# Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of microorganisms obtained through synthetic biology

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# Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of microorganisms obtained through synthetic biology

EFSA Scientific Committee,
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## Abstract

EFSA was asked by the European Commission to evaluate synthetic biology (SynBio) developments for agri-food use in the near future and to determine whether or not they are expected to constitute potential new hazards/risks. Moreover, EFSA was requested to evaluate the adequacy of existing guidelines for risk assessment of SynBio and if updated guidance is needed. The scope of this Opinion covers food and feed risk assessment, the variety of microorganisms that can be used in the food/feed chain and the whole spectrum of techniques used in SynBio. This Opinion complements a previously adopted Opinion with the evaluation of existing guidelines for the microbial characterisation and environmental risk assessment of microorganisms obtained through SynBio. The present Opinion confirms that microbial SynBio applications for food and feed use, with the exception of xenobionts, could be ready in the European Union in the next decade. New hazards were identified related to the use or production of unusual and/or new-to-nature components. Fifteen cases were selected for evaluating the adequacy of existing guidelines. These were generally adequate for assessing the product, the production process, nutritional and toxicological safety, allergenicity, exposure and post-market monitoring. The comparative approach and a safety assessment per se could be applied depending on the degree of familiarity of the SynBio organism/product with the non-genetically modified counterparts. Updated guidance is recommended for: (i) bacteriophages, protists/microalgae, (ii) exposure to plant protection products and biostimulants, (iii) xenobionts and (iv) feed additives for insects as target species. Development of risk assessment tools is recommended for assessing nutritional value of biomasses, influence of microorganisms on the gut microbiome and the gut function, allergenic potential of new-to-nature proteins, impact of horizontal gene transfer and potential risks of living cell intake. A further development towards a strain-driven risk assessment approach is recommended.



**Keywords:** agri-food use, food, feed, genetically modified microorganism (GMM), guidance, risk assessment, synthetic biology

**Requestor:** European Commission

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## Summary

Synthetic Biology (SynBio) is an interdisciplinary field at the interface of molecular engineering and biology, aiming to develop new biological systems and impart new functions to living cells, tissues and organisms. SynBio microorganisms (SynBioM) have potential applications in agri-food systems, requiring a pre-market authorisation in the European Union (EU). This implies an EFSA risk assessment in the context of applications for Feed additives, Food additives, enzymes and flavourings, Novel Foods, Health claims, Feed material, Food, Plant protection products and Plant biostimulants. Four categories of products have been defined by EFSA: Categories 1 and 2 are products free from DNA and cells (e.g. amino acids, vitamins, enzymes), Category 3 are products containing DNA but no viable cells (i.e. biomass) and Category 4 are products containing viable cells (e.g. probiotics, food starter cultures).

This Opinion addresses four Terms of Reference (ToRs) requested by the European Commission on the food and feed risk assessment of SynBioM and complements the EFSA Scientific Committee's Opinion on microbial characterisation and environmental risk assessment (EFSA Scientific Committee, 2020): (1) identification of sectors/advances in the agri-food system considered among SynBioM developments (excluding bioremediation, de-extinction, bioweapons/biopreparedness, medical use, biofuels); (2) identification of potential risks and potential novel hazards that SynBioMs could pose for humans and animals (farmed and pets); (3) evaluation of the adequacy of existing guidelines for risk assessment of current and near-future SynBioMs arriving in the EU market in the next decade and expected in the wider future (xenobiology); and (4) identification of specific areas for which updated guidance is needed.

As a first step, existing guidelines for food and feed risk assessment from various domains were collected and evaluated for relevance for SynBioMs. Second, the existing guidelines were challenged towards a selection of 15 cases representing products that could reach the market in the next decade (and some for the wider future). These cases are representative for the variety of microorganisms that can be used (bacteria, fungi, viruses, bacteriophages and microalgae) and for the whole spectrum of techniques that can be used for SynBio as there are no clear criteria for differentiating between a genetically modified microorganism (GMM) and a SynBioM. A variety of exposure routes to humans and animals and a variety of phenotypic traits were covered by the selected cases, to challenge the existing guidelines that have been developed so far within EFSA and other organisations. As a third step, an overall gap analysis was performed capturing gaps disconnected from the existing guidelines and from the selected cases. EFSA consulted EU Member States and interested parties during a public consultation and addressed the comments received.

### ToR1: Identification of sectors in the agri-food system considered among SynBioM developments

No other sectors/advances were identified in addition to the six identified by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), Scientific Committee on Consumer Safety (SCCS) and Scientific Committee on Health and Environmental Risks (SCHER): (1) genetic part libraries and methods; (2) minimal cells and designer chassis; (3) protocells and artificial cells; (4) xenobiology; (5) DNA synthesis and genome editing; and (6) citizen science. There are no clear criteria to differentiate between a GMM and a SynBioM. Cases 1 and 4–15 are part of a continuum between classical GMM and SynBioM. From a technical point of view, there are SynBioM applications that could be ready for food and feed use in the EU in the next decade. However, xenobionts, falling within the remit of EFSA, are not expected for practical application in the next decade. Information on new SynBioM products may not be made publicly available, which limits the predictive capacity of this Opinion.

### ToR2: Identification of potential risks and potential new hazards SynBioMs could pose related to their food and feed use

Unusual and/or new-to-nature components (e.g. xenobiotic nucleic acid (XNA), xenoproteins) may include potential concerns regarding their presence, their stability and/or their potential degradation into harmful metabolites, may trigger concern for allergenicity, may cause imbalanced nutrition (e.g. by altering bioavailability) and may cause an adverse effect on the gut environment.

### ToR3: Evaluating the adequacy of existing guidelines for risk assessment of current and near-future SynBioMs

The existing Guidances are generally adequate for assessing the SynBioM products, the production process and the product preparation process, the detection of viable cells and DNA, the nutritional assessment, the toxicity assessment, the evaluation of allergenicity, the calculation of exposure and the post-market monitoring (PMM). Existing guidance describes well the principles of the comparative approach that is also applicable for SynBioM. The use of a comparator in the risk assessment is adequate for those SynBioM (Categories 1–4) with sufficient familiarity to the non-GM counterpart. SynBioM producing new-to-nature products and xenobionts would require a safety assessment per se for the new-to-nature components. In cases when the parental organism of the GMM does not have a history of use in the particular application, conventional food products may still be used as comparators to identify possible compositional changes and to assess their safety implications. Relying on the Qualified Presumption of Safety (QPS) status for the safety assessment of building blocks of SynBioM is valid when there is sufficient familiarity of the SynBioM/chassis with the QPS microorganism.

### ToR 4: Identification of specific areas for which updated guidance is needed

Updated guidance is recommended:

- For the production process of xenobionts containing XNA and/or producing xenoproteins and for non-GM, GM and SynBio protists/microalgae and bacteriophages; for bacteriophages, also the possible formation of phages with transducing properties of genes coding for virulence factors and toxins.
- For the confirmation of the purity of the product, on the detection of non-GM, GM and SynBio bacteriophages, protists/microalgae, XNA and/or xeno amino acids.
- For the assessment of bacteriophages on the gut microbiome.
- For toxicological safety assessment for non-GM, GM and SynBio bacteriophages; especially for those propagated in pathogens. For oral exposure: assessment of non-GM, GM and SynBio plant protection products and biostimulants. Specific indications for tolerance and efficacy in insects as target species of feed additives should be developed for non-GMM, GMM and SynBioM.
- For PMM of potential adverse effects of microorganisms (non-GMM, GMM and SynBioM) future updates would benefit from including descriptions of fit-for-purpose approaches and future updates may expand on bacteriophages.

Development of risk assessment tools is recommended:

- For assessing microbial persistence and colonisation as well as the potential overgrowth/ disturbance of the microbiome balance and gut function and for defining healthy baseline endpoints for interpretation of the results.
- For assessing the impact of horizontal gene transfer (HGT) of sequences of concern.
- For assessing the allergenic potential of new-to-nature proteins.
- For assessing bioavailability of non-GMM, GMM or SynBioM biomasses used for food and feed and for the nutritional assessment of new-to-nature products.

General recommendations:

- The EFSA Scientific Committee recommends a concerted international effort on developing internationally agreed guidance and harmonised frameworks for identifying and addressing living cell intake in the risk assessment process.
- Continuation of research on testing methods for risk assessment, including 'omics' high-throughput experimental studies, and the application of bioinformatics tools.
- As the technique-driven risk assessment has limitations especially for the assessment of SynBioM, a strain-driven approach can be envisaged for all future SynBioM assessments.
- As a way to reduce the amount of data and studies required for the RA of SynBioM and their products, applicants should be encouraged to include food and feed safety aspects throughout the SynBio design.

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# 1. Introduction

Synthetic Biology (SynBio) is an interdisciplinary field at the interface of molecular engineering and biology, aiming to develop new biological systems and impart new functions to living cells, tissues and organisms. SynBio is not a technique, or a combination of techniques, rather a process or strategy comprising also theoretical–experimental approaches. It employs engineering principles such as standardisation, modularity, modelling and computer-aided design to improve the predictability of the bioengineering process and achieve the desired characteristics of the product (EFSA GMO Panel, 2021). By combining molecular engineering, life sciences and computational modelling, SynBio is expanding the range of applications and products that are being developed.

The principles of standardisation and modularity facilitates the engineering process and iterative engineering cycles of 'design–build–test–learn'. So, by bridging engineering, life sciences and computational modelling, the range of applications and products that can be developed expands and the predictability of biotechnological design is improved.

SynBio has potential applications in the food and feed chain that would require under current legislation a pre-market authorisation in Europe. Some of those applications may include the use of engineered organisms and/or their products into the food and feed chain (Figure 1).

The European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Consumer Safety (SCCS) had previously published[1] three Opinions (SCENIHR, SCCS, SCHER, 2014, 2015a,b) on SynBio, addressing six SynBio developments: (1) genetic part libraries and methods; (2) minimal cells and designer chassis; (3) protocells and artificial cells; (4) xenobiology; (5) DNA synthesis and genome editing; and (6) citizen science (Do-It-Yourself Biology). The Opinions addressed the definition of SynBio, risk assessment methodologies and safety aspects, risks to the environment and biodiversity and research priorities in the field of SynBio. The non-food Scientific Committees concluded that new SynBio applications may be assessed using current risk assessment methodology for genetically modified organisms (GMOs). However, the rapidly evolving technologies may require existing methodologies to be revisited at regular intervals and improved when necessary to continue ensuring safety.

Therefore, as a proactive measure, the European Commission requested EFSA for an opinion on GMOs developed using SynBio approaches and the implications, if any, for risk assessment methodologies. EFSA identified a total of six work packages (WP) to be reflected in the development of six Opinions, according to organism group and risk assessment aspects (see Section 1.3). In this context, the Scientific Committee adopted in 2020 a Scientific Opinion evaluating the SynBio developments in microorganisms for deliberate release into the environment and the adequacy of existing guidelines for molecular characterisation (MC) and environmental risk assessment (ERA) (EFSA Scientific Committee, 2020). For plants obtained through SynBio, the GMO Panel also adopted its opinion on the adequacy of existing guidelines for MC and ERA and to determine if updated guidance is needed (EFSA GMO Panel, 2021).

## 1.1. Definitions for SynBio for the Terms of Reference

SynBio has been previously defined as follows by the joint SCENIHR, SCCS and SCHER committees upon request of the European Commission[1]: 'Synthetic biology is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in viable organisms'. Similar to the EFSA Scientific Opinions addressing WP1 [MC and ERA SynBio microorganisms] and WP2 (MC and ERA SynBio plants), this definition is used as a starting point for the present Opinion due to the request of the European Commission to build on the three Opinions of SCENIHR, SCCS, SCHER (2014, 2015a,b).

The Convention on Biological Diversity[2] further clarified that 'While there is no internationally agreed definition of 'synthetic biology', key features of SynBio include the 'de novo' synthesis of genetic material and an engineering-based approach to develop components, organisms and products'. This

[1] SCENIHR, SCCS, SCHER (2014) Synthetic Biology I Definition, Opinion, September 2014. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_044.pdf; SCENIHR, SCCS, SCHER (2015) Synthetic Biology II – Risk assessment methodologies and safety aspects, Opinion, May 2015. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_048.pdf; and SCENIHR, SCCS, SCHER (2015) Synthetic Biology III – Research priorities, Opinion, December 2015. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_050.pdf

[2] https://www.cbd.int/doc/meetings/cop/cop-12/information/cop-12-inf-11-en.pdf

further clarification established the link for the request in the ToRs to EFSA to support the EU in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety (Cartagena Protocol, 2000/2003).

Further background and technical specificities on SynBio are provided in Section 3 of the Opinion on SynBioM (MC and ERA) (EFSA Scientific Committee, 2020).

