermentation can help break these bonds, making the proteins more accessible to digestive enzymes (Siddiqui et al., 2023).

Regarding NFs, most of the studies retrieved explore the fermentation of flours, but also one study on plant by-products and another dealing with macroalgae were found. The combination of cooking and fermentation of the flour of Kariya (*Hildergardia barteri*) resulted in an improvement of IVPD from 63 to 85% (Fawale et al., 2017), while the fermentation of cowpea (*Vigna unguiculata L.*) by *S. cerevisiae* (25°C for 24 h) improved the IVPD from 81.6 to 84.3% (Boye et al., 2012). Similarly, lentil flour fermented by *Pleurotus ostreatus* (28°C for 14 days) presented a higher fraction of digested protein (17%) in comparison to raw product when simulating *in vitro* digestion (Asensio-Grau et al., 2020). Finger millet (*Eleusine coracana*) fermentation by lactic-acid bacteria combined with yeast also improved IVPD from 71.2% (raw) to 79–83.7 (fermented) (Annor et al., 2017). Regarding by-products, date palm wastes,

chopped, soaked and inoculated with mushroom (*Pleurotus florida*) exhibited higher organic matter digestibility in comparison to untreated date palm wastes (El-Waziry et al., 2016). Finally, the macroalgae *Palmaria palmata* underwent a digestibility improvement after physical treatment followed by fermentation. The digestibility improvement was related to the elimination of soluble molecules such as xylan and mineral salts and to the degradation of insoluble fibers (Marrion et al., 2003).

Regarding UFs, the digestibility of *S. cerevisiae* microbial meal for fish nutrition was studied by Langeland et al. (2016). Cell wall disruption was achieved by autolysis and the absence of intact cell walls had a positive effect on digestibility of *S. cerevisiae* for the fishes. Grape pomace waste was also studied as alternative feed for ruminants after fermentation by *Pleurotus cornucopiae* and *Ganoderma resinaceum* (25°C for up to 8 weeks). Fermentation of grape pomace with both white-rot fungi reduced lignin and condensed tannin content and increased crude protein, improved rumen fermentation and dry matter and fiber digestibility. This enhanced concentration of volatile fatty acids and ammonia-nitrogen in the rumen contributes to better microbial crude protein synthesis and metabolizable energy by ruminants (Abid et al., 2023). However, mixing pig feed with water at 1:3 ratio and allowing for fermentation tended to reduce protein ileal digestibility compared with dry feed. Fermentation reduced protein ileal digestibility, but not energy digestibility (Pedersen et al., 2010).

3.3.2.1.4. Ensiling

Ensiling is a method of preserving forage crops by anaerobic fermentation. This process involves the fermentation of plant sugars by lactic acid bacteria, which produce lactic acid and lower the pH of the material. The acidic environment inhibits the growth of spoilage organisms, effectively preserving the forage for long-term storage. Ensiling is widely used in livestock feed processing because it helps maintain the nutritional quality of the forage, reduces losses due to spoilage, and allows for the storage of high-moisture crops that might otherwise be difficult to preserve.

Ensiling is particularly important for ruminants that rely heavily on forage as a significant part of their diet. During ensiling, proteolytic enzymes break down proteins into smaller peptides and amino acids. This process can increase the ruminal degradability of protein, making it more accessible to rumen microbes. The breakdown of proteins can lead to the formation of ammonia. While some ammonia is necessary for microbial protein synthesis in the rumen, excessive amounts can reduce the overall protein quality (Kung and Shaver 2001).

Focusing on UF, the effect of ensiling on the nutritional composition and fermentation characteristics of brown seaweeds as a ruminant feed ingredient (UF) was studied by Campbell et al. (2020). The seaweeds were spread thinly onto a large plastic sheet and wilted for 24 hours. The results showed losses of the Crude Protein (CP, −32%) but a limited effect on the in vitro true dry matter digestibility.

3.3.2.1.5. Enzymatic hydrolysis

Enzymatic hydrolysis is a process in which enzymes facilitate the cleavage of bonds in molecules with the addition of the elements of water (i.e. hydrolysis). It is supposed to play an important role in the digestion of food. Specific enzymes such as proteases, lipases, and amylases catalyse the breakdown of proteins, fats, and carbohydrates, respectively. For

example, proteases break down proteins into peptides and amino acids. One of the significant advantages of enzymatic hydrolysis is its specificity and efficiency. Unlike chemical hydrolysis, which can be harsh and non-selective, enzymatic hydrolysis occurs under mild conditions, preserving the nutritional and sensory qualities of the food. This makes it an ideal method for processing sensitive ingredients like dairy products, where enzymes like lactase are used to produce lactose-free milk.

Regarding NFs, Fleurence et al (2001) studied the use of an enzymatic composition comprising the specific association of two enzymes selected to improve the digestibility of the soluble protein fraction of the macroalga P. palmata. On the subject of UFs, fermenting DDGS prior to feeding with non-starch polysaccharide-degrading enzymes increased digestibility in pigs. The enzymes were a mixture of xylanase and β-glucanase or cellulose. Fermentation led to significantly increased apparent ileal digestibility of non-starch polysaccharides, and apparent total tract digestibility of dry matter and crude protein CP (Jakobsen et al., 2015). In the case of aquaculture feed, the diets that included the hydrolysed ingredients (brewers' spent yeast and grain protein) showed a growing trend in the digestibility comparing to non-hydrolysed ones. Optimum hydrolysis conditions for both brewers' spent grain and yeast were defined by comparing different enzymes combination and hydrolysis conditions at laboratory scale. Afterwards, selected enzymes and conditions were validated at industrial scale (San Martin et al., 2020). Regarding crops, the enzymatic hydrolysis with phytases increased in vivo protein digestibility in soybean meal ($p < 0.05$) evaluated using common carp *Cyprinus carpio* (Watanabe et al., 2016).

On the other hand, Casaretto et al., 2022 studied the in vitro characterization of *Acrocomia totai* defatted kernel meal as a novel raw material in aquaculture (Nile tilapia) feed and the effect of exogenous phytase inclusion over nitrogen and phosphorus bioavailability. The incubation was performed for 30 min at 37°C. Nitrogen and phosphorus bioaccessibility were studied. The greatest solubilisation was registered for the phytase treatment towards the end of the gastric stage and this condition outperformed all the treatments without enzyme. On a crude protein content basis, the relative performance of the treatments with *A. totai* improved.

3.3.2.1.6. Germination

Germination is a natural process where seeds begin to sprout and grow into new plants. This process involves soaking the seeds in water, which activates enzymes that break down complex molecules like starches and proteins into simpler, more digestible forms. Germination improves the nutritional quality of cereals and pulses by increasing nutrient digestibility, reducing the levels or activities of anti-nutritional compounds, boosting the contents of free amino acids, and available carbohydrates, and improving functionality. Germination is widely used in the production of various food products, including malt for brewing, sprouted grains for baking, and even in the preparation of traditional foods like tempeh and miso. Germinated pigeon pea, kidney bean, and fava bean at 25°C during 72 h increased 23%, 110%, and 110% in IVPD (Avezum et al., 2023). Germination of finger millet (*Eleusine coracana*) (30 ºC, 48 h) improved IVPD from 79% (raw) to 92% (germinated) (Annor et al., 2017). The combination process (germination 96 h + autoclaving) applied to *V. unguiculata (L.) Walp* subsp. *unguiculata* improved the IVPD from 71.3 (raw) to 84.9% (Kalpanadevi et al., 2013).

The germination of Faba bean (*Vicia faba L.*) (25°C, 72 h) improved IVPD (%) from 70.8 (raw) to 78.1% (germinated) (Alonso et al., 2000).

3.3.2.1.7. Alkali treatment

Alkali treatment is a common method in food processing used to modify the properties of various food components, particularly starches and proteins. This treatment involves the use of alkaline substances, such as sodium hydroxide or potassium carbonate, to achieve desired changes in texture, flavour, and nutritional value. One notable application is the production of *maize tortillas* and yellow alkaline noodles, where alkali treatment enhances the texture and colour of the final product (Xu et al., 2024). Additionally, alkali treatment may improve the digestibility of proteins and inactivate certain anti-nutritional factors, making the food more nutritious.

Arekemase et al. (2022) studied the alkali treatment of the Egyptian riverhemp (*Sesbania Sesban*) seeds. It was revealed that crude protein content was significantly higher for samples boiled in slake lime and in lye compared to unprocessed sample. IVPD was significantly higher in the treated seeds, following the order: boiling in lye>boiling in slaked lime> soaking in slaked lime>soaking in lye.

3.3.2.1.8. Non-Thermal technologies

Non-thermal technologies in food processing are innovative methods that preserve food without using traditional heat treatments. These technologies aim to maintain the sensory and nutritional quality of food while ensuring safety and extending shelf life.

High Pressure Processing (HPP) involves subjecting food to high pressures usually with the aim to inactivate microorganisms and enzymes without significant heat. This technology may alter protein structures, improving their solubility and functional properties and could also enhance the bioavailability of proteins and reduce allergenicity by modifying protein epitopes. In lentil and faba bean protein concentrates HPP (600 MPa, 4 min) resulted in comparable or greater gastric digestibility than untreated controls, but higher gastric proteolysis than heat treatment. Neither treatment impacted overall IVPD, for either lentil or faba bean protein concentrate. HPP slightly reduced trypsin inhibitor activity (6-8%), while heat treatment led to much greater reductions (78-86%) than untreated controls. Overall, HPP and heat treatment did not negatively impact lentil and faba bean protein quality (Hall et al., 2021). Similarly, to these results, HPP (25-150 MPa) increased lentil protein digestibility (Sridhar et al., 2022).

Microwave processing uses electromagnetic waves to heat food rapidly and uniformly. While typically associated with thermal effects, microwaves can also induce non-thermal effects that alter protein structures. Microwaves could impact the folding kinetics of proteins, leading to changes in their secondary and tertiary structures. This could enhance protein digestibility and modify functional properties like solubility and emulsification. About NF, the microwave cooking of Vigna unguiculata (L.) Walp subsp. unguiculata improved the in vitro protein digestibility from 81.6 (raw) to 92.2% (Boye et al., 2012). Regarding UF for ruminants, microwave treatment (750 W, frequency of 2450 MHz for 240 s) of *Posidonia oceanica* wastes improved the amount of rumen fermentation and digestibility of cell wall polysaccharides and dry matter without altering the fermentation rate (Abid et al., 2023 b).

Gamma irradiation uses high-energy gamma rays to sterilize food and modify its components. This method can induce structural changes in proteins, such as deamination, peptide bond cleavage, and the formation of disulfide bonds. These changes could affect the functional properties of proteins, including their solubility, digestibility, and allergenicity. None of the digestibility coefficients of dry matter or crude protein were affected by feeding irradiated (20 KGy) or non-irradiated olive pulp (by-product) to Japanese quails (Abd El-Moneim et al., 2022). It was concluded that, this by-product both irradiated or not could be used in quail diets up to 10% without any deleterious effects on liver and renal function as well as nutrient digestibility. In case of beam-irradiated lotus (*Nelumbo nucifera*) seeds, the IVPD decreased on irradiation, but it was only significant at 30 kGy ($p < 0.05$). However, a significant increase and higher concentration of essential amino acids (EAA) (threonine, valine, leucine, tyrosine + phenylalanine, and lysine) was detected after EB irradiation (Bhat et al., 2008).