## 1.2. Background and Terms of Reference as provided by the requestor

Building on SCENIHR, SCCS, SCHER (2014, 2015a,b) and taking into account available literature and previous analyses carried out by EU Member States or at the international level, the Commission asked EFSA,[3] in accordance with Article 29(1) of Regulation (EC) No 178/2002, for an opinion on GMOs developed through SynBio and its implications for risk assessment methodologies. The scope of the present mandate is limited to agri-food uses.[4] In this context:

1) EFSA was asked to consider whether and which newer sectors/advances should be considered among SynBio developments, in addition to the six identified by the SCs (ToR1).
2) EFSA was requested to identify, if possible, potential risks in terms of impact on humans, animals and the environment that current and near-future SynBio developments could pose; in this respect EFSA was also asked to identify potential novel hazards compared with those of established techniques of genetic modification[5] (ToR2).
3) EFSA was requested to determine whether the existing guidelines for risk assessment are adequate and sufficient for current and near-future SynBio developments or whether there is a need for updated guidances (ToR3).
4) In the latter case EFSA was requested to identify the specific areas for which such updated guidances are needed (ToR4).

EFSA was also requested to provide technical and scientific expertise on risk assessment of GMOs obtained through SynBio to support the EU in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety (2000/2003).

The mandate received from the EC was split in six WPs by EFSA to be reflected in six Opinions:

1) Microbial characterisation and ERA of GMM developed through SynBio (EFSA Scientific Committee, 2020).
2) Molecular characterisation and ERA of GMP developed through SynBio (EFSA GMO Panel, 2021).
3) Food and feed risk assessment of GMM developed through SynBio (current opinion).
4) Food and feed risk assessment of GMP developed through SynBio (ongoing).
5) Molecular characterisation and ERA of GM animals developed through SynBio.
6) Food and feed risk assessment of GM animals developed through SynBio.

The current opinion addresses WP3 and it is intended to complement the Scientific Committee (SC) Opinion on MC and ERA of SynBio Genetically Modified microorganisms (EFSA Scientific Committee, 2020).

## 1.3. Interpretation of the Terms of Reference

The following interpretations of the ToRs were previously made in agreement with the European Commission[6] (EFSA Scientific Committee, 2020). This interpretation is considered applicable to this Opinion with an adjustment for ToR2 in line with the food/feed scope of this Opinion:

- Not all of the six developments previously identified by the SCs were considered relevant: citizen science was excluded as a concept not being linked to a technique or a product.
- 'Near future': for this mandate, this is interpreted as having the potential of reaching the EU market within the next decade. This is reflected in Section 2.5 when selecting 13 out of 15

[3] See mandate M-2018-0205 in https://open.efsa.europa.eu/questions/EFSA-Q-2021-00052
[4] For the purpose of this mandate agri-food uses means agri/food/feed products falling within the remit of EFSA.
[5] For the purpose of this mandate the term 'established techniques of genetic modification' refers to various genetic engineering techniques that have been significantly used over the last 30 years to produce genetically modified organisms, such as those that have been authorised under Directive 2001/18/EC and Regulation (EU) No 1829/2003.
[6] See correspondence under mandate M-2018-0205 in the EFSA register of questions: https://open.efsa.europa.eu/questions/EFSA-Q-2020-00768

case studies. Due to the specific biology of microorganisms, the wide variety of organisms that can be used (including viruses and algae) and the fast research development in this field, two case studies for xenobiology were included which are expected in the wider future.
- 'Agri-food uses': on footnote 5 of the mandate 'For the purpose of this mandate agri-food uses means agri/food/feed products falling within the remit of EFSA', further clarification was needed to determine which applications fell within the remit of EFSA, within this mandate and within the available time frame. The limited time frame led to the explicit exclusion of bioremediation applications from this mandate. By extrapolation, the following applications are also excluded from this mandate: de-extinction, bioweapons/biopreparedness, medical use and biofuels.
- For this Opinion, ToR2 is focused on the evaluation of potential hazards and risks for humans and animals (farmed and pets) that could be posed by food and feed from microorganisms obtained through current and near-future SynBio approaches. The MC and ERA were addressed in WP1 and the published Opinion (EFSA Scientific Committee, 2020), see Section 1.4.
- 'Existing guidelines for risk assessment': see Section 2.3.
- The terms 'Adequate' or 'Not fully adequate' were used in this opinion. If not fully adequate, this means that the guidance is considered not sufficient and, accordingly, needs for updates were indicated. In some situations, guidance is also "not applicable".

This Opinion is produced not only to support the European Commission, but is also meant for the public, scientific community, stakeholders, companies and institutions, interested in or dealing with safety of SynBioM developments, all of which were given the opportunity to provide comments on the final draft opinion during the public consultation (see Section 2.2).

### 1.4. Summary of the previous opinion on MC and ERA of SynBioM (EFSA Scientific Committee, 2020)

A horizon scan showed that SynBioM applications could be ready for deliberate release into the environment of the EU in the next decade. However, xenobionts are only expected in the wider future. For the MC and the ERA, the existing EFSA guidances are useful as a basis. The extent to which existing guidances, which are based on the comparative approach, can be used depends on the familiarity of the SynBioM with non-modified organisms. Among the recommendations for updated guidance, are the range of uses of products to be assessed covering all agri-food uses and taking into account all types of microorganisms, their relevant exposure routes and receiving environments. It is suggested that new EFSA guidances for microorganisms address all 'specific areas of risk' as per European Commission Directive 2001/18/EC (European Commission, 2001a). No novel environmental hazards are expected for current and near-future SynBioMs. However, the efficacy by which the SynBioMs interact with the environment may differ. This could lead to increased exposure and risk. Novel hazards connected with the development of xenobionts may be expected in the wider future.

## 2. Data and methodologies

### 2.1. Ad hoc expert Working Group and its methodology

EFSA established an ad hoc expert Working Group of the Scientific Committee for the development of this Opinion on food and feed safety aspects of SynBioMs [from this point onwards referred to as the SynBioM FF Working Group (WG)].[7] In delivering its Scientific Opinion, the Scientific Committee, together with the SynBio FF WG, considered:

- the current legislation and corresponding (EFSA) guidance documents (see Section 2.3 and Table 2);
- the use applications of SynBioM that fell within the remit of EFSA (see Section 2.4);
- available published information for the identification of relevant case studies (see Section 2.5 and Table 3).

The WG has adopted a methodology based on a three-phase approach, as represented in Table 1.

[7] https://ess.efsa.europa.eu/doi/doiweb/wg/685310

**Table 1:** Methodology of the SynBioM FF WG to address the Terms of Reference of the mandate

| | Phase 1 evaluation | Phase 2 evaluation | Phase 3 evaluation |
|---|---|---|---|
| **Aim** | ToRs 2 and 3: Evaluation, section by section, of the adequacy and applicability of available risk assessment approaches for current and near-future SynBioM developments and to identify potential new hazards | ToRs 2 and 3: Test the adequacy of existing guidance documents in a realistic/most relevant scenario and identify potential new hazards | ToR4: Perform an overall gap analysis that could not be captured by the previous phases |
| **Approach** | Analysis of existing EFSA guidances and underlying EU legislation, having a broad spectrum of microorganisms and applications in mind | Identification and selection of case studies focusing on the current status of SynBioM developments. In total, 15 cases were selected to challenge the existing guidelines and to identify possible limitations | Identification of gaps disconnected from the existing guidance documents listed in Section 2.3 or disconnected from the selected cases |
| **Outcome** | Table 2 and Section 3 | Section 2.2<br>The results of Phase 2 are prevalently presented in table format after each section in Section 3. It is underlined that these findings do not represent an in-depth and comprehensive risk assessment of the (hypothetical) cases. | Outlooks for the future in Section 4 |

## 2.2. Consultations

In line with its policy on openness and transparency, EFSA consulted EU Member States and interested parties by an online public consultation. Between 19 January and 20 March 2022, stakeholders were invited to submit their comments on the draft Scientific Opinion.[8] Following this consultation process, the document was revised by the SC and the members of the SynBioM FF WG. The comments received were considered and were, when appropriate, incorporated into the current Opinion. The outcome of the public consultation is reported in detail and will be published on EFSA's website as supporting document together with the final Scientific Opinion as adopted by the Scientific Committee.

## 2.3. Existing guidances and guidelines checked in this Opinion

The WG retrieved to the best of its knowledge all the possible relevant EFSA guidance and statement documents (including the former guidance documents), along with the current legislation and other international recognised guidance documents and guidelines until December 2021. These are presented in Table 2 and it is noted that some of these guidances might meanwhile been updated. For the development of this Opinion, the guidance documents and guidelines presented in Table 2 were screened for their scientific adequacy and sufficiency for the food and feed risk assessment of applications using current and near-future SynBioM developments. A total number of 30 documents was screened for adequacy for this Opinion. How their content has been used in this Opinion is commented in the column 'content' of Table 2. In total, 20 reference documents were taken into consideration for the assessment, with the most prominent ones in bold. Ten documents, which are in italics in Table 2, were not further used for the assessment and/or superseded by more recent guidances.

[8] https://open.efsa.europa.eu/consultation/a0c7U000000IBr0QAG

**Table 2:** Reference documents per sector that have been checked for relevancy for this Opinion, and for adequacy regarding the food/feed safety evaluation of SynBioMs

| Document no. | Reference document per sector in the remit of EFSA | Content explaining the relevance for the present Opinion |
|---|---|---|
| | **GMO** | |
| **1** | **EFSA GMO Panel, 2011 – Guidance on the risk assessment of GMMs and their products intended for food and feed use** | Focuses on the risk assessment of food and feed consisting, containing or produced from GMMs. It includes FF assessment. The aspects on the molecular characterisation of GMMs in this guidance are superseded by the FEEDAP guidance (EFSA FEEDAP Panel, 2018a) and the Statement of the CEP Panel (EFSA CEP Panel, 2019) replaced by the guidance of the EFSA CEP Panel (2021) |
| **2** | EFSA GMO Panel, 2017 – Guidance on allergenicity assessment of genetically modified plants | Provides supplementary guidance on specific topics for the allergenicity risk assessment of genetically modified plants. The topics addressed are non-IgE-mediated adverse immune reactions to foods, *in vitro* protein digestibility tests and endogenous allergenicity |
| | **FEED** | |
| **3** | **EFSA FEEDAP Panel, 2018a – Guidance on the characterisation of microorganisms used as feed additives or as production organisms** | Details the steps for characterisation of microorganisms (including GMMs) used as feed additives or as production organisms and introduced the Whole Genome Sequencing (WGS) analysis for RA for the first time. MC is however already addressed in Opinion 1. The sections regarding the use of WGS data for the characterisation and risk assessment have been updated by the EFSA statement (number 16 of this table) |
| **4** | EFSA FEEDAP Panel, 2018b – Guidance on the assessment of the efficacy of feed additives | Details specifically the assessment of the efficacy of feed additives for use in animal nutrition |
| **5** | EFSA FEEDAP Panel, 2017a – Guidance on the safety of feed additives for consumers | Comprises ADME (absorption, distribution, metabolism, and excretion) and toxicological studies of the FEED additive in the animal with a specific focus on metabolites that can affect the consumer |
| **6** | EFSA FEEDAP Panel, 2017b – Guidance on the assessment of the safety of feed additives for the target species | Details the *in vivo* toxicological studies with multilevel feed additives and its tolerance to and effects on the target animal species (Sections 4, 5 and 6) |
| **7** | EFSA FEEDAP Panel, 2017c – Guidance on the identity, characterisation and conditions of use of feed additives | Covers the identity, characterisation and conditions of use of the additives, including a section on the production process (Section 2.3) |
| **8** | EFSA FEEDAP Panel, 2012a - Guidance on studies concerning the safety of use of the additive for users/workers | Provides guidance on how to conduct studies concerning safety for the user/workers: toxicology, effects on respiratory system, effects on eyes and skin (relevant for the allergenicity section), systemic toxicity and exposure assessment |
| **9** | EFSA FEEDAP Panel, 2021a – Guidance on the renewal of the authorisation of feed additives | Provides guidance on the principles of the assessment of applications for renewal of the authorisation, including the post-market monitoring section |
| ***10*** | *EFSA FEEDAP Panel, 2008a – Technical guidance: Compatibility of zootechnical microbial additives with other additives showing antimicrobial activity* | *Checked for adequacy; superseded by the FEEDAP guidances (EFSA FEEDAP Panel, 2017b, 2018a); addresses efficacy (compatibility in the gut) but does not address safety, therefore is not relevant for this Opinion* |
| ***11*** | *EFSA FEEDAP Panel, 2008b – Technical guidance microbial studies* | *Checked for adequacy; superseded by the FEEDAP guidance (*EFSA FEEDAP Panel, 2018a*) and no longer used in detail for this Opinion* |

| | | |
|---|---|---|
| ***12*** | *EFSA FEEDAP Panel, 2011 – Guidance on the assessment of microbial biomasses for use in animal nutrition* | *Checked for adequacy; superseded by the FEEDAP guidance (EFSA FEEDAP Panel, 2018a) and no longer used in detail for this Opinion* |
| | FOOD improvement | |
| **13** | **EFSA CEP Panel, 2021 – Guidance for the submission of dossiers on food enzymes** | Updates the former guidance on the Submission of a Dossier on food enzymes (EFSA CEF Panel, 2009) taking into account the statements issued on the exposure assessment of food enzymes and the characterisation of microorganisms used in the production of food enzymes (EFSA CEP Panel, 2019) |
| **14** | EFSA FAF Panel, 2021 – Scientific guidance for the preparation of applications on smoke flavouring primary products | Focuses on toxicology and also covers the assessment of potential immunotoxicity |
| **15** | EFSA ANS Panel, 2012 – Guidance for submission for food additive evaluations | Covers exposure toxicokinetics, toxicity, reproductive and developmental toxicity, neurotoxicity. This guidance was meanwhile updated and republished in 2021 (https://doi.org/10.2903/j.efsa.2012.2760). |
| ***16*** | EFSA, 2021 – *Statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain* | *Provides recommendations on the analysis and results of WGS analysis of microorganisms, which should be provided to EFSA in the context of an application. Information on this Statement is included in the guidance of the* EFSA CEP Panel *(2021) and as such this statement per se is no longer used in detail for this Opinion* |
| ***17*** | *EFSA CEF Panel, 2009 – Guidance on the Submission of a Dossier on food enzymes for safety evaluation* | *Provides the requirements for a dossier submission on food enzymes for safety evaluation, including a section on the manufacturing process (Section 1.2) and the toxicological data needed (Section 2). This guidance is superseded by the guidance of the* EFSA CEP Panel *(2021)* |
| ***18*** | EFSA CEP Panel, 2019 – *Statement: Characterisation of microorganisms used for the production of food enzymes* | *Covers production organisms only and is focused on food enzyme applications. This statement was fully addressed in Opinion 1 and is superseded by the guidance of the* EFSA CEP Panel *(2021)* |
| | **BIOHAZARDS/Food Improvement** | |
| **19** | EFSA BIOHAZ Panel, 2010 – Guidance document on the submission of data for the evaluation of the safety and efficacy of substances for the removal of microbial surface contamination of foods of animal origin for human consumption | Mentions in a general way the need for information on the identity of the substance, the manufacturing process and the toxicological potential; allergenicity is not mentioned in the guidance. This document has meanwhile been amended and republished in 2021 (https://doi.org/10.2903/j.efsa.2010.1544) |
| | **NUTRIENTS AND DIETARY PRODUCTS** | |
| **20** | EFSA NDA Panel, 2016a – Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283 | Provides the requirements for an application of a novel food, including the characterisation of microorganisms, fungi and algae (Section 2.2.3), the production process (Section 2.3), the ADME (Section 2.8), the nutritional (Section 2.9) and the toxicological (Section 2.10) assessment. This document has meanwhile been republished in 2021 (https://doi.org/10.2903/j.efsa.2021.6555). |
| **21** | EFSA NDA Panel, 2016b – Guidance on the scientific requirements for health claims related to the immune system, the gastrointestinal tract and defence against pathogenic microorganisms | Focuses on health claims (beneficial effects) which is by definition not a safety assessment. However, the guidance is informative on methods for how to assess/how to test possible clinical endpoints or parameters that maybe useful as intermediate indicators for adversity. |

| | | |
|---|---|---|
| **22** | EFSA NDA Panel, 2014 – Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes | Reviews the database on food allergens and the methods for establishing the potential for food to cause allergic reactions |
| | **PLANT PROTECTION PRODUCTS** | |
| **23** | Regulation (EU) No 283/2013, Annex part B on microorganisms as plant protection products (PPP), Setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of PPPs on the market | Provides data requirements for active substances consisting of microorganisms, including viruses e.g. in Chapters 5 and 6 data are requested on the effects on human health and on residues in or on treated products, food and feed |
| **24** | Regulation (EU) 2019/1009 – New regulation laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003 | Regulation including provisions for the use of (non-GM) microorganisms in the EU and their assessment. This legal framework may be relevant for assessing translocation of the microorganisms and its metabolites to the edible plant parts leading to human exposure through the oral route |
| ***25*** | *OECD, 2019 – Report of the 9th Biopesticides Expert Group Seminar on Test Methods for Microorganisms Series on Pesticides No. 100 (18 June 2018 in Paris, France)* | *Outcome discussion identifying the need to: (i) update the requirements for the registration of microbial biopesticides, (ii) revise existing methods for testing microbials, and (iii) develop more suitable alternative methods. Current methods were classified as technically challenging, relevancy of the administration route debated, appropriateness of the animal model used was questionable and the interpretation of the results deemed complex* |
| ***26*** | *OECD, 2020c – Report of the 10th Biopesticides Expert Group Seminar on Test Methods for Microorganisms Series on Pesticides No. 103 (24 June 2019 in Paris, France)* | *Outcome discussion concluded that is not yet envisioned that the use of genome sequencing, bioinformatics tools and databases would lead to new data requirements, despite possibility to use whole genome sequencing (WGS) to address data requirements for the registration of microbials, particularly for identification and characterisation. Further consideration should be given to the possibility of using WGS as a standard data requirement* |
| ***27*** | *OECD, 2010a – Guidance document on horizontal gene transfer between bacteria* | *Provides information on how to assess potential risks resulting from horizontal gene transfer (HGT)* |
| | **MEDICINAL PRODUCTS** | |
| ***28*** | *EMA, 2010 – Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines* | *Covers immunologic pharmaceuticals or vaccines on how to address these beneficial and desired effects. The potential induction of autoimmunity is mentioned without any indication how to address this. The section on safety does not specify methodological details* |
| ***29*** | EMA, 2012 – *Guideline on requirements for the production and control of immunological veterinary medicinal products* | *For veterinary vaccines, the guideline is intended to supplement Directive 2001/82/EC (European Commission, 2001b)* European Pharmacopoeia (2016a, b)*, and VICH guideline (*EMA, 2016*). The sections on safety and field trials do not specify methodological details* |
| | **FERTILISING PRODUCTS** | |
| **30** | Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products | Microbial plant biostimulants fall within the scope with 4 taxonomic families which can be used (Azotobacter spp., Mycorrhizal fungi, Rhizobium spp., Azospirillum spp). |

## 2.4. Categories of products, use applications covered in this Opinion

To obtain an overview of the SynBio developments in microorganisms (SynBioMs) that are likely to enter the market in the next decade, different use applications of SynBioMs that fall under the remit of EFSA were considered under the scope of this Opinion.

Depending on their nature and their final use, these products can be classified into Categories 1–4 as indicated by the guidance on the risk assessment of GMMs and their products intended for food and feed use (EFSA GMO Panel, 2011):

Cat 1–2: products free from DNA and cells from the production microorganism (e.g. amino acids, vitamins, and enzymes produced by microorganisms).

Cat 3: products containing the DNA of the production microorganism but no viable cells (e.g. biomasses).

Cat 4: products containing viable cells and DNA of the production microorganism (e.g. probiotics, food starter cultures).

In particular, the following applications of GMM/SynBioM as expected by the WG in the near future were considered for this Opinion and an indicative categorisation is given:

- Feed additives produced with microorganisms, such as amino acids, vitamins and enzymes (digestibility enhancers and nutritional additives), generally fall into Category 1 or 2 and feed additives containing viable microorganisms, such as silage additive or probiotics, are Category 4 products. Biomasses (considered as feed materials) consisting of, or containing, inactivated GM/SynBio microorganisms and containing recombinant DNA are Category 3.
- Food additives, Food enzymes and Food flavourings produced with GM/SynBio microorganisms generally belong to Category 1 or 2.
- Novel food produced with GM/SynBio microorganisms (not present in the final product) generally belong to Category 1 or 2.
- Food biomasses consisting of, or containing, inactivated GM/SynBioMs and containing recombinant DNA are Category 3. Viable GM/SynBioMs used in food fermentation (food starter cultures) or as food supplements (e.g. probiotics) belong to Category 4.
- Plant protection products (PPP) and plant biostimulants that may cause epiphytic and endophytic colonisation of edible plant parts. These products, being based on viable cells, are included in Category 4.
- Viable GM/SynBioM potentially covered by health claims regulation also belong to Category 4.

## 2.5. Techniques used in synthetic biology

SynBio has been previously defined as follows by SCENIHR, SCCS and SCHER (SCENIHR, SCCS, SCHER, 2014, 2015a,b) upon request of the European Commission: 'Synthetic biology is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in viable organisms'. This definition is used as a starting point for the present Opinion due to the request of the European Commission to build on the Opinions of SCENIHR, SCCS and SCHER.

Notwithstanding the definition of SynBio, in practice, as show in Figure 1 of Opinion 1 (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2020.6263), there is not a defined distinction between the micro-organisms obtained using the established genetic modification techniques and those obtained using SynBio approaches.

The level of genetic modification in SynBio agents or products might range from being very similar to the ones from GM technology assessed so far or can go (far) beyond with unfamiliar characteristics at the genotypic or phenotypic level, such as the case of xenobionts .

Directed or accelerated evolution, a widely used technique which works through mutagenesis and selection, can also be used in a SynBio design framework.

The term 'Genome editing', although indicated by SCENIHR, SCCS and SCHER as a SynBio development, is not separately addressed in this Opinion. Genome editing refers to a range of techniques that edit the genome in a targeted way by inducing (site-)specific changes with or without targeted insertion of DNA sequences (see European Commission, 2017). Although genome editing is increasingly used in SynBio, because of its capacity to 'edit' the genome in a targeted way, it only refers to some of the techniques available to produce a SynBio product as any other technique addressed in this Opinion.

## 2.6. Selection of case studies

There is no distinct borderline between the microorganisms obtained using existing genetic modification techniques and those derived from SynBio (EFSA Scientific Committee, 2020). Considering this lack of clarity, 15 case studies were selected for Phase 2 of this Opinion (Table 3). Cases 4–15 are part of a continuum between classical genetically modified and SynBio microorganisms, and Cases 1–3 are at the far end of the spectrum as being least familiar. These cases include minimal cells and xenobionts.

In search for useful cases for this Opinion, the WG queried public databases (e.g. PubMed, Scopus and Google Scholar) with strings referring to the use of SynBioM in food and feed (e.g. "synthet* biolog*" AND "food" OR "feed" AND "microorganism name"). No systematic approach was followed in view of the very specific cases the WG was searching for.

For selecting the most useful cases for this Opinion, the following criteria were used:

- different types of microorganisms;
- development has advanced with a possibility to reach the market in the next decade and wider future;
- different routes of exposure (intended use applications) and the anticipation of hazards for humans and animals;
- different techniques used and extent of genetic modification; SynBioMs can be generated by any technique of genetic modification, therefore, the cases are technique independent. The hazard or risk identification of SynBioM is based on extended molecular characterisation of the pertinent GMM/SynBioM strain and not solely on the genetic modification techniques used to obtain it. This is further explained in Section 4.2;
- major phenotypic changes or novel phenotypes.

The reason for these selection criteria is that cases resembling (in complexity) GMMs that are already being assessed by EFSA guidances, would not challenge such guidance and therefore would not be useful to identify possible needs for respective updates. For the identification of novel hazards, phenotypes are selected that have hitherto not been evaluated by EFSA but are realistic in reaching the market in the near or wider future.