Light pulse technology involves the application of intense, short-duration pulses of broad-spectrum light, including ultraviolet (UV), visible, and infrared light, to decontaminate food surfaces and packaging. The aim of high-intensity light pulses is to inactivate microorganisms by causing damage to their DNA and cell structures. However, light pulse could also affect proteins by inducing structural changes, such as denaturation and aggregation, which could alter their functional properties. Pulsed light (PL) processing (between 1 and 10 light pulses of 0.4 J.cm-2) improved digestibility of β-lactoglobulin (BLG). The results show that the treatment seems to facilitate digestibility of the protein network, especially regarding trypsinolysis. Firstly, treatment just barely enhances the enzymatic degradation of BLG by pepsin, which dilutes and weakens the interfacial layer, due to increased hydrophobicity of the protein owing to PL-treatment. Secondly, PL treatment importantly modifies the susceptibility of BLG to trypsin hydrolysis. While it dilutes the interfacial layer in all cases, it strengthens the BLG and weakens the PL-BLG interfacial layer (del Castillo-Santaella et al., 2014).

Pulsed Electric Field (PEF) involve the application of short pulses of high voltage to food products. This technique disrupts cell membranes, leading to microbial inactivation and enhanced extraction of bioactive compounds. However, PEF could cause protein unfolding and aggregation. The intensity and duration of the electric pulses would determine the extent of these changes. In vitro digestibility of plant proteins (Black beans) increased after PEF (1 kV/cm; 10 kJ/kg, 20 μs, 50 Hz) (Kamiloglu et al., 2024).

Cold Plasma (CP) is an ionized gas containing various reactive species. It is used for microbial decontamination and to modify food components and food contact materials. Cold Plasma (170-230 V, 5-15 min) resulted in 3.4-fold increase in IVPD of soy protein (Dabade et al., 2023).

Ultrasound (US) uses high-frequency sound waves to create cavitation bubbles in liquids, which collapse and generate intense local energy. This process can enhance mass transfer, extraction, and microbial inactivation. Ultrasound could break down protein molecules, leading to changes in their solubility, emulsifying, and foaming properties. It can also improve the efficiency of enzymatic hydrolysis of proteins. US treatment of chickpea protein (600 W, 30 min, 25°C) resulted in the highest IVPD (91.36%). The treatment of buckwheat protein (60% amplitude 10 min), increased digestibility from 41.4% (control) to 58.2%. The

treatment of potato protein (600 W, 20 min, 20 kHz) increased 12% IVPD. The treatment of soy protein isolate nanofibrils, (20 kHz, 750 W, 80% amplitude, 10 min) resulted in the highest IVPD in the range studied (20-80%). Thus, in general, the higher the amplitude of the US treatment the higher the IVPD of the plant-based product (Kamiloglu et al., 2024).

3.3.2.1.10. Supercritical fluid extraction

Supercritical fluid extraction uses supercritical fluids, typically carbon dioxide (CO_2), to extract bioactive compounds from food. This technique operates above the critical temperature and pressure of the fluid, allowing it to exhibit properties of both liquids and gases and can selectively extract compounds based on their solubility in the supercritical fluid. The process could affect proteins by causing denaturation. Marine microalgae (*Nannochloropsis granulata*) meals destined for aquaculture feed improved after supercritical fluid extraction (SCFE). SCFE processing at 70 and 90°C showed significantly higher degree of hydrolysis (DH) and predicted apparent digestibility coefficients (ADC) than the untreated base material for rainbow trout with average DH of 4.79% and predicted ADC of 87.0%, compared to 2.5% and 79.1%, respectively (Tibbetts et al., 2020).

3.3.2.1.11. Others

Duijsens et al. (2022) studied the effect of manufacturing conditions on *in vitro* starch and protein digestibility of (cellular) lentil-based ingredients. The product used was pulse powder: Raw Dupuy-type green lentils (*L. culinaris*). Lentil powders with different microstructural properties were produced from a single batch of whole lentils by altering and interchanging manufacturing steps (i.e., mechanical disintegration, thermal treatment, application of a cell concentration method, and the choice of drying method). After drying, the ingredients were stored in a desiccator until use. Raw-milled lentil powder was produced by milling raw, whole lentils until passing through a 500 µm sieve mesh. Isolated cotyledon cells powders were also produced. Raw whole lentils were soaked in an excess of demineralized water (1:10 w/v) for 16 h at 25°C after which the soaking water was discarded. The lentils were cooked in demineralized water (1:10 w/v) for 15 or 60 min at 95°C. At the end of the gastric phase, the amount of hydrolysed readily bioaccessible protein (NH2TCA,hydro) varied from 25 to 30% for powders with cellular intactness to approximately 41% for the raw-milled flour. For the raw-milled flour, upon 120 min of small intestinal digestion, about 92% (NH2TCA,hydro) of the protein, was converted into readily bioaccessible peptides. Interestingly, the ratio of the estimated final extent NH2TCA,hydro to NH2TCA (around 30%) indicates that the bioaccessible protein was mostly made up of peptides with an average polymerization degree of around 3. In comparison, around 65% of whole lentil and 80% of Isolated cotyledon cells protein was rendered bioaccessible during 120 min of small intestinal digestion. On the other hand, the effect of cooking and that of the effect of drying on proteolysis was limited.

3.3.2.1.12. Summary/Conclusions

In summary, heat/thermal processing generally improves the IVPD of plant-based products, mainly by denaturing proteins and modifying protease inhibitors. However, the extent of improvement varies with the type of plant and the specific heat treatment applied. For example, cooking and autoclaving significantly enhance the digestibility of legumes, while high-temperature treatments may reduce the digestibility of quinoa albumin due to amino

acid degradation. Apropos insects, while protein content remains relatively stable after most heat treatments, digestibility varies, with proteins from raw and fried mealworms being less digestible compared to those subjected to other cooking methods. In the particular case of frying, this effect would probably be linked to the increase in lipid levels. Overall, thermal processing can enhance the digestibility of proteins in both plant-based products and insects, but the effectiveness depends on the specific type of food and the conditions of the heat treatment. Risk assessors will need to consider the specific processing methods used, as the effectiveness of these methods can vary significantly depending on the type of food and the conditions applied and the species (livestock, pets or humans) for which the product is intended. This requires detailed documentation and validation of processing techniques in NF/UF applications. Additionally, the variability in digestibility improvements highlights the importance of tailored processing methods to optimize the nutritional value and safety of these foods.

Drying methods, such as freeze-drying, vacuum oven drying, and oven drying, can also impact the digestibility of plant-based proteins but, again, the direction and magnitude of the effect largely depend on the type and parameters of the drying process. For example, freeze-dried hempseed meal shows the highest IVPD compared to vacuum oven- and oven-dried meals, likely because the enzymes have better access to the cleavage sites. Furthermore, mild heating during vacuum and oven drying can cause reactions that reduce digestibility. In addition, the effect of drying on protein digestibility also seems to depend on the type/characteristics of the NF/UF product. Thus, for microalgae like *C. vulgaris*, methods such as ATFD at temperatures up to 98°C maintain higher nitrogen solubility and protein digestibility compared to other methods like solar drying, spray drying, or freeze-drying. Regarding insects, such as BSF larvae, drying methods applied to them, do not significantly alter protein digestibility, although they can improve feed acceptance in animals like fish. This indicates that drying would be a viable method for processing insect proteins without compromising their nutritional value. Overall, drying methods can effectively preserve and sometimes enhance the digestibility of proteins in plant-based products, microalgae, and insects, but again the specific method and conditions used are critical to achieving optimal results.

Regarding fermentation, it significantly improves the IVPD of several plant-based products and by-products. These improvements have been attributed to the breakdown of proteins, reduction of anti-nutritional factors, and formation of bioactive peptides. Fermentation also improves the digestibility of macroalgae eliminating soluble molecules and degrading insoluble fibers, resulting in better protein digestibility. This makes macroalgae a more viable protein source for various applications. Similarly, fermentation enhances the digestibility of microbial meals, improving protein accessibility and digestibility, making it a more efficient feed ingredient. In the case of ruminants, the use of fermented vegetal wastes as feed leads to better microbial protein synthesis and metabolizable energy by ruminants. However, fermentation of pig feed mixed with water tends to reduce protein ileal digestibility compared to dry feed, although it does not affect energy digestibility. This indicates that while fermentation can improve some aspects of feed quality, it may not enhance protein digestibility in all contexts.

Enzymatic hydrolysis significantly enhances the digestibility of plant-based proteins by breaking down complex proteins into simpler, more digestible forms. The use of specific enzyme combinations improves the digestibility of macroalgae proteins, DDGS for pigs, brewers' spent yeast and grain protein for aquaculture feed and also the digestibility and bioavailability of nutrients in novel raw materials. Thus, it can be stated that, overall, enzymatic hydrolysis is a versatile and effective method for improving protein digestibility across various food and feed matrices, enhancing their nutritional value and making them more suitable for consumption.

Germination also significantly improves the IVPD of various cereals, herbs and pulses by increasing nutrient digestibility, reducing antinutrient content, and boosting free amino acids and available carbohydrates. Alkali treatment can also enhance the IVPD and crude protein content of seeds depending on the specific procedure.

Finally, both non-Thermal Technologies (including HPP, microwave, gamma irradiation, pulsed light, PEF, cold plasma, and ultrasound) and supercritical fluid extraction, seem to improve protein digestibility in general. However, the available evidence regarding these technologies is particularly reduced and further work would be required for validation.

Table 9: Impact of processing on the digestibility of novel protein sources. The rows highlighted in green indicate that the treatment leads to an increase in digestibility, those in red indicate a decrease, and the yellow ones indicate that there would either be no effect or the result would be variable.

Processing Technology	Product	Processing parameters	Outcome	Reference
Heat/Thermal treatment	Legumes. *Phaseolus vulgaris L.*	80°C up to 9 min in a water bath	Increased degree of hydrolysis (DH) of protein from 62.3 to 73.6%	Li et al., 2023
	Legumes. Black grams, chickpea, lentil, red and white kidney bean	Autoclaving at 121°C for 10 min	Increased protein digestibility by 96–105% when compared to raw legumes	Avezum et al., 2023
	Legumes. *Vigna unguiculata* (L.) Walp subsp. *unguiculata*	100°C – 30 min	Increased *in vitro* protein digestibility (IPDV) from 71.3 (raw) to 78.7%	Kalpanadevi and Mohan, 2013
		121°C - 20 min	Increased IVPD from 81.6 (raw) to 89.7%	Boye et al., 2012
	Legumes. Chickpea *(Cicer arietinum)*	Autoclaving (120°C for 50 min)	Increased IVPD from 71.8 (raw) to 83.5%	Clemente et al., 1998
	Legumes. Faba bean *(Vicia faba L.)*	Autoclaving (121°C for 30 min)	Increased IVPD from 64.6 to 73.7%	Khalil et l., 1995
	Legumes. Velvet bean (*Mucuna pruriens*)	Autoclaving (120°C for 30 min)	Increased true digestibility from 48.5 to 81.6%	Siddhuraju et al., 1996

Table 9 *(cont.)*

Processing Technology	Product	Processing parameters	Outcome	Reference
Heat/Thermal treatment	Herbaceous plant finger millet (*Eleusine coracana*)	Cooking, temperature and time were not reported	Increased IVPD from 79 (raw) to 84.7–86.3%	Annor et al.,2017
	Seeds of flowering plant amaranth (*Amaranthus hybridus*)	100°C - 10 min	Increased true digestibility from 84.4 to 92.0%	Suffo Kamela et al., 2016
	Quinoa albumin	80, 100 and 121°C	Reduced hydrolysis ratio and loss of AA content	Yang et al., 2022
	Novel cereal *Glabrous Canary* seed	Roasting (dry-heat in an oven at 176°C for 12 min) / boiling (in water at 98°C for 12 min)	Increased digestibility (roasting, boiling). Altered the protein electrophoretic profile (roasting)	Rajamohamed et al., 2013
	Whole grain red sorghum (*Sorghum spp*)	Extrusion (182°C, 14% moisture)	Increased IVPD from 53.2 (raw) to 70.0% (extruded)	Llopart et al., 2014
	Distillers dried grains with solubles (DDGS)	Extrusion (120°C, 30% moisture)	Increased IVPD by 15%	Liu et al., 2024
	Insects. Mealworms (*Tenebrio molitor*)	Vacuum cooking (74.0°C for 60 min) / Pan frying (1 min) / Boiling (100.0°C for 1 min)/ Oven cooking (70.0°C for 30 min)	Proteins from raw and fried insects were less digestible than proteins from all other treatments	Megido et al., 2018
Drying/a_w reduction	Microalgae. *Chlorella vulgaris*	Agitated thin film drying (ATFD) up to 98°C with a residence time of 10 min	Increased N-solubility after ATFD (80.3%) compared to the other drying methods (solar, spray or freeze-drying (64.7–69.8%)	Van De Walle et al., 2024
	Hempseed (*Cannabis sativa L.*)	Freeze-drying (FD), vacuum oven (VOD) and oven drying (OD)	FD meal had the highest IVPD (88.2%), followed by OD meal (84.4%) and VOD meal (84.1%)	Duijsens et al., 2023