**Table 3:** Selected cases that include different microorganisms, different use applications and different SynBio techniques used for the Phase 2 evaluation of this Opinion. For each case the most applicable Guidance is listed in column 2 and those form the basis for the assessment part of this opinion and the further tables therein. (a) See Section 2.4 for cathegorisation and use applications

| Case # Cat. (a) | Use application Most applicable Guidance | Reference and description | Development of GM/SynBioM |
|---|---|---|---|
| **1 Cat. 1/2** | Food enzyme Feed additive (enzymes) EFSA CEP Panel guidance, 2021; EFSA FEEDAP Panel guidances, 2017a,b, c, 2018a | Aguilar Suárez et al. (2019): The use of a *B. subtilis* 168 derivative with a 36% genome reduction for production of proteins difficult to be expressed and secreted in heterologous hosts. This strain lacks 332 prophage- and AT-rich island-encoded genes and its genome contains 2,648 genes instead of the 4,253 found in the parental strain. The pool of deleted genes contained also the secreted proteases, that limit the heterologous protein production. The production of surfactin has been radically limited, by deleting specific regulation factors. The minimised strain is able to express and secrete heterologous proteins of staphylococcal origin that cannot be obtained with the parental strain. | **Genome minimisation through deletions.** *B. subtilis* genome minimisation obtained by a series of deletion steps, by using double recombination with a non-replicative plasmid and a selection/ counter-selection strategy. Gene coding for selected proteins were inserted into the *amyE* on the chromosome, under the control of an inducible high-level expression system. Secretion was driven by the signal peptide of the *Bacillus* xylanase gene *xynA*, fused with the proteins difficult to express. |

| Case # Cat. (a) | Use application Most applicable Guidance | Reference and description | Development of GM/SynBioM |
|---|---|---|---|
| **2 Cat. 1/2** | Food additive EFSA CEP Panel guidance 2021 | Karbalaei-Heidari and Budisa (2020): Description of the incorporation of non-canonical amino acids (ncAAs) during translation of the lantibiotics, a category of antimicrobial peptides that includes also the food additive nisin (E234). Different ncAAs were incorporated in the lantibiotic amino acid sequences, e.g. thio-ether groups, alfa-hydroxy-acids, 1,3- or 1,2-aminothiol reactive groups. Some of these new peptides present an increase antimicrobial activity. The resulting organisms lack the capacity for biosynthesis of the tRNA-ncAA and would therefore be auxotrophic for them. | **Xenobiology.** New-to-nature peptides were designed and derived by the insertion of ncAAs during protein translation. The ncAAs were built in the lantibiotic sequences by the stop-codon suppression (SCS) or the selective pressure incorporation (SPI) method using new-to nature tRNA-ncAA configurations. |
| **3 Cat. 1–4** | Different use applications are possible EFSA GMO Panel guidance, 2011; EFSA CEP Panel guidance, 2021; EFSA FEEDAP Panel guidances, 2017a,b,c, 2018a; EFSA NDA Panel guidance, 2016a | Malyshev et al. (2014): Introduction of new-to-nature triphosphates (d5SICSTP and dNaMTP) into *Escherichia coli*, the accurately replication of the so created XNA (xenonucleic acids) and the acceptation of this XNA by the DNA repair pathways. The resulting bacterium is propagating stably an expanded genetic alphabet. | **Xenobiology.** New-to-nature proteins due to the design of xenonucleic acid (XNA). The resulting xenobiont with two additional base pairs would lack the capacity to synthesise the xeno nucleotides, and so their replication would depend on the addition of these two compounds. |
| **4 Cat. 3** | Food EFSA GMO Panel guidance, 2011; EFSA CEP Panel guidance, 2021 | Ding et al. (2019): Development of a genetically modified *Aspergillus oryzae* strain with improved genetic stability and a higher biomass in the endproduct, a reduced cellulose and pectinase activity and a higher and more variable content of important flavour components in the soy sauce. As the microorganisms are killed the resultant soy sauce is a Category 3 product, containing a high concentration of nucleic acids from the genetically modified *A. oryzae* strain. | **Intragenesis in a new food product from non-QPS source.** Natural mutants *A. oryzae* producing mono-nuclear spore, having genetic stability compared with the parental organism, were selected. The gene coding for the regulatory promoter CreA from *A. oryzae* was inserted into the *A. oryzae* genome. This regulatory protein affects the general cell metabolism, limiting the expression of secreted enzymes including the cellulase and pectinase. |
| **5 Cat. 3** | Feed material/Food ingredient (Biomasses) EFSA GMO Panel guidance, 2011; EFSA FEEDAP Panel guidances, 2017a,b, c, 2018a; EFSA NDA Panel guidance 2016a | Gassler et al. (2020): The metabolism of *Pichia pastoris* (reclassified as *Komagataella phaffii*), a yeast species qualified for QPS for enzyme production, was turned from heterotrophy to autotrophy, being capable of growth on $CO_2$. With the insertion of eight heterologous genes and the deletion of three native genes, methanol metabolism | **Reprogramming of metabolic engineering trough different techniques (trans-genesis/deletions).** The six introduced genes were inserted into the *Pichia* genome. The C-terminal peroxisomal-targeting signal (PTS1)25 |

| Case # Cat. (a) | Use application Most applicable Guidance | Reference and description | Development of GM/SynBioM |
|---|---|---|---|
| | | pathway was converted to a $CO_2$-fixation pathway resembling the Calvin–Benson–Bassham cycle. The resulting strain can grow continuously with $CO_2$ as the sole carbon source. Theoretically, this yeast strain can be used to produce biomass for feed and food purposes, by transforming $CO_2$ in organic matter. | was fused to each of the heterologously expressed enzymes. In this case a SynBio approach was used to reprogram the energy metabolism of a yeast |
| **6 Cat. 3** | Feed material/Food ingredient (Biomasses) EFSA GMO Panel guidance, 2011; EFSA FEEDAP Panel guidances, 2017a,b, c, 2018a; EFSA NDA Panel guidance, 2016a | Sun et al. (2018): Advancements and potential of cyanobacterial chassis for the production of chemicals and biomass for biofuels (eventually also to be used as food and feed source). Cyanobacteria as *Synechococcus* spp., *Synechocystis* spp., *Anabaena* spp. are studied as model organisms. The interest in cyanobacterial chassis is based on their capability to directly use sunlight and $CO_2$ as the sole energy and carbon sources for biomass production. | **Chassis concept and targeted mutagenesis.** Although SynBio tools are not yet evolved as far as for the other bacterial and yeast chassis, the tools for genetic modifications to tune gene expression, carbon flux re-direction and genome-wide manipulations are increasingly developed for cyanobacteria with promoters, riboswitches, ribosome binding site engineering, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 systems, small RNA regulatory tools and genome-scale modelling strategies. |
| **7 Cat. 4** | Feed additives, Food EFSA GMO Panel guidance, 2011; EFSA CEP Panel guidance, 2021; EFSA FEEDAP Panel guidances, 2017a,b,c, 2018a; EFSA NDA Panel guidance, 2016a | Son et al. (2020): Description of the use of genome editing to modulate the biosynthesis of riboflavin in *Leuconostoc citreum* (a hetero-fermentative lactic acid bacterium widely used in food fermentations). The expression levels of two genes (*ribF* and *folE*) were downregulated (3.3-fold and 5.6-fold decreases, respectively) and, in addition, the co-expression of the rib operon was introduced. All in all, the production of riboflavin increased more than 1.5 times compared with the unmodified strain. | **Design of metabolic pathway via CRISPR interference system technology.** The article reports the development of a CRISPR interference (CRISPRi) system for engineering the expression of a bioactive compound in this microbial species. The system used to down-regulate the expression of two genes involved in the riboflavin pathway was the synthetic single guide RNA (sgRNA) and the deactivated Cas9 of *Streptococcus pyogenes* (SpdCas9) constructed in a bi-cistronic design (BCD) expression platform using a high-copy-number plasmid. Co-expression of the rib operon was also achieved. |
| **8 Cat. 4** | Food EFSA GMO Panel guidance, 2011; EFSA CEP Panel | Xu et al. (2019): Metabolic engineering of *Lactococcus lactis* commonly used as starter in the | **Metabolic engineering by gene simultaneous co-expression.** |

| Case # Cat. (a) | Use application Most applicable Guidance | Reference and description | Development of GM/SynBioM |
|---|---|---|---|
| | guidance, 2021; EFSA NDA Panel guidance, 2016b | dairy fermentation industry, for co-expression of high-levels of glutathione (GSH) and *S*-adenosylmethionine (SAM) to enable their simultaneous production. In addition, an adhesion factor was co-expressed in the engineered strain to increase its adhesive ability to the human gastrointestinal tract. The highest accumulation of SAM (9.0 mg/L) and GSH (17.3 mg/L) was achieved after 17 h cultivation of the engineered strain which also showed improved auto-aggregation and hydrophobicity capabilities. | In this study metabolic engineering was achieved by cloning the key genes encoding the enzymes related to the biosynthesis of GSH (GSH synthase gene *gshF*) and SAM (SAM synthase gene *metK*) and the adhesion factor encoding gene from *Lactiplantibacillus plantarum*. Genetic modification was carried out by construction of several inducible vectors introduced in the modified strain, thus allowing a linear dose–response between inducer and protein expression level. |
| **9 Cat. 4** | Food EFSA GMO Panel guidance, 2011; EFSA CEP Panel guidance, 2021; EFSA NDA Panel guidance, 2016b | Xin et al. (2018): Description of a genome engineering strategy for metabolic engineering of *Lacticaseibacillus casei* for acetoin production. The genome engineering approach was used to delete with high efficiencies three genes (*pflB*, *ldh* and *pdhC*) involved in energetic metabolism and negatively affecting acetoin production. The yielding quadruple mutant could produce a ~ 18-fold higher amount of acetoin than the wild-type and converted 59.8% of glucose to acetoin in aerobic systems. | **Metabolic engineering by single-plasmid genome editing.** A plasmid containing prophage recombinase operon driven by the nisin-controlled inducible expression system and the site-specific recombinase gene *Cre* under the control of the promoter of the lactose operon was constructed. Integration of a *hicD3* gene linear donor cassette (up-lox66-cat-lox71-down) was catalysed by the LCABL_13040–50-60 recombinase and the *cat* gene was excised by the Cre/lox system. Using this single-plasmid system, four different genes (*hicD3, pflB, ldh,* and *pdhC*) responsible for acetoin biosynthesis were subsequently deleted to investigate the feasibility of high level of acetoin production. |
| **10 Cat. 4** | Food (decontaminant) EFSA GMO Panel guidance, 2011; EFSA BIOHAZ Panel guidance, 2010 | Dunne et al. (2019): Bacteriophages were designed with extended host range to control *Listeria monocytogenes* serovars. This was achieved by changing and tuning the receptor-binding protein (RBP) on the base plate of the phage. Phages with a chimeric RBP were able to | **SynBio strategies.** The specific *L. monocytogenes* phage PSA is reprogrammed by a combination of SynBio strategies, structure-guided design of the phage RBP and artificial intelligence |

| Case # Cat. (a) | Use application Most applicable Guidance | Reference and description | Development of GM/SynBioM |
|---|---|---|---|
| | | specifically interact with a defined subset of foodborne *Listeria* serovars. These designed phages can be used to specifically infect and kill *Listeria* serovars. | approaches. The code for the designed chimeric RBP (Gp15) is engineered into phage DNA and the chimaeric RBP was directed to the base plate of the phage. |
| **11 Cat. 4** | Feed additive EFSA GMO Panel guidance, 2011; EFSA FEEDAP Panel guidances 2017a,b,c, 2018a | Jester et al. (2022): The photosynthetic cyanobacterium spirulina (*Arthrospira platensis*) was modified to express camel antibodies (nanobodies) against flagellin A of the foodborne pathogen *Campylobacter jejuni*. Genetic engineering methodology was developed specifically for spirulina, in the current case. The recombinant spirulina is designed for oral delivery and was demonstrated to prevent enteric *Campylobacter* infection in an animal model. | **Transgenesis in microalgae.** The transgene was transformed by natural competence and integrated specifically into the spirulina chromosome by double crossover events, as is typical for natural transformation. |
| **12 Cat. 4** | Feed additive EFSA GMO Panel guidance, 2011; EFSA FEEDAP Panel guidances 2017a,b,c, 2018 | Wang et al. (2020): Description of the construction of a *Lactococcus lactis* expressing a variant infectious bursal disease virus (IBDV) envelope protein VP2 on its surface. CVP2 antigen was fused N-terminally to a surface protein from *Salmonella* Typhimurium, that blocks the complement response in chicken ['resistance to complement killing' (*RCK*) gene]. This strain provides protection against IBDV when orally fed to chicken. | **Transgenesis.** The *CVP2* antigen gene was fused to the outer membrane protein H peptide to anchor the VP2-RCK fusion protein onto the surface of *L. lactis*. Genetic modification was executed using a plasmid containing the nisin-controlled inducible expression and harbouring the fused gene coding for the avVP2-RCK fusion protein. The case was included to assess a category 4 organism expressing heterologous proteins that is active in food producing animals. |
| **13 For this Opinion, this is considered as Cat. 4** | Biostimulant Plant growth promoting bacteria Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products, EFSA GMO Panel guidance 2011 | Shulse et al. (2019): Refactored phytase genes were expressed in the root-colonising bacteria *Pseudomonas simiae*, *P. putida* and *Ralstonia* spp. The best performing 12 engineered strains with improved P-solubilising activity, were selected and demonstrated to confer a growth advantage on plants in P-limiting conditions. | **SynBio strategies.** A combinatorial SynBio approach was applied to refactor 82 phylogenetically diverse phytases, from across eight bacterial phyla. Refactoring was aimed to increase the expression in Proteobacteria. Genes were integrated, by conjugation, in a modified genetic locus on the genome of three soil proteobacteria. |
| **14 Cat 4** | Plant Protection Product Commission Regulation (EU) No 283/2013 Annex | Hajeri et al. (2014): double stranded RNA (dsRNA) expressed in a Citrus tristeza virus (CTV) vector to tackle | **Transgenesis** to construct a category 4 plant virus expressing dsRNA to be |

| Case # Cat. (a) | Use application Most applicable Guidance | Reference and description | Development of GM/SynBioM |
|---|---|---|---|
| | part B, EFSA GMO Panel guidance, 2011. Background documents related to RNAi risk assessment (EFSA, 2014; EFSA GMO Panel, 2018) | *Diaphorina citri*, a phloem-sap sucking insect vector of bacterial citrus greening disease (citrus huanglongbing). The CTV vector contains a truncated fragment of the endogenous abnormal wing disc-like protein (Awd) gene of *D. citri*. During replication CTV accumulates abundant amounts of dsRNAs in the phloem of plants which are ingested by the phloem-sap sucking insect *D. citri*, causing impaired ability of *D. citri* to fly and is expected to limit the vectoring of the bacterial pathogens causing citrus greening disease. | applied on citrus plants. The dsRNA causes impaired ability to fly of the insect through RNAi. This case study was used for ingestion of the citrus fruit that will carry the modified virus. |
| **15 Cat 4** | Feed additive EFSA GMO Panel guidance, 2011; EFSA FEEDAP Panel guidances, 2017a,b,c, 2018a. Background documents related to RNAi risk assessment (EFSA, 2014; EFSA GMO Panel, 2018) | Leonard et al. (2020): dsRNA expressed in *Snodgrassella alvi*, a core member of the conserved gut microbiota in honeybees. *S. alvi* was engineered to induce eukaryotic RNA interference (RNAi) by express dsRNA fragments corresponding to the Deformed Wing Virus (DWV) genome and to essential genes of the *Varroa* mite honeybees inoculated with the engineered *S. alvi* strains showed improved survival of DWV-injected bees. Moreover, an increased killing of the *Varroa* mite was observed. | **Transgenesis** to construct a category 4 gut bacterium harbouring plasmids expressing dsRNA in honeybees. The dsRNA causes protection of the honeybees to infection with the DWV virus and the *Varroa* mite through RNAi. This case study is used to assess the effect of a feed additive designed to interact with bee as target animal and the effects on derived food products such as honey. |

# 3. Assessment

This section 'Assessment' follows the structure of the EFSA GMO Panel (2011) guidance that is the primary document to be checked for adequacy. This structure was complemented in the current document with information from other guidances per topic when necessary.

## 3.1. General outline of risk assessment for genetically modified microorganisms

The risk assessment for GMMs, subject to an authorisation in the EU under specific regulations, as described in the relevant EFSA guidances, is based on a stepwise approach that can be summarised in the following main phases:

1) Microbial and molecular characterisation: aimed to identify the GMM and its parental organism and to identify and characterise related hazards (e.g. antimicrobial resistance (AMR), virulence, pathogenicity, toxin production).
2) The safety of the genetic modification: focused on the intended and predicted unintended effects of the genetic modification and potential additional hazards derived from the GMM.
3) The ERA: targeted to assess potential adverse effects to humans, animals and the environment resulting from the deliberate release of the GMM into the environment. ERA is further complemented with post-market environmental monitoring.
4) Safety for humans and animals, including intended and unintended effects.

The first three points have already been extensively addressed in Opinion 1 on SynBioMs Molecular characterisation and ERA (EFSA Scientific Committee, 2020). The different EFSA guidances dealing

with the 4th point – Safety for humans and animals – will be evaluated for adequacy in the current Opinion.

## 3.2. Assessment of the 'Categorisation of the GMMs and their products for risk assessment purposes' of the EFSA GMO Panel Guidance (2011)

**Phase 1 evaluation**

The guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011) identifies four different categories of GMM:

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

This categorisation is seen as a pragmatic approach to optimise the risk assessment of GMM and specific examples are reported in the EFSA GMO Panel (2011) guidance.

**Phase 2 evaluation**

**Table 4:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for the categorisation of the GMMs and their products for risk assessment purposes of the EFSA GMO Panel (2011) guidance. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case Cat. | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1 Cat. 1/2** | The absence of viable cells and DNA of the production strain, required by the existing guidance, this is an evaluation criterion that applies to this SynBioM enzyme producer with minimised genome. | Adequate. It should be noted that Categories 1 and 2 are not distinguished in practice. | Merge Categories 1 and 2 into one single category in future updates of the guidance |
| **2 Cat. 1/2** | The antimicrobial xenopeptide contains new-to-nature non-canonical amino acids. The absence of viable cells and DNA of the production strain is the prerequisite to include the products in Cat. 1 or 2. | Adequate. It should be noted that Categories 1 and 2 are not distinguished in practice. | Merge Categories 1 and 2 into one single category in future updates of the guidance |
| **3 Cat. 1–4** | The XNA-containing bacterial cells may be present in the end-product; however, the organism is not able to propagate without external addition of xenonucleotides and the XNA is not able to be stabilised after horizontal gene transfer. If absence of viable cells and XNA of the production strain is demonstrated the products falls into Cat. 1 or 2. The absence of viable cells should be demonstrated for Cat. 3. | Adequate | No update needed |

| Case Cat. | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **4 Cat. 3** | *A. oryzae* has been modified to improve the soy sauce fermentation. Heat treatment during food processing eliminates viable cells but not DNA. | Adequate | No update needed |
| **5 Cat. 3** | Yeast, able to growth on $CO_2$, can be used for food/feed biomasses production. The absence of viable cells should be demonstrated to accomplish the Cat. 3 requirements. | Adequate | No update needed |
| **6 Cat. 3** | The cyanobacterium metabolism has been refluxed to increase biomass production for food/feed product. The absence of viable cells should be demonstrated to accomplish the Cat. 3 requirements. | Adequate | No update needed |
| **7 Cat. 4** | Engineered viable *Leuconostoc citreum* bacterium highly expressing riboflavin. The bacterium can be added to fermented bioactive products. | Adequate | No update needed |
| **8 Cat. 4** | Engineered viable *Lactococcus lactis* bacterium highly expressing GSH and SAM and with increased adhesive capacity. | Adequate | No update needed |
| **9 Cat. 4** | Engineered live *Lacticaseibacillus casei* bacterium improved in acetoin production to be used in dairy fermentation. | Adequate | No update needed |
| **10 Cat. 4** | To be used as decontaminating agent, being based on viable bacteriophages reprogramed to broaden the host range against *Listeria* spp. | Not fully adequate. | Update recommended: This section of the guidance should be extended to include viable bacteriophages |
| **11 Cat. 4** | Viable cyanobacterium cells expressing antibodies against *Campylobacter* spp. | Adequate | No update needed |
| **12 Cat. 4** | Viable *Lactococcus lactis* expressing viral antigens aimed to improve animal welfare by protecting chickens against IBDV. | Adequate | No update needed |
| **13 Cat. 4** | Viable *Pseudomonas simiae*, *P. putida* and *Ralstonia* sp. cells able to improve P-solubilisation in soil at the root level. | Adequate | No update needed |
| **14 Cat.4** | Viable CTV expressing dsRNA in the phloem of plants aimed to inactivate an insect vector of a plant pest. | Adequate | No update needed |
| **15 Cat.4** | *Snodgrassella alvi* colonisation of the gut of the honeybee as defence against infections with the DWV virus and the *Varroa* mite. | Adequate | No update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: The categorisation is applicable to SynBioMs and their products expected to reach the EU market in the near and wide future. Currently, Categories 1 and 2 are not distinguished in practice.

*Need for updates*: Categories 1 and 2 should be merged.

## 3.3. Microbial characterisation including QPS evaluation

The adequacy of the existing guidances on microbial characterisation for SynBioMs was already addressed in the first Opinion (EFSA Scientific Committee, 2020). The section on the application of the QPS (EFSA, 2007; Herman et al., 2019) was evaluated further in view of (1) specific animal and human health considerations for the SynBioM, and (2) the extended scope of this Opinion to products belonging to Categories 1–4 and not only Category 4 as in Opinion 1 (of WP1).

As this Opinion deals with the adequacy of existing guidance for food and feed, the QPS approach was re-considered and reconfirmed to be a fundamental tool for the RA of microorganisms in relation to food and feed use. The application of QPS in the safety assessment of microorganisms and their products may reduce the requirements for safety assessment, as clearly described in the guidance for the preparation of applications on food enzymes (EFSA CEP Panel, 2021), Section 1.1.10.1 'Toxigenicity and pathogenicity' and Section 4.1. 'Exemptions from toxicity testing'. This guidance describes a specific approach for the risk assessment that applies to those species of microorganisms included in the list of recommended biological agents for QPS status (EFSA, 2007; EFSA BIOHAZ Panel, 2020a,b).

The QPS evaluation is based on extensive literature searches to reveal the body of knowledge and identify possible safety concerns for humans, animals and the environment related to their release. Those strains qualifying for the QPS approach are presumed safe for target species, consumer and the environment, encompassing possible effects on human and animal health. Safety concerns identified for a taxonomic unit (TU) are, when possible, confirmed at strain or product level, reflected as 'qualifications' that should be assessed at the strain level by EFSA's Scientific Panels. The qualification 'for production purpose only' implies the absence of viable cells of the production organism in the final product and can also be applied to food and feed products based on microbial biomass (EFSA BIOHAZ Panel, 2020a,b). The QPS status is also applicable to GMMs if the recipient strain qualifies for the QPS status, and if the genetic modification does not indicate a concern (EFSA BIOHAZ Panel, 2020a,b). For details on the evaluation of possible concerns raised by the genetic modification, see the guidance of the CEP and FEEDAP Panels (EFSA FEEDAP Panel, 2018a,b; EFSA CEP Panel, 2021).

For production strains, meeting the criteria for a QPS approach to safety assessment, toxicological studies will only be required in relation to possible safety concerns identified elsewhere in the assessment process, e.g. manufacturing. The QPS approach for risk assessment can be followed when the taxonomic identity of the production strain confirms that it belongs to a QPS TU and that all qualifications are met. The QPS concept and approach (Herman et al., 2019) is worthwhile to consider as a basis for the risk assessment of chassis of SynBioM and could be applied in a safety-by-design approach for SynBioM. In the case of chassis obtained by genome minimisation, attention should be paid to the potential consequences for safety. Although genome minimisation is used in the safe-by-design approach (Grosjean et al., 2021) to create safer chassis organisms, there are some reports where increased virulence was associated with evolutionary genome reduction (Diop et al., 2018; Murray et al., 2021).

**Phase 2 evaluation**

**Table 5:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for the microbial characterisation including QPS evaluation. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | *B. subtilis* is included in the QPS list with two qualifications, the absence of toxigenic potential and lack of antimicrobial resistance genes. The | The QPS approach is adequate. | No update needed |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| | minimised *B. subtilis* PG10 lacks surfactin production, the cyclic peptide associated with the rare case of *B. subtilis* intoxication. It harbours antimicrobial resistance genes introduced during the genetic modification for heterologous protein production. | | |
| **2 and 3** | XNA and xenoproteins obtained after translation are not covered by QPS. | The QPS approach cannot be used to assess new-to-nature compounds due to lack of body of knowledge. | No update needed |
| **4** | *Aspergillus oryzae* is not included in the QPS list (EFSA BIOHAZ Panel, 2021a). | Filamentous fungi are excluded from the QPS approach | No update needed |
| **5** | *Komagataella (Pichia) pastoris* is included in the QPS list (EFSA BIOHAZ Panel, 2021a). If the genetic modification does not cause safety concerns, the final autotrophic strain can follow the QPS assessment | The QPS approach is adequate. | No update needed |
| **6 and 11** | Cyanobacteria can be assessed for inclusion in the QPS list (EFSA BIOHAZ Panel, 2021a) and the genetic modifications need to be evaluated for possible safety concerns. | The QPS approach is adequate. | No update needed |
| **7** | *Leuconostoc citreum* is included in the QPS list (EFSA BIOHAZ Panel, 2021a). The engineered strain can follow the QPS assessment if the genetic modification does not introduce safety concerns. | The QPS approach is adequate. | No update needed |
| **8 and 12** | *Lactococcus lactis* is included in the QPS list (EFSA BIOHAZ Panel, 2021a). The engineered strain can follow the QPS assessment if the genetic modification does not introduce safety concerns. | The QPS approach is adequate. | No update needed |
| **9** | *Lacticaseibacillus casei* is included in the QPS list (EFSA BIOHAZ Panel, 2021a). The engineered strain can follow the QPS assessment if the genetic modification does not introduce safety concerns. | The QPS approach is adequate. | No update needed |
| **10** | Bacteriophages are not considered for QPS evaluation (EFSA BIOHAZ Panel, 2021b). | The QPS approach is not applicable. | No update needed |
| **13** | *Pseudomonas simiae, P. putida* and *Ralstonia* sp. can be assessed for inclusion in the QPS list (EFSA BIOHAZ Panel, 2021a) and the genetic modifications need to be evaluated for possible safety concerns. | The QPS approach is adequate. | No update needed |
| **14** | The CTV virus can be assessed for potential inclusion in the QPS list and the genetic modifications need to be evaluated for possible safety concerns. | The QPS approach is adequate. | No update needed |
| **15** | The *Snodgrassealla alvi* bacterium can be assessed for potential inclusion in the QPS list and the genetic modifications need to be evaluated for possible safety concerns. | The QPS approach is adequate. | No update needed |

### Conclusions on Phase 1 and Phase 2 evaluations

*Conclusions on adequacy*: Relying on the QPS status for the safety assessment of building blocks of SynBioM is valid when there is sufficient familiarity of the SynBioM/chassis with the QPS organism.