Table 9 *(cont.)*

Processing Technology	Product	Processing parameters	Outcome	Reference
Drying/a_w reduction	Solid herbal waste as alternative feed for ruminants	Different drying methods	Gas production, methane, NH3, microbial protein, *in vitro* degradability of dry matter (IVDMD) and organic matter (IVDOM) in silage and dried samples were lower ($p < 0.05$) compared to sun dried and freeze dried.	Kisworo et al., 2017
	Insect. Black soldier fly larvae	Oven drying (130°C, 1 h or 80°C, 23 h)	No changes on digestibility, but better feed acceptance for fish	Rawski et al., 2020
Fermentation	Flour of Kariya (*Hildergardia barteri*)	Combination of cooking and fermentation, 30°C for 5 days	Increased IVPD from 63 to 85%	Fawale et al., 2017
	Cowpea (*V. unguiculata L.)*	*Saccharomyces cerevisiae* (25°C, 24 h)	Increased IVPD from 81.6 to 84.3%	Boye et al., 2012
	Lentil flour	*Pleurotus ostreatus* (28°C for 14 days)	Increased fraction of digested protein (17%) in comparison to raw	Asensio-grau et al., 2020
	Finger millet (*Eleusine coracana*)	Lactic-acid bacteria combined with yeast	Increased IVPD from 79 (raw) to 71.2–83.7% (fermented)	Annor et al., 2017
	By-products, date palm wastes	*Pleurotus florida* inoculated	Increased organic matter digestibility	El-Waziry et al., 2016
	Macroalgae *Palmaria palmata*	*Trichoderma reesei* inoculated	Increased digestibility from 63 to 85%	Marrion et al., 2003
	S. cerevisiae microbial meal for fish nutrition	Autolysis	Increased apparent digestibility by 15%	Langeland et al., 2016
	Grape pomace waste as alternative feed for ruminants	*Pleurotus cornucopiae* and *Ganoderma resinaceum* (25°C for up to 8 weeks)	Increased crude protein and improved rumen fermentation	Abid et al., 2023
	Pig feed	Watering at 1:3 ratio and allowing for fermentation	Reduced protein ileal digestibility as compared with dry feed	Pedersen et al., 2010

Table 9 *(cont.)*

Processing Technology	Product	Processing parameters	Outcome	Reference
Ensiling	Brown seaweeds as a ruminant feed	The seaweeds were spread thinly onto a large plastic sheet and wilted for 24 h	Losses in Crude Protein (CP, −32%) but a limited effect on the *in vitro* true dry matter digestibility	Campbell et al., 2020
Enzymatic hydrolysis	*P. palmata*	Xylanase, cellulose. 50°C, 24 h	Increased protein digestibility	Fleurence et al., 2001
	DDGS	Mixture of xylanase and β-glucanase or cellulose (25°C, 48 h)	Increased apparent total tract digestibility of dry matter and crude protein	Jakobsen et al., 2015
	Brewers' spent yeast and grain protein	Different enzymes combination and hydrolysis conditions (50-60°C, 2-24 h)	Increased *in vitro* digestibility	San Martin et al., 2020
	Crops: Soybean Meal	Phytases (two types) or pepsin added. Incubated at 35°C for 5 days	Increased *in vivo* protein digestibility in common carp (*Cyprinus carpio*)	Watanabe et al., 2016
	Acrocomia totai defatted kernel meal	Exogenous phytases, 37°C for 30 min	Increased nitrogen bioaccessibility, greatest solubilisation at the end of the gastric stage, improved relative performance on a crude protein content basis	Casaretto et al., 2022
Germination	Pigeon pea/kidney bean/fava bean	Germination at 25°C for 72 h	Increased IVPD by 23 in pigeon and 110% in both kidney and faba bean	Avezum et al., 2023
	Finger millet (*Eleusine coracana)*	30°C for 48 h	Increased IVPD from 79 (raw) to 92%	Annor et al., 2017
	V. unguiculata (L.) Walp subsp. *unguiculata*	96 h	Increased IVPD from 71.3 (raw) to 84.9%	Kalpanadevi et al., 2013
	Faba bean (*V. faba* L.)	25°C for 72 h	Increased IVPD from 70.8 (raw) to 78.1% (germinated)	Alonso et al., 2000

Table 9 *(cont.)*

Processing Technology	Product	Processing parameters	Outcome	Reference
Alkali treatment	Egyptian riverhemp (*Sesbania Sesban*)	Boiled or soaked in slake lime (BSL/SSL), in lye (BL/SL)	Increased crude protein content for BSL and BL, compared to unprocessed. Increased IVPD in the treated seeds, following the order: BL > BSL > SSL > SL.	Arekemase et al., 2022
Non-thermal technologies	Lentil and faba bean protein concentrates	HPP at 600 MPa for 4 min	Increased gastric proteolysis than heat treatment, slight reduction in trypsin inhibitor activity (6-8%). No impact on overall IVPD	Hall et al., 2021
	Lentil protein	HPP at 25-150 MPa, 15 minutes	Increased protein digestibility	Sridhar et al., 2022
	V. unguiculata (L.) Walp subsp. *unguiculata*	Microwave cooking (750 W, 2450 MHz, 5 min)	Increased IVPD from 81.6 (raw) to 92.2%	Boye et al., 2012
	Posidonia oceanica wastes	Microwave treatment at 750 W and 2450 MHz for 240 s	Increased rumen fermentation and digestibility of dry matter	Abid et al., 2023b
	Olive pulp (by-product)	Gamma irradiation at 20 KGy	No effect on digestibility coefficients of dry matter or crude protein	Abd El-Moneim et al., 2022
	Lotus (*Nelumbo nucifera*) seeds	Beam irradiation at 30 KGy	Decreased IVPD. Increased concentration of essential amino acids	Bhat et al., 2008
	β-lactoglobulin (BLG)	Pulsed light (PL) processing between 1 and 10 light pulses of 0.4 J.cm^{-2}	Increased digestibility. Enhanced enzymatic degradation by pepsin and trypsin	del Castillo-Santaella et al., 2014
	Black beans	Pulsed Electric Field (PEF) at 1 kV/cm; 10 kJ/kg, 20 µs, 50 Hz	Increased IVPD	Kamiloglu et al., 2024
	Soy protein	Cold Plasma (CP) at 170-230 V (input) for 5-15 min	Increased IVPD (3.4-fold)	Yang et al., 2024

Table 9 *(cont.)*

Processing Technology	Product	Processing parameters	Outcome	Reference
Non-thermal technologies	Chickpea protein	Ultrasound (US) treatment at 600 W, frequency 20 kHz, 70% amplitude, 30 min, 25°C	Highest IVPD (91.36%)	Kang et al., 2022
	Buckwheat protein	US treatment at 600 W, frequency 20 kHz 60% amplitude for 10 min	Increased digestibility from 41.4 (control) to 58.2%	Kamiloglu et al., 2024
	Potato protein	US treatment at 600 W, frequency 20 kHz 70 % amplitude for 20 min	Increased IVPD by 12%	Kamiloglu et al., 2024
	Soy protein isolate nanofibrils	US treatment at 20 kHz, 750 W, 80% amplitude for 10 min	The IVPD increased by approximately 3.4-fold	Kamiloglu et al., 2024
Supercritical fluid extraction	Marine microalgae (*Nannochloropsis granulata*) for aquaculture	SCFE processing at 70 and 90°C	DH increased from 2.5 to 4.8 in SCFE samples. Predicted apparent digestibility coefficients (ADC) increased from 79.1 % to 87.0% in SCFE samples.	Tibbets et al., 2020
Others	Raw-milled lentil powder	Milling raw, whole lentils until passing through a 500 µm sieve mesh	92% protein converted into readily bioaccessible peptides after 120 min of small intestinal digestion	Duijsens et al., 2022
	Isolated cotyledon cells powders (ICC)	Soaking in demineralized water (1:10 w/v) for 16 h at 25°C. Cooking for 15 or 60 min at 95°C	80% protein rendered bioaccessible during 120 min of small intestinal digestion	Duijsens et al., 2022

2.2.1.1 Impact of processing on food/feed nutritional properties

In the same way as for digestibility, the impact of processing on the nutritional properties of novel protein sources is reviewed below and summarized in Table 10.

3.3.2.2.1. Heat/Thermal treatments

Severe thermal treatment can influence the amino acids content and improve or worsen the quality and nutritional values of different food/feed products such as legume seeds. Examples

in both directions include for instance the reported increase in the phenylalanine content of kidney beans or the loss of essential amino-acids to Maillard products (Ohanenye et al., 2022).

White sorghum (*Sorghum bicolor* (L.) grains and flour were heat treated in a conventional hot air oven to evaluate the effect of heat moisture treatment (100°C for 4 h at 17% adjusted moisture content). The crude protein (7.22–9.77 g/100 g depending on sorghum variety) decreased in treated samples compared to the control (Perraulta Lavanya et al., 2021). Bayukcapar et al. (2006) studied raw and heat-treated culban (*Vicia peregrina*) seed as protein source for aquaculture feed (mirror carp fingerlings). The ground seed was heat-treated in an autoclave at 121°C for 10 min. The diets that included the higher percentage of treated seeds were better on the basis of the specific growth rate, feed conversion ratio and protein efficiency ratio in comparison to those including raw seeds. Whole body fat content of the fish fed the diets containing the higher levels (>10%) of raw *V. peregrina* was significantly lower than in fish in the other treatments. Thus, the seed should be heat-treated if considerable inclusion rates to the diet are formulated. In *in vivo* studies, it is often difficult to ascertain the exact mechanisms at play. This complexity arises because multiple physiological processes and interactions within the organism can influence the outcomes. While heat treatment may improve the digestibility and nutritive properties of the seeds, the overall impact on fish growth and health can be affected by other factors.

3.3.2.2.2. Fermentation

Vasilica et al. (2022) investigated the use of a *Lactobacillus plantarum* strain on insect (*Acheta domesticus*) flour fermentation. Fatty acids, amino acids, minerals, and aroma volatile compounds were analyzed. Fermentation improved the nutritional quantity of the bioactive compounds, mainly after 24 h of fermentation, where they reached higher extended values. Alanine, Valine, Leucine and Methionine increased their values by 1.76-, 3.67-, 1.99- and 2.89-fold higher after fermentation (48 h, 37°C). Thus, the process led to an enriched insect flour sourdough that could be further used in the manufacturing of new products. Similarly, Jaeger et al. (2024) studied the lactic acid fermentation (*Lactobacillus amylovorus*, 50°C for 20 h) as a valorising agent for brewer's spent yeast. Protein profiles showed significant protein degradation, and free amino acid content was greatly increased following fermentation, from 2.8 ± 0.2 to 10.5 ± 0.4% w/w.

3.3.2.2.3. Ensiling

According to Moshely et al. (2015) *Hedychium gardnerianum* silage receiving molasses plus urea with inoculant, revealed the highest crude protein value (183.6 g/kg DM) and the maximum digestibility (56.31% over control). On the other hand, Ayemele et al. (2024) reported differences in amino acid composition between non ensiled and ensiled *Calotropis gigantea* (Giant milkweed). Furthermore, regarding palatability, non-ensiled GM is unpalatable for cows and drastically reduces the animal's feed intake, whereas ensiled Giant Milkweed has better palatability and does not reduce milk yield and milk protein.

3.3.2.2.4. Extraction

Saraiva et al. (2022) studied the bitter extraction process for brewing by-product. They heated a mixture of water and trub powder (25 g/L) at 90°C for 20-60 minutes, with the pH ranging from 7 to 13. This process resulted in an 85.70% increase in protein content. The use

of alkaline pH during extraction likely promotes protein unfolding, exposing hydrophobic groups and increasing protein-protein interactions, which reduces solubility. Additionally, the high temperature helps stabilize these hydrophobic interactions, which are endothermic. These conditions altered the technological properties of the proteins.

3.3.2.2.5. Others: size reduction

During the processing of algae milling is a usual step. Protein content, amino acid profile, and other nutritional components can become affected through milling and, therefore, vary across the different powder grades produced by the milling process. Healy et al. (2022 and 2023) maintained drying temperature at an average of 38-40°C to achieve a powder with a moisture content of about 10% after different post-harvest steps. These authors concluded that nutritional value depends on the particle size, finer particles tend to have higher surface area, which can enhance the digestibility and bioavailability of nutrients. Also, the protein concentration is different depending on post-harvest processing. For instance, milling and sieving can concentrate or dilute the protein content based on the separation of different components.

3.3.2.2.6. Summary/Conclusions

Thermal treatments can both improve and worsen the nutritional quality of legume seed proteins depending on the particular matrix and treatment parameters (temperature and time). High-temperature treatments like autoclaving usually improve the nutritional value and digestibility, however, excessive heat can lead to the Maillard reaction, which may reduce protein nutritional quality. The same conclusion can be drawn for cereals and insects. Thus, frying may reduce protein concentration due to increased lipid content, whereas other methods like boiling or oven cooking may better preserve nutritional value. By contrast, one article dealing with the heat treatment of seeds used in aquaculture feed reported an improvement in their nutritional quality and digestibility, as evidenced by better growth rates and protein efficiency in fish. This highlights the importance of selecting appropriate heat treatments to optimize the nutritional properties of feed ingredients.

Other processes such as fermentation, ensiling and extraction processes (particularly under alkaline conditions and high temperatures) have been proven to improve the nutritional quality of products that could be similar to the NF/UF such as insect flour (fermentation), certain forages (ensiling), and brewing by-products (fermentation and extraction).

In addition, and at least in one case, it has been reported that nutritional value can also be influenced by particle size and post-harvest processing methods such as milling and sieving.

Lastly, it should be pointed out that given the significant physiological differences between species (consuming these food/feed products), these conclusions/generalizations should be approached with caution as will be further discussed below.

Table 10: Impact of processing on the nutritional properties of novel protein sources. The rows highlighted in green indicate that the treatment leads to an increase in nutritional value, those in red indicate a decrease, and the yellow ones indicate that there would either be no effect or the result would be variable.

Processing Technology	Product	Processing parameters	Outcome	Reference
Heat/Thermal treatment	White sorghum (*Sorghum bicolor* (L.)	100°C for 4 h at 17% adjusted moisture content.	Decreased crude protein content although the values for the treated samples are not reported.	Perraulta Lavanya et al., 2021
	Culban (*Vicia peregrina*) seed	Autoclave at 121°C for 10 min	Diets including the higher percentage of treated seeds were better on the basis of the specific growth rate, feed conversion ratio and protein efficiency ratio of fish	Bayukcapar et al., 2006
Fermentation	Fermented Insect (*Acheta domesticus*) flour	*Lactobacillus plantarum,* 48 h, 37°C	Increased Alanine, Valine, Leucine and Methionine values: 1.76, 3.67, 1.99 and 2.89 hold higher, respectively.	Vasilica et al., 2022
	Fermented Brewer's Spent Yeast	*Lactobacillus amylovorus*, 50°C for 20 h	Increased free amino acid levels from 2.8 ± 0.2 g/100 g to 10.5 ± 0.4 g/100 g	Jaeger et al., 2024
Ensiling	*Hedychium gardnerianum*	Silage receiving molasses plus urea with inoculant (Lactic acid bacteria)	Increased Crude Protein from 138.0 to 183.6 g/kg DM	Moshely et al., 2015
	Calotropis gigantea (Giant milkweed)	Ensiled with fermentative bacteria and sucrose	Increased Crude Protein from 120 to 150 g/kg DM	Ayemele et al., 2024
Size reduction	Macroalgae and seaweed	Milling and sieving	Finer particles can enhance the digestibility and bioavailability of nutrients. Milling and sieving can concentrate or dilute the protein content based on the separation of different components.	Healy et al., 2022 and 2023

2.2.1.1 Impact of processing on gut microbiota

The methodology used only allowed for finding/identifying one document that studied the impact of processing on the effect of new protein sources on the gut microbiota. Weththasinghe et al. (2022) studied the modulation of Atlantic salmon gut microbiota composition and the predicted metabolic capacity by feeding diets with processed insect meals and fractions. Black soldier fly (*H. illucens*) larvae was processed into three meals (full-fat, defatted and de-chitinized) and two fractions (oil and exoskeleton). The inclusion of insect meals and fractions decreased abundance of proteobacteria and increased abundance of firmicutes in salmon gut. The diets that contained insect chitin, i.e., insect meals or exoskeleton diets, increased the abundance of chitinolytic bacteria including lactic acid bacteria and actinomyces in salmon gut, with fish fed full-fat meal diet showing the highest abundances. The diets that contained insect lipids, i.e., insect meals and oil diets, enriched bacillaceae in fish gut. The fish fed diets containing full-fat insect meal had a unique gut microbiota composition dominated by beneficial lactic acid bacteria and actinomyces, and showed a predicted increase in mucin degradation compared to the other diets. In summary, the dietary inclusion of insect meals and/or its fractions can differently modulate the composition and predicted metabolic capacity of gut microbiota in Atlantic salmon pre-smolts. Thus, the use of full-fat black soldier fly larvae meal in diets for salmon would be more favourable for beneficial modulation of gut microbiota than larvae processed by separation of lipid or exoskeleton fractions. In any case, these findings highlight the relevance of non-protein components and suggest that enriching the diet of salmon in (insect) protein content might have a negative effect on its gut microbiota. However, this effect would not probably be linked to the insect protein processing *per se*.

Given the limited information on the effect of processing on the gut microbiota, no relevant conclusions can be drawn. Therefore, this would be one of the areas where the greatest research effort should be made, given the well-known relevance of gut microbiota not only for digestive health but for overall health.

2.2.1.2 Impact of processing on food/feed toxicity

Most of the documents retrieved in the SR related to the effect of processing on the toxicity of novel protein sources did not deal with toxic compounds of a protein nature. In addition, those articles related with toxic proteinaceous compounds were limited to thermal treatments or extrusion. The results provided in these documents is discussed below and summarized in Table 11.

Thermal treatment can significantly affect the toxicity of foods in both positive and negative way. A well-known example of the first case is the degradation/removal of natural toxic proteins contained in many foods such as lectins in beans which can cause gastrointestinal distress and can be reduced or removed by proper cooking. On the other hand, heat treatment may cause the formation of new harmful compounds such as acrylamide, heterocyclic amines (HCAs) but also degrade nutrients, increase oxidation and inactivate detoxifying enzymes (Mehta 2015; Micali and Fiorino, 2016). Interestingly, the recently published EFSA guidance on NFs states that the impact of processing on the compositional profile of the NF (e.g., occurrence of heat-induced processing contaminants) should also be considered (EFSA NDA Panel, 2024).

Omosebi et al. (2018) studied the effect of extrusion on antinutritional factors (ANF). Extrusion at 170–180°C reduced the amount of trypsin inhibitor, in a meal derived from maize, soybean concentrate and cassava starch. In this sense, according to Bessada et al. (2019) different processing methodologies, including thermal processing, have also been successfully investigated, to reduce/eliminate ANF in pulses. On the other hand, and as an example of increased toxicant presence due to processing, the acrylamide concentrations significantly increased in plant-based proteins during pan-frying (160 and 200°C for 5 min on each side), especially in samples with high contents of glucose and asparagine. Given its relevance and the limited amount of information about this hazard in new food/feed products, investigations into the formation of food contaminants in novel processed foods have been suggested (Pospiech et al., 2024).

Regarding NEPs in GM crops, the data included in Table 11 indicate that the effect of temperature and pH as part of stability studies led to the loss of function (enzymatic activity) of all the NEPs evaluated, although the treatment conditions to achieve this vary depending of the NEP investigated (Herouet et al., 2005; EFSA GMO Panel, 2004, 2007a,b, 2011, 2012a,b, 2017a,b , 2018, 2020, 2021, 2023, 2024; De Luis, 2008, 2009; Wang et al., 2016; Omosebi et al., 2018; Pospiech et al., 2024). It should also be noted that higher temperatures might be required in order to make the NEPs non immunodetectable (de Luis et al., 2009). On one hand, by assuming that the outcome of these studies could be extrapolated to final products derived from typical GM crops, it could be expectable that crops processed under certain conditions (thermal processing, changes in pH, reducing agents, mechanical shearing) could lead to a loss or reduction of functional activity of introduced insecticidal or herbicide-tolerant proteins (Hammond and Jez, 2011). In line with this observation, active Cry proteins could be detected in grain and plant tissues (raw agricultural commodities) from GM corn, but neither in processed food products (Margarit et al., 2006; de Luis et al., 2009) nor in wet milled fractions and in corn mash from the dry-grind ethanol process (Dien et al., 2002). On the other hand, processing may also affect the stability of proteins to digestion by reducing or increasing their liability to protease degradation depending on the type of protein, food matrix and processing conditions. However, given the diversity of food matrices and food processing procedures, knowledge of their effects on susceptibility of proteins to digestion is still limited. Consequently, the effects of processing and of the food matrix on the susceptibility of a particular protein to digestion are difficult to predict (EFSA GMO Panel, 2017a; EFSA GMO Panel, 2021b).

In summary, results obtained indicate that thermal treatments/extrusion can significantly impact food toxicity by destroying natural toxins, such as trypsin inhibitors. Heating also deactivates NEPs/enzymes in GM crops, reducing their activity. However, pan-frying plant-based proteins at high temperatures can increase acrylamide concentrations.

Table 11: Impact of heating/thermal treatments on the toxicity of novel protein sources. The rows highlighted in green indicate that the treatment leads to a decrease in toxicity, those in red indicate an increase, and the yellow ones indicate that there would either be no effect or the result would be variable. For NEPs the expression host is indicated in brackets.