*Need for updates*: No updates needed.

## 3.4. Information relating to the product, information relating to the production process and information relating to the product preparation process (several guidances)

### Phase 1 evaluation

Different EFSA guidances address the assessment of the production process as a key section of the risk assessment of microbial products. Therefore, the production process, including fermentation, downstream processing and product formulation, determines the composition and purity of the end-product and is therefore a critical factor for the risk assessment in relation to product applications as food and/or feed.

Guidance is provided in the EFSA GMM guidance (2011) Section 2, covering all four categories, in the guidances for feed additives regarding the identity, characterisation and conditions of use of feed additives Section 2.3 (EFSA FEEDAP Panel, 2017c) and characterisation of microorganisms used as feed additives or as production organisms Sections 2–5 (EFSA FEEDAP Panel, 2018a), in the guidance for the risk assessment of food enzymes (EFSA CEP Panel, 2021, Section 1.2), and in the guidance for authorisation of novel foods (EFSA NDA Panel, 2016a, Section 2.3). For safety, the description should include information on potential by-products, impurities or contaminants. Information should also be provided on the culture conditions for microorganisms, including microalgae. The description of the cultivation of microorganisms should also include information on the use of antimicrobial agents. Information on substances used in the manufacturing process, e.g. identity of the extraction solvents, ratio of extraction solvent to the material, reagents, residues remaining in the final product and any special precautions (light and temperature) should be provided.

In the Commission Regulation (EU) No. 283/2013, setting out the data requirements for pesticide active substances, the degree of purity of the active substance is defined. The method of manufacture and information concerning the impurities should be provided (Part A, section A Identity of the actives substance). Full information on how the microorganism is produced must be provided (Part B, 1.2 and 3.4).

These guidances were designed to assess cellular organisms, mainly bacteria and fungi and did not provide indication on the safety assessment of bacteriophages and other viruses. For phages, the assessment of the manufacturing process can be based on: (1) the virulence and pathogenic potential of the propagating microorganism and the presence on the genome of temperate bacteriophages, (2) the growth medium ingredients, and (3) the downstream processing. This approach was followed by the EFSA BIOHAZ Panel for the bacteriophage Listex P100 (non-GMM) to control *L. monocytogenes* (EFSA BIOHAZ Panel, 2016b). More recently, the same approach was followed by the EFSA FEEDAP Panel, in the assessment of the safety and efficacy of Bafasal® (four bacteriophages) to control *Salmonella enterica* serovar Gallinarum (EFSA FEEDAP Panel, 2021b).

### Phase 2 evaluation

**Table 6:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents on production and manufacturing processes. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The minimised *B. subtilis* strain expressing a heterologous protein is expected to be grown in the typical fermentation conditions used for bacilli. The downstream processes for the protein purification do not differ from those of enzymes produced in GM strains of *B. subtilis*. | Adequate | No update needed |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **2 and 3** | The production process of xenobionts (containing XNA and producing xenoproteins) needs the provision of xenocomponents. | Not fully adequate | Update is recommended for xenobionts containing XNA, XNA precursors and producing xenoproteins |
| **4** | The *Aspergillus oryzae* strain is produced following the typical fermentation conditions used for fungi; the organism is killed during downstream processing. | Adequate | No update needed |
| **5** | The production of autotrophic yeast is an innovative process requiring adaptations from current fermentation processes. | Adequate | No update needed |
| **6 and 11** | The production of cyanobacteria is not covered by the current guidance. | Not fully adequate | Update is recommended for protists/microalgae |
| **10** | The production of bacteriophages is not covered by current guidance. | The BIOHAZ Panel guidance (2010) is not detailed and hence not sufficient. | Update is recommended for bacteriophages and can be done based on gained experience |
| **7, 8, 9, 12, 13 and 15** | To scale up the production of the engineered strains, regular fermentation conditions are expected to be used. | Adequate | No update needed |
| **14** | The production process of plant viruses is covered by Regulation (EU) No 283/2013. | Adequate | No update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: The existing EFSA guidances are generally adequate for assessing the product, the production process and the product preparation process and are applicable for the different SynBioMs products.

*Need for updates*: Update is needed for xenobionts containing XNA and/or producing xenoproteins to assess potential concerns regarding their presence, their stability and/or their potential degradation into harmful metabolites. The specificities of the manufacturing and purification processes for non-GM, GM and SynBio protists/microalgae and bacteriophage fermentation need to be taken into account and, for bacteriophages, also the possible formation of phages with transducing properties of virulence and toxin factors.

## 3.5. Presence of SynBioM and SynBioM DNA or XNA in the product

**Phase 1 evaluation**

The presence of organisms (viable and/or inactivated) and/or their genetic information (DNA or XNA) are key elements for the safety evaluation of GMMs, including SynBioMs (as was provided for in the GMM guidance, 2011). For Categories 1 and 2, the product may not contain either viable cells, viable but non-cultivable cells (VBNC), spores or non-viable physically intact microorganisms, including xenobionts. It also may not contain DNA or XNA. Category 3 products may contain DNA/XNA but no microorganisms in any stage of viability and Category 4 products may contain both microorganisms in any stage of viability and DNA/XNA.

The most recent guidance for testing the presence of viable microorganisms and DNA is provided in the EFSA FEEDAP Panel (2018a) guidance Section 3, EFSA CEP Panel statement (2019) Section 2, EFSA CEP Panel guidance (2021) Section 1.3.4. Guidance for testing the presence of inactivated physically intact cells is only described in the EFSA GMO Panel guidance (2011), Section 2.2.2.

The existing EFSA GMM guidance (2011) for testing the different viability stages and forms of viable bacteria and fungi are applicable for SynBio bacteria and fungi. It is well elaborated with proposing

culture and culture-independent methods. Detection of stressed cells and endospores is also included. Guidance is missing on detection of SynBio bacteriophages, protists and microalgae. Guidance for detection of SynBioM with xeno nucleic acids (XNA) and/or xeno amino acids (XAA) would need extra methodological guidance on adapted culturing conditions and culture-independent detection.

The presence of DNA forms a possible hazard because of the horizontal transfer of genes of concern and their spread and maintenance in human or animal microbiota. Guidance is provided to demonstrate the absence of DNA in Categories 1 and 2 products. Detection is based on polymerase chain reaction (PCR) amplification of fragments with a maximum length of 1 kb or of the size of the smallest gene of concern. Focusing only on gene length and not on the detection of certain genes of concern is acceptable because the stability of DNA is dependent on DNA length and is not sequence dependent. This general principle remains valid in case of SynBioM.

The guidances cited above are relevant for detecting the presence of DNA from SynBioM in food and feed products. Guidance is missing on detection of XNA, which may not be able to be amplified by conventional PCR.

**Phase 2 evaluation**

**Table 7:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for the presence of SynBioM and SynBioM DNA or XNA in the product. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The ability of the minimised genome strain to survive in open environment is reduced in comparison with the parental strain (e.g. lack of the sporulation ability). No differences are expected, in terms of potential horizontal gene transfer, between a GM strain of *B. subtilis* and the strain with a minimal genome. | Adequate | No update needed |
| **2 and 3** | For xenobionts with XNA and/or incorporation of xeno amino acids in xenoproteins the presence of XNA and/or xenobionts in the product needs to be assessed. | Not fully adequate | Guidance update is recommended to develop testing for the presence of XNA and/or xenobionts in the product |
| **4–6** | In this Cat. 3 product the presence of viable cells needs to be assessed. | Adequate | No update needed |
| **7–15** | Cat. 4 products in which viable cells/ virus and DNA are expected to be present. | Not required | No update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: The guidances cited above are adequate for detecting the different viability stages of bacteria and fungi, including spores, and the presence of DNA from SynBioM in food and feed products.

*Need for updates*: Guidance is missing on detection of non-GM, GM and SynBio bacteriophages, protists and microalgae. Guidance for detection of SynBioM with XNA and/or xeno amino acids would need additional methodological guidance on adapted culturing conditions and culture-independent detection. Guidance is missing on detection of XNA, which would not be able to be amplified by conventional PCR.

## 3.6. Comparative approach of the EFSA GMO Panel GMM guidance 2011

This section of the EFSA GMO Panel (2011) guidance focuses on the comparative approach as a key general principle in the RA of GMMs. For the choice of comparator, the following provisions are provided for in the legislation and are quoted in the GMM guidance:

'Regulation (EC) No 1829/2003 defines the comparator (conventional counterpart) as a **similar food** or feed produced without the help of genetic modification (as defined in European Commission Directive 2001/18/EC) and for which there is a **well-established history of safe use**'.

In line with these legal provisions, according to the above GMM the guidance effects of the GMM are compared with those of the non-GMM that **is applied under similar conditions**. A comparator for the RA of GMMs is therefore **generally considered to be the non-GM microorganism**. Several situations can arise:

- **When the parental/recipient strain is already *'a similar food or feed produced without the help of genetic modification'***, the use of a comparator in the risk assessment is adequate for those SynBioM (Categories 1–4) with sufficient familiarity to the non-GMM counterpart. In this respect also the QPS concept could be used (see Section 3.3). Comparators should be selected on a case-by-case basis and depending on the purpose of the test. The choice of the comparator should be explained.
- When the recipient strain is **not yet used as food or feed**, i.e. is not *'a similar food or feed produced without the help of genetic modification'*, the GMM guidance of 2011 foresees that *'When the recipient strain does not have a history of safe use, the choice of a **different strain** of the same species or a **phylogenetically close** relative as comparator must be justified. All the available information should be provided and evaluated on a case-by-case basis'*.
- For **SynBioM without sufficient familiarity**, other comparators could be used, such as strains derived from the same chassis with similar traits or functionalities of comparable GMMs/ SynBioMs with a history of (safe) use for similar applications (familiarity). The GMM guidance of 2011 foresees that, in cases when the parental organism of the GMM does not have a history of use in the particular application, conventional food products may still be used as comparators to identify possible compositional changes and to assess their safety implications.

When there is a **lack of a comparator or comparable product,** such cases trigger requirements for extra data to form conclusions on potential adverse effects on human and animal health. Also, for **complex changes** in the composition of the genetically modified food or feed, the GMM guidance foresees that '*Where no comparator can be identified for the GMM and/or its product, a comparative safety assessment cannot be made and a comprehensive safety assessment should be carried out*'. As SynBioMs may be redesigned by modification in several genetic loci resulting in the combination of several novel traits, the comparative approach may not equally be sufficient for SynBioMs. A novel type of SynBio product or new-to-nature components with no conventional counterpart produced with a SynBioM with insufficient or no familiarity to a non-GMM counterpart or an already adopted GMM product would require a safety assessment per se as provided for already in the GMM 2011 guidance. This would include toxicological and/or (anti)nutritional assessment based on *in vitro* and *in vivo* studies and would also include testing of pathogenicity, allergenicity and gut–environment interactions.

It should be noted that the most recent EFSA guidances (EFSA, 2021, statement on WGS; EFSA CEP Panel, 2021) for RA of GMM base the safety evaluation of bacteria and yeast on the analyses of the WGS. This approach, aimed at the identification of the genetic determinants for virulence factors, toxins and of other genes of concern, focuses directly on the microorganism under evaluation and should be performed independently from the comparative approach.

### Phase 2 evaluation

**Table 8:** Phase 2 summary on the adequacy testing of existing guidance documents for the comparative approach of the EFSA GMO Panel (2011) guidance. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1–15** | In all Cases 4–15 a comparator can be identified. For 1–3 the guidance stipulates an assessment *per se*. | The existing guidance is adequate. | No update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: The EFSA GMO (2011) guidance describes well the principles of the comparative approach that is also applicable for SynBioM. The use of a comparator in the risk assessment is adequate for those SynBioM (Categories 1–4) with sufficient familiarity to the non-GMM counterpart. SynBioM producing new-to-nature products and xenobionts would require a safety assessment per se for the new-to-nature components as provided for already in the GMM 2011 guidance. The EFSA GMO Panel (2011) guidance foresees that, in cases when the parental organism of the GMM does not have a history of use in the particular application, conventional food products may still be used as comparators to identify possible compositional changes and to assess their safety implications.