Product/Protein	**Processing parameters**	**Outcome**	**Reference**
Crops: maize, soybean, cassava	Extrusion: 170-180°C, screw speed 230 rpm, 20% moisture content	Reduced trypsin inhibitors	Omosebi et al., 2018
Plant-based protein	Pan frying (160-200°C, 5 min)	Increased acrylamide concentration	Pospiech et al., 2024
AAD-12 (Microbial)	30 min at 50, 70 and 95°C in a phosphate-based buffer solution	At all heating conditions (50–95°C) the enzymatic activity was eliminated and the protein lost more than 99% of its immunoreactivity.	EFSA GMO Panel, 2017b
AtHB17Δ113 (Microbial)	15-30 min at 75-95ºC	Partial loss of activity at 75ºC. Increased degradation at 95ºC.	EFSA GMO Panel, 2018
AvHPPD-03 (Microbial)	30 min at 37ºC 30 min at 65ºC	25% decrease immunoreactivity Activity below limit of detection	EFSA GMO Panel, 2020
CP4 EPSPS (Microbial)	15-30 min at 25-75ºC	≤ 45°C no effect ≥65ºC completely inactivated the enzyme	EFSA GMO Panel, 2004
Cry14Ab-1 (Microbial)	30 min at 4°C, 25°C, 37°C, 55°C,75°C and 95°C	Unstable, undetectable and has no activity after incubation at temperatures ≥ 75°C.	EFSA GMO Panel, 2021a
Cry1A(b) (Corn grain)	Nixtamalization (100ºC/5 min or 85ºC/60 min)	Decreased immunoreactivity (40 to 70%)	De Luis et al., 2009
	Porridge (75ºC/3 min)	90% decrease of immunoreactivity	
	Griddled and fried tortillas (180 and 190ºC 5-25 s)	No immunoreactivity after 25 s at 180 ºC (griddled) or 5 s at 190ºC (fried)	
Cry1A(c) (Soy)	15.5 min at 190ºC	>94% reduction in the quantity of immunodetectable protein	EFSA GMO Panel, 2012a
Cry1A(b) (Corn leaves)	2 min at 75-77ºC	Decreased immunoreactivity by 40% (75ºC) and 70% (77ºC)	De Luis et al., 2008
Cry34Ab1 and Cry35Ab (Microbial)	30 minutes at 60°C, 75°C and 90°C	Loss of biological activity	EFSA GMO Panel, 2007b
DMO (Microbial)	15 and 30 min at 25, 37, 55, 75 and95°C	At temperatures of 55°C and above for 15 and 30 min, a loss of functional activity below the limit of detection	EFSA GMO Panel, 2017c

Table 11 *(cont.)*

Product/Protein	**Processing parameters**	**Outcome**	**Reference**
DMO (Microbial)	15 or 30 min at 25, 37,55, 75 and 95°C	No or marginal activity after incubation at temperatures ≥ 55°C.	EFSA GMO Panel, 2023.
DMO (Soybean)	55 ºC	Purified recombinant DMO enzymes were deactivated	Wang et al., 2016
EPSPS (Soy)	30 min at 190 ºC	More than 97% reduction in the quantity of immunodetectable CP4 EPSPS	EFSA GMO Panel, 2012a
GAT (Microbial)	36-60 ºC	Decreased enzymatic activity over 49.5ºC. No enzymatic activity over 56.1ºC	Delaney et al., 2008b
GAT4601(Microbial)	15 min at 50ºC and 56ºC	Reduction of 40% after incubation at 50ºC. Enzyme was practically inactivated at 56 ºC	EFSA GMO Panel 2011
Glycine max-HRA (Microbial)	15 min at 44ºC and 50ºC	Reduced activity (50 %) at 44 ºC Enzyme was practically inactivated at 50ºC	EFSA GMO Panel 2011
HPPD-4 (Microbial)	30 min at 4°C, 25°C, 37°C, 55°C,75°C and 95°C	Instability upon temperature treatments of ≥ 55°C. At 65°C no detectable activity was observed	EFSA GMO Panel, 2021a
IPD079Ea (Microbial)	~30 min at 25, 50, 75 and 95°C	Protein was inactive after incubation at temperatures≥ 50°C..	EFSA GMO Panel, 2024
mEPSP (Microbial)	30 min at 25, 37, 65 and 95ºC	At 25 and 37ºC there was no or only a slight influence on activity, whereas at 65 and 95°C the enzyme was completely inactivated.	EFSA GMO Panel, 2007a
PAT (Microbial)	30 min at 50, 70 and 95°C in a buffer solution	At temperatures ≥ 55°C, > 99% of the enzymatic activity was lost with no residual activity detected above 75°C. At temperatures ≥ 37°C, the soluble PAT protein lost ≥ 91% of its immunoreactivity	EFSA GMO Panel, 2017b
PAT (Microbial)l	15 and 30 min at 25, 37, 55, 75 and 95°C	At 55°C for 15 and 30 min a loss of functional activity of, respectively, 76% and 60% was observed, exceeding 90% at temperatures of 75 and 95°C for 15 and 30 min.	EFSA GMO Panel, 2017c
PAT (Microbial)	15 or 30 min at 25, 37,55, 75 and 95°C	No or marginal activity after incubation at temperatures ≥ 55°C.	EFSA GMO Panel, 2023

Table 11 *(cont.)*

Product/Protein	**Processing parameters**	**Outcome**	**Reference**
PAT (Corn)	10-60 min at 60-90 ºC	No enzymatic activity after 10 min/55ºC	Herouet et al., 2005
PMI 105 (Microbial)	25, 37, 65 and 95 ºC	No loss of enzymatic activity at 25°C and 37°C resulted in. Incubation at 65°C resulted in loss of enzymatic activity below the enzymatic assay limit of quantitation, and incubation at 95°C resulted in no detectable enzymatic activity.	EFSA GMO Panel 2012b
PMI-0198 (Microbial)	30 min at 65ºC (pH 7.0)	Activity almost completely lost (98 % reduction)	EFSA GMO Panel 2012b
Vip3Aa20 (Microbial)	30 min at 4,25, 37, 65 and 95°C (pH 10.5)	After incubation at 65ºC for 30 minutes at pH 10.5, no activity was detected.	EFSA GMO Panel 2012b

2.2.1.1 Impact of processing on food/feed allergenicity

Finally, the impact of processing on the allergenicity novel protein sources will be discussed below.

3.3.2.5.1. Heat/Thermal Treatments

It is known that the effect of heat on food allergens may depend on various factors, such as the type of food, the heating method, temperature, duration, and the specific protein involved (Pi et al., 2024). Heat may affect food allergenicity through protein denaturation. This could lead to the reduction in allergenicity because most of allergens are proteins, and when they are denatured (unfold and lose their three-dimensional structure), their ability to bind to antibodies like Immunoglobulin E (IgE) can be reduced, thus decreasing their allergenic potential. This would be the case of egg allergenic proteins (such as ovalbumin) that can be denatured by cooking, which is why some people allergic to raw eggs can still eat cooked eggs. In clinical settings, the baked egg challenge recipe is used under medical supervision to assess whether individuals with egg allergies can tolerate eggs that have been extensively heat treated. The process involves baking eggs within a food matrix, such as muffins or cakes, typically at 160-180°C for at least 30 minutes. This extensive heating alters the protein structure of the egg, reducing its allergenicity by denaturing the proteins and making them less likely to trigger an allergic reaction. If the challenge is successful, patients are often advised to regularly include baked egg in their diet to maintain tolerance (Lemon-Mulé et al., 2008). However, some allergens are more resistant to heat and may retain their allergenicity even after heating. This incomplete denaturation occurs in certain milk proteins (e.g., casein) and peanut allergens (e.g., Ara h 1) which are relatively heat-stable and may not lose their ability to cause allergic reactions even after heating. On the other hand, heating may expose new epitopes or even produce the formation of new ones (neoallergens) thus increasing allergenicity. This happens when proteins aggregate or form new bonds after heat treatment.

For example, in peanuts, roasting can increase allergenicity by causing the formation of more stable protein structures or by facilitating Maillard reactions, which can produce compounds that enhance immune recognition (Maleki et al., 2000; Beyer et al., 2001). In summary, the impact of denaturation on allergenicity is complex and can vary depending on the specific protein and the conditions of denaturation (Mills et al., 2019).

Analogously, some other processes, such as gluten deamidation, Maillard reaction and formation of advanced glycation end products (AGEs) can have opposite effects on the allergenicity of this protein depending on factors like pH, temperature and the specific allergen (Mondoulet et al., 2005; Wieser et al., 2009; Hilmenyuk et al., 2010; Denery-Papini et al., 2012; EFSA GMO Panel, 2022).

Plant based proteins

In the case of NFs/UFs that are fruits or vegetables, it is known that pollen-food syndrome may occur. This syndrome causes allergenic reactions to raw fruits or vegetables due to cross-reactivity with pollen allergens. In that case, heating often reduces allergenicity because the responsible proteins are heat-sensitive (Popescu, 2015). However, specific studies would be needed for the particular plant-based NFs because published articles, to the best of our knowledge, are scarce with the scope and inclusion/exclusion criteria of our SR.

Insects

Several articles dealing with the effect of processing on insect allergenicity were found in the SR.

Broekman et al. (2015) studied the effect of thermal processing on mealworm (*Tenebrio molitor*) allergenicity. Raw mealworms (50 g) were heat processed by various methods: Blanching for 1 min at 100°C, boiling in 300 mL water for 10 min at 100°C, baking for 3.5 min at 1000 Watt on an induction cooker, or frying for 30 s at 180°C in peanut oil. Thermal processing of the insect did not change its IgE binding in a basophil activation test nor in the skin reaction in a skin prick test (crustacean-allergic patients were used), although the solubility of several proteins was altered. Similarly, Van Broekhoven et al. (2016) studied the influence of processing and *in vitro* digestion on the allergic cross-reactivity of three mealworm species (*T. molitor*, *Zophobas atratus* and *Alphitobius diaperinus*). Larvae were either boiled for 5 min in tap water (40 g larvae in 500 mL water) or fried for 5 min at 180°C in vegetable frying oil (120 g larvae in 2 L oil). Mealworm proteins, including tropomyosin (TM), alpha-amylase and muscle myosin, cross-reacted with serum from patients with previous house dust mites or crustacean allergy. Heat treatment only reduced in some cases but did not eliminate the allergenicity of mealworms in samples taken from these patients. This IgE cross-reactivity was significantly attenuated by the frying treatment (180°C, 5 min), while their IgE cross-reactivity was increased by the boiling treatment (100°C, 4 min). *In vitro* digestion also diminished but did not eliminate allergenicity (i.e. IgE cross-reactivity) of house dust mites or TM. Therefore, results of both articles warned that individuals allergic to house dust mites or crustaceans might be at risk when consuming mealworms, even after heat processing.

Following that, Sokol et al. (2017) studied TM as the cause of grasshopper anaphylaxis in patients allergic to house dust mites, cockroach, and crustaceans. Results suggested that the IgE cross-reactivity of grasshopper proteins in shrimp allergic patients is weakened by the boiling treatment (100°C, 5 min) or frying treatment (180°C, 3 min). Importantly, the frying process eliminated the IgE cross-reactivity of grasshopper proteins. As a traditional food processing method, heat processing may reduce the allergenicity of insect proteins by changing the structure of protein, but the reduction effects seem to be highly related to the thermal stability, species, and treatment. Frying and boiling are the main insect processing methods. In most cases, the SDS-PAGE analysis bands of insect proteins did not change significantly during the boiling treatment. The effects of frying on reducing the potential allergenicity of insect proteins seems more significant, from these recent studies in grasshopper and mealworm.

More recently, De Marchi et al. (2021) assessed the allergenicity of the edible cricket *Acheta domesticus* in terms of thermal and gastrointestinal processing and IgE cross-reactivity with shrimp. Edible cricket flour was used to prepare biscuits that were cooked in a static oven at a temperature of 180°C for about 15 min until they turned brown. Using IgE of allergic patients to crustaceans, authors examined the immunoreactivity of the proteins of the insect flour used to make biscuits (heat treated), compared with those of the shrimp *Litopenaeus vannamei*. Once again, TM was identified as the most relevant IgE-binding protein, and its cross-reactivity with shrimp TM was demonstrated by ELISA. While shrimp TM showed scarce stability to gastric digestion, cricket TM withstood the whole digestion process. The sarcoplasmic calcium-binding protein, specifically detected in shrimp, showed exceptional stability to gastrointestinal digestion. IgE-binding proteins in a model of enriched baked products were partially protected from proteolysis. In conclusion, the ingestion of *A. domesticus* proteins poses serious concerns to the crustacean-allergic population because there is a risk of cross-allergic reaction. Furthermore, high stability of cricket TM represents a severe risk of primary sensitization.