*Need for updates*: No updates needed.

## 3.7. Toxicology

**Phase 1 evaluation**

In general terms, the part of the EFSA GMO Panel (2011) guidance on GMM is in place to cover toxicology and applies for SynBioMs and products produced by SynBioM production organisms. In addition to proteins, also new constituents other than proteins, as well as any anticipated changes in specific metabolic pathways due to the modification, are to be evaluated. This may include toxicological testing on a case-by-case basis.

In addition to the GMM guidance, other guidances apply as well, i.e. the guidance of the EFSA NDA Panel (2016a) on Novel Foods, the guidances from the EFSA FEEDAP Panel (2017a, b) and the guidance for the submission of dossiers on Food Enzymes of the EFSA CEP Panel (2021).

Exemptions from toxicity testing are provided for food enzymes obtained from microbial sources (GM and non-GM) **which meet the requirements of the QPS approach** (EFSA, 2007; Herman et al., 2019) (see Section 3.3) and in addition no safety issues are raised by the manufacturing process. For those cases in which the strain belongs to a species that is included in the QPS list, but harbours acquired AMR genes, toxicity testing may still be waived if no viable cells and DNA are present (Category 1/2 products).

For bacteriophages, safety assessment (case-by-case) has been based on the characterisation of the phage, bioinformatics analysis to detect the presence of possible virulence factors harboured in the viral genome, and on a 90-day oral toxicity study. This approach was followed for the bacteriophage Listex P100 (non-GMM) to control *L. monocytogenes* as evaluated by the EFSA BIOHAZ Panel (2016b) based on the EFSA guidance document on carcass decontamination (EFSA BIOHAZ Panel, 2010). In addition to bioinformatics and a 90-day oral toxicity study, the assessment of the safety of the feed additive Bafasal® (a cocktail of bacteriophages against *Salmonella enterica*) also included *in vitro* mammalian cell gene mutation tests and *in vitro* micronucleus assays (EFSA FEEDAP Panel, 2021b).

Further information on the toxicological tests to be applied to GMMs when needed according to EFSA guidances and other international guidelines are reported in Appendix A. These approaches, which include genotoxicity, systemic toxicity and effect on the immune systems, are considered adequate for toxicological assessment of SynBioMs.

**Phase 2 evaluation**

**Table 9:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for toxicology. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The minimised genome *B. subtilis* strain derives from a QPS parental strain. The production of surfactin has been depressed and the genetic modification is not expected to raise additional concerns. | Adequate | No update needed |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **2 and 3** | The toxicology of the presence of XNA and xenoproteins need to be assessed. It is considered that toxicity of xenocompounds can be detected as for other (chemical) compounds or proteins. | Adequate | No update needed |
| **4** | DNA of the *Aspergillus oryzae* remains in the product as may be the secondary metabolites produced by the production organisms. | Adequate | No update needed |
| **5** | The production strain is enlisted as QPS and autotrophy is not expected to change this QPS status. | Adequate | No update needed |
| **6** | Some cyanobacteria may produce cyanotoxins. In principle cyanotoxins and other microbial toxins (e.g. secondary metabolites including mycotoxins) can be detected using the standard toxicological approaches (genotoxicity and rodent studies). | Adequate | No update needed |
| **7–9** | The species is QPS granted. Therefore, no toxins or (geno)toxic metabolites are expected, and *in vitro* genotoxicity testing can be waived. | Adequate | No update needed |
| **10** | The product consists of bacteriophages. | The BIOHAZ guidance is general and not detailed, but there are two examples that applied an approach for non-GM bacteriophages that are adequate | Update of the general guidances on the basis of gained experience is needed |
| **11** | Some cyanobacteria may produce cyanotoxins. In principle cyanotoxins and other microbial toxins (e.g. secondary metabolites including mycotoxins) can be detected using the standard toxicological approaches. | Adequate | No update needed |
| **12** | The parental species/strain is QPS. Therefore, no toxins or toxic (genotoxic) metabolites are expected. However, this product is designed to express viral and *Salmonella* antigens. Therefore, interactions with immune function should be checked. | Adequate | No update needed |
| **13** | The engineered strains [*Pseudomonas simiae*, *P. putida* and *Ralstonia* (*Pseudomonas*)] are non-QPS organisms and toxicological studies may be necessary. | Adequate | No update needed |
| **14** | Regarding the oral uptake by humans and non-insect animals, dietary non-coding RNAs are generally rapidly degraded in the gastrointestinal tract and barriers | Adequate for toxicological assessment of human or non-insect animals.<br>Guidance for testing off-target RNAi effects in insects is provided in EU | No update needed<br>No update needed |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| | (e.g. mucus, cellular membranes) limiting their cellular uptake in the gastrointestinal cells. Hence, the amount of RNAs taken up and absorbed after oral ingestion is considered negligible (EFSA, 2014; EFSA GMO Panel 2018) and therefore the dsRNA producing CTV viruses are not considered as hazardous.<br>Oral ingestion by insects could trigger an off-target RNAi effect. | Regulation No 283/2013 and is adequate | |
| **15** | The SynBioM *Snodgrassella alvi* (producing dsRNA) is expected to be present in bee products (such as honey) similarly to the natural strain. Regarding the oral uptake by humans, dietary non-coding RNAs are generally rapidly degraded in the gastrointestinal tract and barriers (e.g. mucus, cellular membranes) limiting their cellular uptake in the gastrointestinal cells. Hence, the amount of RNAs taken up and absorbed after oral ingestion is considered negligible (EFSA, 2014; EFSA GMO Panel 2018) and therefore the dsRNA producing bacterium is not considered as hazardous.<br>The potential adverse effect of the feed additive on honeybee should be assessed. Specific guidance for testing adverse effect of agents on insects do exists in other areas (e.g. PPP and GMO), however they have different aims in line with the purpose of the product. | Adequate.<br>Specific indications for tolerance and efficacy in insects as target species of feed additives are not available in the FEEDAP Guidances 2018 and 2017. | No update needed<br>Update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: In general terms, existing guidances sufficiently cover the genotoxicity and systemic toxicity assessment of SynBioM products, including those produced from and with XNA and/or xeno amino acids. Guidance for assessing SynBioM products for effects on the immune system, including inadvertent immunomodulation can be based on existing guidances from EFSA (EFSA NDA Panel, 2016b) and OECD (2018a, TG443, and 2018b, TG408), respectively.

*Need for updates*: Guidance for the toxicological safety assessment of non-GM, GM and SynBioM bacteriophages is needed; especially for those propagated in pathogens. This can be based on gained experience of already evaluated non-GM bacteriophages. Specific indications for tolerance and efficacy in insects as target species of feed additives should be developed for non-GM, GM and SynBioM.

## 3.8. Gut microbiome and horizontal gene transfer

**Phase 1 evaluation**

**Available guidances:** Despite its importance, limited information about the risk assessment of microorganisms or their products present in food and feed in relation to the gut environment is available in existing EFSA guidances. The most updated guidances referring to the need of studies are

Chapter 4 of the EFSA GMO Panel (2011) guidance, Chapter 4 of the EFSA FEEDAP Panel (2018a) guidance and Section 8 of the EFSA FEEDAP Panel (2017b) guidance.

The GMM guidance (EFSA GMO Panel, 2011) refers to the potential enhancement of the ability of the GMM to persist in the human gastrointestinal tract (GIT), which is of particular relevance for safety evaluation. SynBio microorganisms may **be engineered to deliver specific functions** and successfully compete in the GIT but, conversely, this competitive advantage can also have unexpected adverse effects on the balance of the gut microbiota.

The EFSA GMM guidance (EFSA GMO Panel, 2011) on the risk assessment of GMMs and their products intended for food and feed uses specifies the need for 90-day rodent studies when previous assessment, primarily based on the molecular characterisation of the genetic modification, the composition and the assessment of the identified intended and unintended effects, points out the need for such animal studies. This guidance refers to the impact of GMM on the gastrointestinal microbiota, with particular attention to the ability of the GMM to persist in the GIT and to interact with the gut microbiota, in several of its sections:

- In the Characteristics of the recipient strain: data on its ability to colonise and persist in the GIT of humans and animals should be provided, in particular for Category 4 products.
- In the Information related to GMM. For Category 4, the effect of genetic modification on the persistence in the gut environment and the impact on the gut microbiome should be reported.
- The Toxicology section for Categories 3 and 4 GMMs states that particular attention should be paid to interactions with the gut microbiota including human studies when appropriate.
- In the Category 3 product evaluation: focus is on the fate of recombinant DNA in environments including the GIT of humans or animals.
- In the Category 4 product evaluation: the interaction of GMMs with biotic environments is addressed, including gut and on the analysis of HGT.

The approach described above is also applicable to Categories 3 and 4 SynBioMs, covering both the intended effects, when the modification was specifically designed to exert an action in the GIT, and unintended effects. However, with the exception of the 90-day rodent feeding study no other indications on how to perform the analysis of the impact of GMM on the gastrointestinal microbiota were provided. It should be noted that, since the EFSA GMO Panel GMM guidance publication (2011), several scientific papers dealing with the gut microbiome analyses mainly by using 'omics' approaches have been published, increasing the body of knowledge on the gut microbiome and the way that effects on it can be tested. A recent publication on factors influencing the gut microbiome composition and function of a large cohort of Dutch individuals, shows that the environment (including diet and socioeconomics), early-life factors and cohabitation, primarily shape the human gut microbiome (Gacesa et al., 2022). Furthermore, the study showed that consistent microbiome–disease patterns found across a number of diseases, enable to pinpoint shared microbiome signatures between seemingly unrelated diseases. Despite this remarkable advancement in knowledge, a lack of consensus on what a healthy microbiome is still exists, potentially limiting the use of microbiome analyses and their interpretation in current risk assessment.

The assessment of potential overgrowth or shedding of potential pathogens is performed for feed additives and described in the guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a) and mentioned in the guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b). The assessment is required for those feed additives:

- that in the tolerance test applied to animals give an indication of an adverse effect related to digestive tract disturbances;
- in which an adverse effect on the gut microbiota can otherwise be anticipated;
- which are ionophoric coccidiostats;
- which are specifically designed to reduce the numbers of enteropathogens and potential for carcass/product contamination.

The approach described above is also applicable to SynBio microorganisms, if they fall under any of the above-mentioned provisions. Indications about how to assess the impact of the additive on the gut microbiome are primarily focused on the potential effect on pathogenic agents and limited e.g. to the target animals and pathogenic strain(s) used/studied.

Perturbation of the gut microbiome structure and microbial metabolism can also have consequences on the gastrointestinal (including metabolic, barrier defence and immune) function. Gut microbiome

imbalances can impact epithelial integrity and, therefore, trigger adverse immune responses and inflammation. This can be of particular relevance in infants during the first months of life when severe disturbances of the gut microbiome balance and gut function may trigger chronic diseases at this point or later in life.

In general, the impacts on the gut microbiome can be anticipated as being more complicated for Categories 3 and 4 (see examples in Appendix B), considering that entire cells or their DNA can have diverse types of impact on the microbial ecosystem (including displacement due to enhanced adhesion, microbial imbalance, decrease of microbial diversity and HGT). However, in line with future/current provisions for advancing risk assessment of regulatory products, the impacts on the gut microbiome might be needed also for Categories 1 and 2 products when it is expected that the SynBio modification may lead to products that have (or are designed to have) effects on the gut microbiome.

### Horizontal gene transfer

The transfer of genetic information from a Category 4 SynBioM, or from Category 3 containing DNA, into other food microorganisms and/or intestinal microbial community may have consequences for human and animal health. DNA from the SynBioM may be transferred to members of the natural microbiomes through mechanisms such as conjugation, transformation or transduction. Genes of concern, encoding harmful traits, may spread in the microbiota providing a selective advantage to one or some of their members, and reducing or displacing other microorganisms with beneficial properties.

Potential risks as a consequence of HGT of sequences of concern are specifically addressed in the EFSA GMM guidance (EFSA GMO Panel, 2011) for microorganisms and no gaps are identified for GMMs or SynBioMs. With respect to methodology, the GMM guidance (EFSA GMO Panel, 2011) does not report methods how to assess and quantitatively measure the HGT of sequences of concern present in GMM products. This also applies to SynBioMs. An extensive risk assessment on the horizontal transfer of antimicrobial marker genes from GM plants to gut and environmental microbiota was published (EFSA, 2009; presenting the joint work of the GMO and BIOHAZ Panels). This assessment extensively documented the mechanisms and the most important environmental factors for the HGT process and the frequencies by which it may occur in the environment and in the human and animal gut. For assessing HGT of GM plants to microorganisms by means of double homologous recombination (DHR), EFSA has issued an Explanatory note (EFSA, 2017). The use of bioinformatics analysis for measuring HGT potential of GM plants is equally applicable to GMMs and SynBioMs.