Even more recently, Traynor et al. (2024) analysed food safety of novel insect proteins. The scientific studies that the authors collected investigating the risk from allergens and their detection concluded, in line with the previously commented studies, that the potency of allergens found in differing insect proteins respond in a different way under different processing techniques. Pali-Schöll et al. (2019) had determined that when exposed to severe heat treatments or enzymatic hydrolysis, the immunoreactivity of migratory locust was eliminated, whereas studies by De Marchi et al. (2021) and Leni et al. (2020) highlighted the inefficiencies of such treatments on the immunoreactivity of insect proteins found in house crickets and black soldier fly. The authors stated that it is important to consider the impact of the food matrix on the allergenic potency of insect proteins, as due to consumerism barriers posed by Western consumers, insect proteins are often added to familiar food products such as pasta or bread to enrich their nutrient qualities.

TM is a major allergen found in both shrimp and insects, and its solubility plays a crucial role in determining the allergenic potential of these foods, especially after processing. However, the solubility of TM can vary between shrimp and insects, influencing how processing methods affect its allergenicity. In shrimp, TM is highly soluble and remains stable under various processing conditions, such as boiling and frying. This stability means that even after cooking,

the protein could still trigger allergic reactions in sensitive individuals (Cheng et al., 2021). In contrast, the solubility of TM in insects would be more variable, depending on the species and the specific processing methods used (Xu et al., 2019).

From the studies included in this SR, it is apparent that further research into the allergenicity of specific insect proteins is required to determine the route of sensitization, and the minor and major allergens associated with each of the four insect species approved as a NF and with the potential species that could be added to the list in the near future. As more insect-based foods trickle into the EU food market, it is imperative that the effect of processing on the safety of insect-based foods needs further investigation to provide a representative risk profile of the consumption of insect proteins on human health, with focus on allergenic hazards (Traynor et al., 2024).

GM crops

Cao et al. (2010) assessed the safety of Cry1C protein from genetically modified rice according to the national standards of PR China for a new food resource. The stability was assessed at a temperature of 100°C for periods of 10, 30, and 60 min. On SDS–PAGE, the Cry1C protein was clearly visible at about 67 kDa at time zero. When incubated at 100°C for 60 min, the Cry1C protein persisted and was still detectable. Therefore, it was not degraded or modified in a way that would affect its migration on SDS–PAGE after exposure to 100°C for 60 min. Focusing on hazard, they primarily focused on the stability of allergens rather than their allergenicity. More stable proteins tend to be more allergenic because they are less likely to be broken down during digestion, allowing them to interact with the immune system. However, to date, EFSA's allergenicity risk assessment for approved GM crops has not identified any additional hazard (Fernández et al., 2024).

3.3.2.5.2. Enzymatic hydrolysis

Plant based proteins

Enzymatic hydrolysis (sequential action of Alcalase® and Flavourzyme®) was reported to be effective in attenuating allergenicity of legume proteins and may be employed for preparing hypoallergenic food extracts (Kasera et al., 2015). Watanabe (1993) developed a process for producing hypoallergenic rice in grain form based on almost complete removal of the major allergenic proteins by proteolysis with added protease, while retaining half or more of the principal rice seed protein, glutelin. The allergenicity of many plant-based proteins can be attenuated by processing exposure to heat, enzyme hydrolysis, or high pressure (Aimutis, 2022).

Insects

Pali-Schöll et al. (2019) studied the cross-recognition of IgE from crustacean- and house dust mite allergic patients, and the potential reduction of allergenicity by food processing. Four different commercially available food-grade enzyme preparations (Alcalase®, Neutrase®, Flavourzyme®, and papain) were used. Hydrolysis was conducted for 2 h at 50°C and pH 7.0 in a shaking water bath. For heat treatment experiments, aliquots were heated at 80 and 100°C for 10 min in a water bath. Enzymatic hydrolysis and thermal treatment eliminated

cross-reactivity and allergenicity of insect extracts (*L. migratoria*), as tested by immunoblots and skin prick test.

Fei et al. (2016) assessed the sensitizing capacity and allergenicity of enzymatic cross-linked arginine kinase, the crab allergen. The treatment consisted of exposure to peroxidase from horseradish (HRP) and tyrosinase (Tyr) from mushroom, at 37 ℃ for 8 h. Enzymatic digestion of tropomyosin resulted in a gradual decrease in its IgE-binding capacity at degree hydrolysis (DH) levels by 15-40%, and complete elimination of IgE-binding occurring at DH levels of 50-85%. However, it should be noted that enzymatic treatment under conditions with a DH below 50% may expose additional tropomyosin epitopes, potentially leading to an increase in allergenicity. The use of Tyr and caffeic acid (TM-Tyr/CA) in enzymatic cross-linking of tropomyosin effectively inhibits mast cell degranulation and reduces allergic symptoms in mice. This leads to lower levels of IgE and histamine in serum. Similarly, cross-linked thermal polymerized arginine-kinase (AK) reduces the allergenicity of AK and induces oral tolerance in mice. Enzymatically cross-linked AK induces oral tolerance and reduces allergenicity in mice, but it is more resistant to gastrointestinal digestion compared to native AK, as indicated by in vitro digestion experiments.

Leni et al. (2020) assessed allergenicity of lesser mealworm, black soldier fly and their protein hydrolysates trough shotgun proteomics, *in-silico* evaluation and immunoblotting assays. Protease from *Bacillus licheniformis* (≥2.4 U/g; EC Number 3.4.21.62) was used in order to produce a peptide rich fraction from grinded lesser mealworm and black soldier fly larvae. The hydrolysis reactions were carried out overnight on 5 g of ground insects, 45 mL of a phosphate buffer (Na_2HPO_4 10 mM) and 1% of enzymes. While IgG-immunoblotting demonstrated the loss of immunoreactivity for both insect hydrolysates, IgE-immunoblotting showed a partial immunoreactivity preservation, also after hydrolysis, in the case of black soldier fly hydrolysate, and a total loss of immunoreactivity for lesser mealworm hydrolysate. These results indicated that different immunoreactivity can still remain in different species, even when subjected to the same enzymatic hydrolysis. Moreover, the simple measure of degree of hydrolysis is not enough to assess hypoallergenicity and IgE reactivity and possibly in vivo challenges are needed.

3.3.2.5.3. Fermentation

Handoyo et al. (2006) developed a hypoallergenic buckwheat flour preparation by *Rhizopus oligosporus* fungi and applied it to soba noodle. Fermentation was carried out at 30°C and 85% relative humidity up to 72 h. Western blot analysis showed that the allergenic proteins appeared in the control (no fermentation), but it disappeared during the time course of fermentation. Allergenic proteins in buckwheat were almost degraded after 16 h fermentation and completely degraded to LMW peptides (amino acids or small peptides) after 24 h fermentation.

3.3.2.5.4. Nonthermal Technologies

Several research reports suggest that treatments with high pressure, ultrasound and pulsed light can reduce the activity of allergens in soybean (Dong et al., 2020).

Kato et al. (2000) studied the release of allergenic proteins from rice grains induced by HHP (100–400 MPa). The major proteins released were identified as a 16 kDa albumin, α-globulin

and 33 kDa globulin, all of which are major rice allergens. The partial destruction of endosperm cells by pressurization enhances the permeation of the surrounding solution into the rice grains. The removal of allergens by pressurization alone was insufficient to reduce allergenic activity, which was almost completely eliminated by pressurization in the presence of proteolytic enzymes. Similar results were obtained by Jankiewicz et al. (1997) who studied the influence of food processing on the immunochemical stability of celery allergens. Thus, high pressure treatment (600 MPa, 20°C) was not sufficient to destroy the IgE-binding capacity of the Bet v 1-like allergen, Api g 1, from celery, nor pulsed electric field processing at 10 kV at 50 Hz or g-irradiation (10 kGy).

US can break the structural integrity of the food products, as well as protein structures but this highly depends on the intensity of application. Thus, recovery of total protein as in the case of soy products may increase after ultrasound treatment, but the allergenicity might not be necessarily reduced in the processed soy products (Bernardi et al., 2021). However, in some other cases, it has been reported that ultrasound can reduce the allergenicity of food products, such as roasted peanut extracts (Nayak et al., 2017).

Finally, it has also been reported that high doses of radiation (>10 kGy) of insects induce structural changes of TM (unfolding or aggregation), which result in the reduction of the IgE-binding capacity of TM (Costa et al., 2022).

3.3.2.5.6. Summary/Conclusions

The processing technologies, products involved, processing parameters, the outcomes regarding allergenicity after the processing and the references are summarized in Table 12.

Different processing methods (e.g., heating, fermentation, hydrolysis) could have varied effects on allergenicity. The lack of standardized protocols for processing and testing can lead to inconsistent results across studies, making it difficult to draw definitive conclusions. However, as a summary of the impact of heat/thermal treatments on the allergenicity of different types of products or matrices, we can conclude that heating often reduces the IgE reactivity of plant-based proteins, especially for heat-sensitive proteins responsible for pollen-food syndrome in fruits and vegetables. For insects, thermal processing such as boiling, frying, and baking could alter IgE reactivity, but the effects vary: mealworms show reduced but not eliminated IgE reactivity, grasshoppers have significantly reduced IgE reactivity when fried, crickets retain some allergenic proteins even after baking, and silkworm pupae exhibit increased IgE reactivity for certain proteins after heat treatment. In GM crops, no new hazards have been identified for approved GM crops up to date.

Regarding enzymatic hydrolysis, it significantly impacts the IgE reactivity of various products. For plant-based proteins, such as legumes and rice, enzymatic hydrolysis using specific enzymes effectively reduces IgE reactivity, making these proteins a potential application for developing hypoallergenic food extracts. In insects, enzymatic hydrolysis combined with thermal treatment could eliminate cross-reactivity and IgE reactivity, as seen in migratory locusts. For crab allergens like TM, enzymatic digestion reduces IgE-binding capacity, with complete elimination at higher degrees of hydrolysis, and cross-linking with tyrosinase and caffeic acid inhibits allergic reactions in mice. However, some insect proteins, like those in Black Soldier fly hydrolysate, may retain partial IgE reactivity even after hydrolysis. Overall,

enzymatic hydrolysis could be effective in reducing IgE reactivity, but the degree of reduction varies depending on the specific protein and hydrolysis conditions, highlighting the need for further *in vivo* testing to ensure safety. Hydrolysis reduces the allergenic potential of proteins by breaking them into smaller fragments, but some of these fragments (peptides) can still retain allergenic properties if they are large enough to be recognized by the immune system. Generally, peptides that are shorter than nine amino acids are considered safe because they are too small to be recognized by IgE antibodies, which are responsible for triggering allergic reactions. This principle is used in the production of hypoallergenic food products, where extensive hydrolysis is employed to ensure that the resulting peptides are small enough to minimize the risk of allergenic responses (Verhoeckx et al., 2015).

Similarly, it has been reported that fermentation with *R. oligosporus* effectively degrades allergenic proteins in buckwheat flour, opening the possibility of making it hypoallergenic within 24 hours. However, as it has been mentioned, further studies with more robust methodologies or combinations of in vitro and in vivo assays, studying the complexity of food matrix are necessary to draw more definitive conclusions.

The impact of non-thermal technologies on allergenicity (IgE reactivity) varies across different products. For plant-based proteins like soybeans, treatments with high pressure, ultrasound, and pulsed light could reduce IgE reactivity, though the effectiveness depends on the intensity and specific conditions. In rice, high hydrostatic pressure alone is insufficient to reduce allergenic activity but combining it with proteolytic enzymes could almost completely eliminate IgE reactivity. For celery, high pressure, pulsed electric field processing, and gamma irradiation are not sufficient to inhibit binding activity. Ultrasound treatment could break protein structures, potentially reducing IgE reactivity in some cases, such as roasted peanuts, but not necessarily in all soy products. In insects, high doses of radiation could induce structural changes in proteins like tropomyosin, reducing their IgE-binding capacity. The effect is technology/product dependent but, overall, only HHP might seem as a consistent option. Furthermore, the effectiveness of methods with potential in reducing IgE reactivity and thus may be allergenicity would vary depending on the specific food matrix and processing conditions. This highlights the need for tailored approaches and further research to ensure safety for specific food.

In any case, these generalizations should be treated with caution, particularly when establishing equivalences between the results obtained and the allergenic potential of different foods under real consumption conditions, for several reasons -some specific to this type of study and others more general, which will be discussed in greater detail in Section 3.3.2.6-.

Regarding those that exclusively or particularly affect allergenicity studies, it should be noted that s tudying the impact of processing on food and feed allergenicity involves several methodologies, each with its own limitations, including *in vitro* assays, *in vivo* animal models, and human clinical trials. *In vitro* assays such as enzyme-linked immunosorbent assays (ELISA) and immunoblotting are commonly used to measure changes in IgE-binding capacity of proteins after processing. However, these methods do not always correlate with clinical allergenicity. Most of the articles mentioned in the present report involve studies in which IgE reactivity was used rather than actual allergenic potential. *In vivo* animal models, like those

using mice or rats, can provide insights into the sensitization potential of processed proteins, but these models are not always predictive of human responses due to species-specific differences in immune systems. This can result in data that is not fully applicable to humans, affecting the reliability of the conclusions drawn. Human clinical trials, including double-blind placebo-controlled food challenges, are considered the gold standard for assessing allergenicity. Yet, these trials are resource-intensive, ethically complex, and not always feasible, thus to the best of our knowledge articles using these kinds of methods are very scarce. Even if human studies were available, the small sample sizes and variability in individual responses could limit the generalizability of the results. The lack of *in vivo* context means that the assays reported in the present SR might miss interactions that occur in a living organism, leading to over- or to under-estimation of allergenic potential. Therefore, there is a need for more comprehensive studies that combine these methodologies to better understand the effects of processing on allergenicity. The development of robust, integrated methods for risk assessment remains a critical area for future research (Verhoeckx et al., 2015). Apart from that, another limitation detected is the lack of comprehensive studies. Most of the studies retrieved on effect of the different processing technologies focus on specific aspects of allergenicity of the food involved, such as IgE-binding capacity, without considering other factors like the ability to induce sensitization. This narrow focus could result in an incomplete understanding of the overall impact of processing on allergenicity and thus the conclusions drawn could be biased.

Table 12: Impact of processing on the allergenicity of novel protein sources. The rows highlighted in green indicate that the treatment leads to a decrease in allergenicity, those in red indicate an increase, and the yellow ones indicate that there would either be no effect or the result would be variable.

Processing Technology	Product	Processing parameters	Outcome	Reference
Heat/Thermal treatments	Fruits or vegetables	Boiling (100°C) or baking (180°C)	Reduced IgE reactivity	Popescu, 2015
	Insect: Mealworm (*T. molitor*)	Blanching (100°C, 1 min); Boiling (100°C, 10 min); Baking (induction, 1000 W, 3.5 min); Frying 180°C, 30 s)	No change in IgE binding in a basophil activation test nor in the skin reaction in a skin prick test	Broekman et al., 2015
	Insect: three mealworm species *(T. molitor, Z. atratus and A. diaperinus)*	Boiling (100°C, 5 min); Frying (180°C, 5 min)	Decreased IgE cross-reactivity (crustacean allergic patients) by the frying. Increased IgE cross-reactivity (crustacean allergic patients) by the boiling	Van Broekhoven et al., 2016
	Insect: Grasshopper proteins	Boiling (100°C, 5 min); frying (180°C, 3 min)	Decreased IgE cross-reactivity in shrimp allergic patients	Sokol et al., 2017
	Insect: Edible cricket (*A. domesticus*)	Oven (180°C, 15 min)	Increased cricket TM stability	De Marchi et al., 2021
	Insect: Migratory locust	60-100°C, 10-30 min	Decreased binding of cross-reactive IgE antibodies	Pali-Schöll et al., 2019
	Insect: Black soldier fly and lesser mealworm	60°C, 60 min (plus protease)	Reduced immunoreactivity of these insects (but for the black soldier fly, it was partially preserved)	Leni et al., 2020
	GMO rice	100°C for periods of 10, 30, and 60 min	Cry1C protein persisted and was still detectable on SDS–PAGE. The protein did not induce significant levels of specific IgG and IgE antibodies in rats.	Cao et al., 2010
Enzymatic hydrolysis	Plant based proteins	Sequential action of Alcalase® and Flavourzyme®	Reduced IgE reactivity of legume proteins	Kasera et al., 2015
	Soybeans and peanuts	Proteases, 37°C, 2 h	Reduced allergenic potential	Watanabe, 1993

Table 12 *(cont.)*

Processing Technology	Product	Processing parameters	Outcome	Reference
Enzymatic hydrolysis	Soybeans and peanuts	Proteases, 37°C, 2 h	Reduced allergenic potential	Watanabe, 1993
	Insect extracts *(L. migratoria)*	Alcalase®, Neutrase®, Flavourzyme®, and papain; 2 h at 50°C and pH 7.0	Reduced (eliminated) cross-reactivity and allergenicity as tested by immunoblots and skin prick test.	Pali-Schöll et al., 2019
	Arginine kinase, the crab allergen	Peroxidase from horseradish and tyrosinase from mushroom, at 37°C for 8 h.	Reduced IgE-binding activity of the crab allergen	Fei et al., 2016
	Lesser mealworm (LM), black soldier fly (BSF) and their protein hydrolysates	Protease, overnight, 1% of enzyme	While IgG-immunoblotting demonstrated the loss of immunoreactivity, IgE-immunoblotting showed a partial immunoreactivity preservation	Leni et al., 2020
Fermentation	Buckwheat flour	*R. oligosporus* fungi; 30°C and 85% RH up to 72 h.	Western blot analysis showed that the allergenic proteins were completely degraded to LMW (amino acids or small peptides)	Handoyo et al., 2006
High hydrostatic pressure	Rice	100–400 MPa, up to 30 min	Major allergens were released but was insufficient to avoid IgE reactivity	Kato et al., 2000
	Cellery allergens	600 MPa, 20°C, 15 min	Not sufficient to destroy the IgE-binding capacity of the allergen Api g 1	Jankiewicz et al., 1997
Pulsed electric field	Cellery allergens	10 kV, 2000 µs	Reduced allergenicity but not sufficient to destroy binding capacity	Jankiewicz et al., 1997
γ-irradiation	Cellery allergens	10 kGy, 30 min	Reduced allergenicity but not sufficient to destroy binding capacity	Jankiewicz et al., 1997
	Edible insects	Greater than 10 kGy	Reduced IgE-binding capacity	Costa et al., 2022
Ultrasound	Soy	20 kHz, 10-30 min, 2 5°C	Treatment can increase the solubility and extraction efficiency of proteins, but it does not necessarily reduce their allergenicity	Bernardi et al., 2021
	Roasted peanut extracts	20 kHz, 15 min, 25°C	Reduced IgE reactivity	Nayak et al., 2017

2.2.1.2. Other (general) considerations

This section reviews some aspects to be considered when interpreting the results from the studies included in the SR and the conclusions drawn.

Impact of protein digestibility on other traits

Protein digestibility is a factor that can affect the nutritional value, toxicity, and allergenicity of proteins. In this regard, and as a way of example, a relation between protein digestibility and allergenicity has been reported in the literature (Koidl et al., 2023). Thus, heat can make some allergens more digestible leading to less allergenic fragments. However, heat treatment can also make some allergens more resistant to digestion by altering their structure, which could enhance their ability to provoke an immune response. In this sense, Jiménez-Saiz et al. (2015) pointed out that within the field of food allergy, it is essential to evaluate the digestion resistance of processed proteins, also as a part of the matrix in which they are usually consumed, as well as the ability of the fragments generated upon gastro-intestinal digestion, ideally *in vivo* or, at least, *in vitro* by using reliable models that mimic physiological conditions, to retain biologically relevant IgE epitopes. Consequently, protein digestibility and/or protein stability are considered important factors in the allergenicity assessment of GM crops/novel foods for which the allergenic potential is unknown (EFSA GMO Panel, 2021b; EFSA NDA Panel, 2024).

Significant physiological differences between species

Due to the significant physiological differences between species, the conclusions drawn along the text should be approached with caution. For example, just as the toxic effect of a compound varies significantly depending on the organism that ingests it, the impact of applying a technology/process on the hazards associated to a food/feed product may also differ based on the species consuming this product. Therefore, the most accurate approach would be to draw conclusions within species, although in most cases, the scientific data/evidence would be insufficient to extract not only robust conclusions but even to draw hypotheses. More details on how the significant physiological differences between species could influence how food and feed processing affects the outcomes studied in the present SR are discussed below.

Regarding digestibility, species-specific enzymes exist which mean that different species have varying types and levels of digestive enzymes. For example, ruminants like cows have a complex stomach with multiple chambers and a unique microbial population that helps break down fibrous plant material, whereas monogastric animals like pigs and humans rely more on their own digestive enzymes. Thus, processing (such as heat treatment, fermentation, and enzymatic hydrolysis) can enhance protein digestibility in some species but may not have the same effect in others. For instance, fermentation improves protein digestibility in ruminants by breaking down complex proteins into simpler forms that are more accessible to their gut microbes. However, the same process might not be as effective in monogastric animals which are generally less able to utilize sources of nitrogen compared to ruminants. This is particularly relevant because, as indicated above, protein digestibility might also impact other protein-safety related aspects.

Concerning nutritional properties, the different species have distinct nutritional requirements. For example, the amino acid profile needed for optimal health in poultry differs from that of fish or humans. Processing methods that enhance the nutritional value of feed for one species might have the opposite effect for other. For example, thermal processing can degrade certain vitamins and amino acids that might be more critical or relevant for some species than others. Regarding specific technologies such as ensiling and fermentation, these can enhance nutrient availability in ruminants in which are usually applied but might not have the same effect in non-ruminants.

Regarding gut microbiota, although just one article has been retrieved (Weththasinghe et al., 2022), it is obvious that the gut microbial composition varies significantly between and even within species. Ruminants have a complex microbial ecosystem that aids in the fermentation of fibrous plant material, while monogastric animals or humans have a simpler gut microbiota. In any case, microbiota composition in humans is also a huge scientific field of study and the analysis of each study regarding processing effect on microbiota would need to consider many aspects, making difficult to draw general conclusions.

Regarding toxicity, detoxification mechanisms differ among species with some having the capacity to detoxify and to tolerate harmful compounds, although each combination of toxin and species should be considered on a case-by-case basis. Ruminants can detoxify certain plant toxins through microbial fermentation in their rumen, while monogastric animals or humans might be more susceptible to these toxins. As it has been described above, some processing may reduce or remove toxins. For example, thermal treatment can reduce the levels of natural lectins in beans, making them safer for consumption. However, other toxic compounds like acrylamide formed during high-temperature processing can pose a risk to all species, though the degree of susceptibility might vary.

Regarding allergenicity, the immune response to allergens can differ between species. In particular, humans might develop allergic reactions to certain proteins that are not allergenic to other animals and likewise, allergic reactions vary among animal species. As it has been described, treatment and enzymatic hydrolysis can reduce the allergenicity of proteins by denaturing them or breaking them down into smaller peptides. However, the effectiveness of these methods would vary and, while treatment might reduce the allergenicity of certain proteins in humans, it might not have the same effect in pets or livestock. Furthermore, data on allergies in animals are limited, and conclusions drawn from one species cannot always be extrapolated to others, particularly in the context of how feed processing influences animal allergies. Each species has unique physiological and immunological responses to allergens, which means that a processing method that reduces allergenicity in one species might not have the same effect in another. For example, the digestive systems of ruminants like cows differ significantly from those of monogastric animals like pigs and poultry, affecting how they process and react to feed allergens. Additionally, the immune system's response to allergens can vary widely between species, making it challenging to predict cross-species outcomes. This variability underscores the need for species-specific research to understand the impact of feed processing on allergenicity. Without comprehensive data across multiple species, it is difficult to develop universally applicable guidelines for feed processing to mitigate allergies in animals (Pali-Schöll et al., 2017; Verhoeckx et al., 2015).

In summary, the physiological differences between species imply that a tailored approach is necessary when studying the effect of food and feed processing on protein safety. This highlights the importance of species-specific research considering the unique physiological traits of each species.

Maillard reaction

The Maillard reaction, a non-enzymatic browning process that occurs when amino acids of proteins and reducing sugars are heated together, produces Maillard reaction products (MRPs) that, although may contribute to the taste, smell, and colour enhancing their sensory appeal, they may have complex effects on food traits. This reaction may significantly influence various aspects of food, including digestibility, nutritional properties, gut microbiota, toxicity, and allergenicity.

The Maillard reaction could impact protein digestibility in several ways. The reaction can cause proteins to form cross-links and aggregates, which may make them more resistant to digestive enzymes which could reduce the overall digestibility of proteins. Furthermore, the Maillard reaction could modify protease inhibitors, which are compounds that inhibit the activity of digestive enzymes. This modification could either enhance or reduce the digestibility of proteins depending on the specific conditions and the type of food being processed. In the case of legumes and other plant-based proteins, heat treatments, which often involve Maillard reactions, have been shown to improve the digestibility by denaturing proteins and modifying protease inhibitors. However, on the contrary, excessive heat could lead to the formation of resistant protein structures, reducing digestibility (Gilani et al., 2012).

Regarding nutritional properties, the Maillard reaction can have both positive and negative effects. The reaction could lead to the degradation of essential amino acids, such as lysine, reducing the nutritional quality of proteins. This is particularly significant in foods subjected to high temperatures for extended periods. In addition, reaction may produce the formation of bioactive compounds. Some Maillard reaction products have antioxidant properties, which can be beneficial. However, the overall nutritional impact depends on the balance between beneficial and detrimental compounds formed during processing. Likewise, the reaction could reduce the availability of certain nutrients by binding them in complex structures that are less accessible to the body (Tamanna et al., 2015).

Regarding gut microbiota, some Maillard reaction products could act as prebiotics, promoting the growth of beneficial gut bacteria, although the specific effects depend on the types of compounds formed and their interactions with the gut microbiota. In addition, the products of the reaction could be metabolized by gut bacteria, potentially influencing the composition and activity of the gut microbiota having downstream effects on gut health (ALJahdali et al., 2017).

Special mention should be made of the effect of the Maillard reaction on toxicity because it can lead to the formation of potentially harmful compounds. Acrylamide and heterocyclic amines are well-known toxicants formed during the Maillard reaction, particularly at high temperatures. They have been associated with increased cancer risk and other health issues. Likewise, Advanced Glycation End Products (AGEs) are compounds that can contribute to oxidative stress and inflammation, which are linked to various chronic diseases, including

diabetes and cardiovascular disease. Furthermore, the Maillard reaction can inactivate detoxifying enzymes, reducing the ability of organisms to neutralize harmful compounds (Friedman, 2003).

Regarding food/feed allergens, the Maillard reaction could alter the allergenic potential of proteins. Heat-induced denaturation could reduce the allergenicity of some proteins by altering their structure and reducing their ability to bind to IgE antibodies. Conversely, the Maillard reaction could create new allergenic epitopes (neoallergens) by forming new bonds and structures, increasing the allergenic potential of certain foods. There is a reported relationship between protein digestibility and allergenicity, and the Maillard reaction can make some proteins more resistant to digestion, potentially enhancing their ability to provoke an immune response. The impact of MRPs on allergenicity is influenced by various factors, including the type of food, the specific proteins involved, and the conditions under which the Maillard reaction occurs, such as temperature and pH. For example, high temperatures and prolonged heating can enhance the formation of AGEs, a subset of MRPs that have been linked to increased immunogenicity and allergenicity. These AGEs can interact with immune cells, potentially triggering allergic reactions (Teodorowicz et al., 2017; Gou et al., 2022).

To sum up, the Maillard reaction plays a complex role in food processing, influencing digestibility, nutritional properties, gut microbiota, toxicity, and allergenicity. While it can enhance flavours and create beneficial compounds, it can also reduce nutrient availability, form harmful substances, and alter allergenic potential. Understanding these effects is crucial for evaluating food processing methods and their effects on food/feed traits. Further research is needed to fully understand the balance of these effects and to assess the processing techniques that maximize benefits while minimizing risks.

Food matrix effects

Special mention should be made to the food matrix effects. The complexity of food matrices could influence the effect of processing on food traits. A food matrix refers to the physical and chemical environment in which food components, such as proteins, fats, carbohydrates, and micronutrients, are embedded. This environment can affect how these components interact with each other and with external factors like heat, enzymes, and pH changes during processing (McClain et al., 2014).

In a complex food matrix, proteins could interact with other macronutrients like fats and carbohydrates, as well as with micronutrients and bioactive compounds. These interactions could alter the structure and stability of proteins, potentially affecting their digestibility, nutritional value or toxic/allergenic properties. For example, proteins bound to fats may be more resistant to denaturation during heating, affecting their allergenic potential. Furthermore, different processing methods can have varied effects on allergenicity depending on the food matrix. Heating can cause proteins to denature and aggregate, which might reduce their solubility and alter their allergenic properties. However, in a complex matrix, these changes might also be mitigated or enhanced by the presence of other components. Similarly, the composition and structure of the food matrix can affect how easily nutrients are broken down and absorbed in the gastrointestinal tract, with factors like the physical form of food and the presence of dietary fibers playing crucial roles (Lund and Ray 2017; Nursten 2005; Teuber, 2002; Akkerdaas et al., 2022).The food matrix also affects the presence and

impact of toxic compounds, influencing the formation of harmful substances during processing and modulating the absorption of toxins (González-Mulero et al., 2021; Lund and Ray 2017). Understanding these interactions would be essential for evaluating the food processing effects and minimize risks.

Regarding analytical challenges and methodologies used in the experimental studies, the complexity of food matrices poses challenges for analytical methods used to assess digestibility, nutritional composition, toxicity or allergenicity (Zheng and Xiao, 2022). In particular, for allergenicity, standard extraction methods may not efficiently isolate all allergenic proteins, especially those that are tightly bound within the matrix. This can lead to underestimation of allergenic potential in processed foods (EFSA Panel NDA, 2014).

In summary, food matrix may significantly influence the digestibility, nutritional properties, and potential toxicity of proteins, as well as the effect of the different processes/technologies. In this sense it should be highlighted that the simplified models used in studies (flours, protein hydrolysates or isolated proteins) may not accurately represent these complex interactions, which might lead to incomplete or misleading results.

3 Conclusions

The Scoping Review, online survey and stakeholder workshop allowed the creation of a repository of novel protein-containing food and feed products including: (a) the composition of 97 NFs, nine UF categories and food and feed products derived from the eight main crops, (b) a flowchart depicting the corresponding production process(es) and (c) a table including the operational conditions for each processing step, when available (Objectives 1 and 2).

Comparison of the maps constructed in Objectives 1 and 2 with the OECD consensus documents for each crop allowed the identification of more than 40 new food and feed products (and their corresponding production processes) that might be considered in addition to those included in the OECD consensus documents (Objective 3).

The main conclusions from the systematic review are presented below, in relation to the impact of processing on protein safety concerning toxicology, immunotoxicity, allergenicity, nutrition, digestibility, and the gut microbiota of the host for these novel protein sources (Objective 4):

-Certain processes/technologies, such as thermal treatments, fermentation, enzymatic hydrolysis, and germination, can significantly enhance protein digestibility across various food and feed matrices, although some exceptions have also been reported. Overall, their effectiveness varies based on the food category/formulation, processing method, and treatment parameters/conditions. Non-thermal technologies and drying methods can also improve protein digestibility, but more research is needed to validate their effects, especially regarding innovative methods like supercritical fluid extraction.

-The impact of thermal treatments on the nutritional properties of legume seeds, cereals, and insects varies depending on temperature, time, and processing method. While methods like autoclaving and boiling can enhance digestibility, excessive heat (e.g., frying) may reduce protein quality due to reactions like the Maillard reaction. Fermentation, ensiling, and

extraction processes have been shown to improve nutritional quality in various products (e.g., insect flour, forages, brewing by-products).

-Thermal treatment can reduce the activity of the newly expressed proteins (NEPs) from GM crops. However, further research is needed since the effect of other technological processes is hardly reported.

-The data gathered indicate that heating often reduces IgE reactivity in plant-based proteins but has varying effects on insects. Additionally, enzymatic hydrolysis can reduce IgE reactivity in plant-based proteins, insects, and crustaceans, although its effectiveness depends on the specific protein and conditions.

In any case, these general conclusions, and specially making extrapolations from them, should be approached with caution due to various factors: i) the food matrix, which may significantly influence digestibility, nutritional properties, and potential toxicity of proteins, as well as the impact of processing ii) the physiological differences across species, which can influence the effect of processing on protein safety, and iii) the limitations of the studies consulted to predict human allergic responses.

Finally, it should be noted that, in some cases, the information gathered was insufficient to draw solid conclusions, such as on the effect of processing on protein safety regarding the gut microbiota of the host or the effect of treatments others than heating on protein toxicity.

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Abbreviations

ADC	Apparent digestibility coefficients
AGEs	Advanced glycation end products
AK	Arginine-kinase
ANF	Antinutritional factors
AP	Alternative Processes. In the context of this project: processes not reported in the OECD consensus documents but leading to crop products already included in them.
ATFD	Agitated thin film drying
CP	Cold Plasma
DDGS	Distillers dried grains with solubles
DH	Degree of hydrolysis
DM	Dry matter
DMO	Dicamba mono-oxygenase
EC	Exclusion criterium/criteria
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assays
EU	European Union
FD	Freeze-drying
GIT	Gastro-Intestinal Tract
GM	Genetically Modified
GMO	Genetically Modified Organisms
HHP	High Pressure Processing
HRP	Peroxidase from horseradish
IgE	Immunoglobulin E
IVDMD	*in vitro* degradability of dry matter
IVDOM	*in vitro* degradability of organic matter
IVPD	*in vitro* protein digestibility
LMW	Low molecular weight
NDA	Novel Foods and Food Allergens
NEP	Newly expressed proteins
NF	Novel Foods
NP	New Products. In the context of this project: crop products not reported in the OECD consensus documents.

OD	Oven drying
OECD	Organisation for Economic Co-operation and Development
PEF	Pulsed Electric Field
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
SCFE	Supercritical fluid extraction
SR	Systematic Review
TM	Tropomyosin
TRL	Technology Readiness Level
TYR	Tyrosinase
UF	Unconventional Feed
US	Ultrasound
UV	Ultraviolet
VOD	Vacuum oven
WoS	Web of Science

List of Appendixes and Annexes to this report

Appendix A – Workshop Program

Appendix B – Scoping review keywords and search strings

Appendix C1 – Scoping review: List of included scientific documents

Appendix C2 - Scoping review: List of excluded scientific documents

Appendix D1 – Systematic review: List of included scientific documents

Appendix D2 – Systematic review: List of excluded scientific documents

Appendix E – Systematic review data extraction form

Appendix F – Systematic review risk of bias assessment

Appendix G – Objective 3 Supplementary Material

Annex A1 - Repository of (GM) crops production processes

Annex A2 - Repository of Novel Food production processes

Annex A3 - Repository of Unconventional Feed production processes

Annex A4 - List of terms found/glossary