### Phase 2 evaluation

**Table 10:** Phase 2 summary on the adequacy testing of existing guidance documents for the impact on the gut microbiome and HGT. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | Designed to have an effect on gut environment: NO.<br>The product is a food/feed enzyme, no assessment on the effect on the gut microbiome should be made. | Not required because the product is not designed to have an effect in gut | No update needed |
| **2** | Designed to have an effect on gut environment: YES.<br>As lantibiotics have an antimicrobial activity, their presence will also influence the gut microbiome. The effect of the presence of xeno amino acids on the microbial interactions in the gut also needs to be assessed. | Not fully adequate | The effect of the presence of xeno amino acids may need special attention |
| **3** | Designed to have an effect on gut environment: NO.<br>Microorganisms with XNA will be consumed and will not be able to | Not fully adequate | General update needed on the presence of XNA. |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| | multiply in the gut without supply of the xeno nucleotides. This is a component of the safety by design approach for this product, that limits the persistence of xenobionts. HGT is not expected. The effect of the presence of xeno nucleotides on the microbial interactions in the gut need to be assessed. | | |
| **4, 5 and 6** | Designed to have an effect on gut environment: NO.<br>Biomass of Category 3 product not containing viable SynBioM cells. Need for assessment of potential for HGT. | Not fully adequate | Updates needed on methodology to assess on impact on the microbiome structure and functionality |
| **7, 9 and 13** | Designed to have an effect on gut environment: NO.<br>No adverse effect on gut environment is expected. HGT should be assessed. | Adequate | No update needed |
| **10** | Designed to have an effect on gut environment: NO.<br>SynBio phage specifically designed with extended host range to control *L. monocytogenes* in carcass decontamination. Residual bacteriophage may persist in food and may exert action in the gut. Taking into consideration the stability of the designed host range, this action is not expected to negatively impact gut environment. HGT should be assessed. | Adequate for HGT.<br>For phage-specific issues, the existing guidances are general and not specific | Update of the general guidances on the basis of gained experience is needed for phages |
| **8, 11 and 12** | Designed to have an effect on gut environment: YES.<br>*Lactococcus lactis* with enhanced adhesion ability or SynBio cyanobacteria and *Lactococcus lactis* expressing antibodies or antigen respectively, should be assessed for potential adverse impact on gut function. | Not fully adequate | Updates needed on methodology to assess on impact on gut function |
| **14** | Designed to have an effect on gut environment: NO<br>No adverse effect on gut environment is expected. | Adequate | No update needed |
| **15** | Designed to have an effect on gut environment: NO<br>This case should be assessed for potential adverse impact on gut function since the bacteria is persistent in the gut. | Not fully adequate | Updates needed on methodology to assess on impact on gut function |

## Conclusions on Phase 1 and Phase 2 evaluations

*Conclusions on adequacy*: The existing guidances define the general framework for the risk assessment of GMM interactions in the gastrointestinal environments. In general terms this is also adequate for SynBioMs. In more detail:

- The **GMM guidance** (EFSA GMO Panel, 2011) on the 90-day rodent studies, describes the importance to assess the viability and the residence time of the GMM in the gut ecosystem. It

also points out the need to study the interactions of the GMMs with the gut microbiota and their effects on digestive physiology and immune responses. Although this is also applicable to SynBio products, it is not designed to assess effects on gut microbiome.
- The **FEEDAP guidances** provide more detailed guidance for assessing the potential effect of feed additives on the growth and persistence of pathogens in the gut environment.
- Guidance on the use of bioinformatics analysis for measuring the potential of HGT is available.

*Need for updates*:

- No guidances are available for **methodologies to study the effects on gut microbiome,** other than those for 90-day rodent studies for Category 4 GMM, and other than those for feed additives, nor for the **interpretation** of the results. There are **no standardised methodologies** available to study the gut microbiome. No consensus exists as to what is a healthy baseline in the analysis of gut microbiota.
- Considering the complexity of the gut microbiome, generally accepted **methodology** is missing **for any product** (non-GM, GMM and SynBioM, new-to-nature compounds) to determine the effect on the microbiome structure and metabolism, as well as potential adverse effects derived from microbiome perturbations on gut functions (including metabolic, barrier defence and immune function). Methods to measure endpoints (e.g. potential persistence and colonisation, effects on the gut microbiome) should be explored to allow correct interpretation of the impact of the product on the host in which it is used. The suitability of such methods depends on the type of microorganism, survival capacity and level of exposure, as well as on the host. It could be envisaged that guidance should be updated with uniform methods for certain endpoints when they become available and experience is gained with the RA of such products. This **would facilitate the interpretation of the results** for microbial persistence and colonisation, as well as the potential overgrowth/disturbance of the microbiome balance (structure and functionality) and gut function.
- Regarding the interpretation of observed effects on microbiome, internationally agreed criteria are needed to establish causality of the experimental observation and their relevance for risk assessment of a given substance.
- The assessment of the effect of bacteriophages on the gut microbiome, which could be based on gained experience. This applies for non-GM, GMM and SynBioM.

## 3.9. Allergenicity

**Phase 1 evaluation**

The relevant existing documents for assessment of allergenicity are: Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011), Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes (EFSA NDA Panel, 2014), Guidance on allergenicity of genetically modified plants (EFSA GMO Panel, 2017), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012a), Scientific Guidance for the submission of dossiers on Food Enzymes (EFSA CEP Panel, 2021).

Food allergens are mostly proteins. They induce the production of specific IgE, and the interaction of the allergenic food with the specific IgE may lead to adverse allergic reactions. The allergenic potential of a food containing no protein (or peptides) is very low. The default assumption for foods containing proteins is that they have **allergenic potential**, and this also applies to foods produced by SynBio. The allergenic potential of the food should be explored by considering its composition, particularly its protein(s), its source (including taxonomic relationships), the production process, and available experimental and human data, including information on cross-reactivity. Appropriate methods to investigate the potential allergenicity of proteins, including those produced through SynBio, include: analysis of the degree of sequence identity with known allergens using a sliding rule of 80 amino acids, and a cut off of 35% identity, and in case of indications of allergenicity *in vitro* immunological tests (e.g. ELISA, western blotting) or clinical tests (skin prick testing, double blind placebo-controlled food challenges) should be performed. Additional investigations i.e. analysis of the protein content in the food, determination of the molecular weight, heat stability, and sensitivity to pH, digestibility by gastrointestinal proteases of the proteins, will help to characterise the allergens. This guidance is also applicable to SynBioM products, with the limitation that for new to nature proteins most allergic reactions cannot be predicted using validated methods at present.

A special case is constituted by **non-IgE mediated allergic reactions** to proteins that may result in coeliac disease. In the guidance on allergenicity assessment of genetically modified plants (EFSA GMO Panel, 2017), for non-IgE-mediated adverse immune reactions to food detailed risk assessment considerations are provided to determine the safety profile of the protein or peptide under assessment for its potential to cause coeliac disease. This assessment includes available information on the source of the transgene and on the protein itself, as well as on data from *in silico* and *in vitro* testing, as and when appropriate. This guidance is also applicable to SynBioM products.

Methods to predict skin and respiratory **sensitising capacity** of agents are available (EFSA FEEDAP Panel, 2012a). In this guidance, the issue of possible allergic reactions is covered for workers and bystanders for skin and respiratory sensitisation. Tests for the skin-sensitising potential should be performed using the appropriate form of the product. Protocols for these studies should comply with OECD guideline TG406 (skin sensitisation) (OECD, 2021) and TG429 (skin sensitisation – local lymph node assay) (OECD, 2010b). Standardised methods are currently not available for respiratory sensitisation. If the product is demonstrated to be a dermal sensitiser then it is assumed, on a precautionary basis and in the absence of other information, that it is also a respiratory sensitiser. If the product is proteinaceous in origin then it is, by default, assumed to be a respiratory sensitiser. *In vitro* models to predict the skin-sensitising capacity of agents are being developed and are well underway, but it remains to be determined on a case-by-case basis if this is a main issue for the products that will have to be assessed. This guidance is also applicable to SynBioM products.

### Phase 2 evaluation

**Table 11:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for allergenicity. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The allergenicity potential of the heterologous protein produced by the minimised genome *B. subtilis* strain can be assessed using the approach developed for food enzymes and GMMs. | Adequate | No updates needed |
| **2 and 3** | The XNA and xenoproteins could trigger allergic reactions. | Not fully adequate | Research is needed for allergenicity assessment of new-to-nature products |
| **4** | Killed SynBio *Aspergillus oryzae* cells remain in the product which are not belonging to a normal diet. | Adequate | No update needed |
| **5** | The biomass contains SynBio yeast cells that may be present in normal diets; the autotrophic nature would not be expected to trigger extra allergenic reactions. | Adequate | No update needed |
| **6, 11 and 13** | The product contains SynBio cyanobacteria (biomass or viable cells) that are not belonging to a normal diet. | Adequate | No update needed |
| **7–9** | The strains engineered in these cases do not introduce new proteins and therefore, they are not expected to trigger allergenic reactions. | Adequate | No update needed |
| **10** | Bacteriophages: the potential for allergenicity can be tested with bioinformatics. | Adequate | No update needed |
| **12** | *Lactococcus lactis* chassis is commonly consumed as part of the normal diet. The heterologous protein contains part of a viral and *Salmonella* antigens. | Adequate | No update needed |
| **14** | Oral intake of the plant virus expressing dsRNA needs to be assessed but is not expected to trigger allergenicity by humans or animals. The | Adequate | No update needed |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| | amount of RNAs taken up and absorbed after oral ingestion is considered negligible (EFSA 2014, EFSA 2018). | | |
| **15** | The honey is expected to contain the *Snodgrassella alvi* bacterium expressing dsRNA, similarly as is the natural strain. The dsRNA is not expected to be present in honey. | Adequate | No update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: For the evaluation of allergenicity, existing guidances for proteins, non-IgE-mediated immune reactions, as well as sensitisation, are also applicable for SynBioM products, with limitations in the predictive tools for allergenic potential of new-to-nature proteins.

*Need for updates*: Continuation of research as well as validation of current results to identify allergenicity and adjuvanticity; the aim is that these scientific advancements should be used in the future to update Guidance for risk assessment. This applies to non-GM, GM and SynBioM (including new-to-nature proteins).

## 3.10. Nutritional assessment

Nutritional assessment is provided for GMM in the guidance of the EFSA GMO Panel (2011) and is applicable to SynBioM. Also, a newer guidance can be consulted (EFSA NDA Panel, 2016) for nutritional assessment of SynBioM food. For animal nutrition, the EFSA FEEDAP Panel (2018b) guidance needs to be considered if the SynBioM is used as a feed additive.

The EFSA GMO Panel (EFSA GMO Panel, 2011) guidance (Section 2.4.3) is applicable for Category 3 and 4 GMM/SynBioM as follows:

- If no corresponding conventional product exists, the estimation of the expected dietary intake is particularly relevant. Information on the anticipated intake and extent of use of the GMM and/or its product, taking into account any possible replacement of existing food, will be required and the nutritional consequences should be assessed to find out whether the nutrient intakes are likely to be altered by the introduction of such products into the food supply.
- In addition to the nutrient content, the bioavailability of nutrient components in the product should be considered.
- If significant changes in the composition of nutrients and/or antinutrients have been identified in the GMM and/or its product, their nutritional relevance should be assessed based on current knowledge and taking into account the anticipated intake.

In the EFSA context of food for human consumption, the guidance on Novel Foods Applications (EFSA NDA Panel, 2016a) can be further consulted and this stipulates that for the evaluation of the food the applicant should demonstrate that the novel food is not nutritionally disadvantageous for consumers under the proposed conditions of use. This applies to SynBio products as well. The content and effect of antinutritional factors in the food (e.g. inhibiting absorption or modifying bioavailability) and other known and suspected interactions with nutrients should also be assessed. Vulnerable subgroups such as young children, pregnant and lactating women, or subjects with particular metabolic or physiological disorders should be specifically considered on a case-by-case basis. When the synthetic food is intended to replace another food, it should be demonstrated that it does not differ in a way that would be nutritionally disadvantageous for the consumer under the proposed conditions of use. In this context, the nutritional value of biomasses (Categories 3 or 4) needs to be evaluated[9]. Microbial biomasses are characterised by

[9] In the transition towards a human diet with less incorporation of animal proteins, single-cell proteins obtained by fermentation would take a certain place. In this respect, also biomass generated by SynBioM could be used as a human food source. The nutritional consequences of such diets should be taken into account and would require a specific safety assessment. In this situation, a risk/benefit evaluation should be balanced. Benefits would also include arguments in the light of the reduction of the environmental burden due to animal production. Processing of these food sources would need to be evaluated considering environmental consequences and human nutritional value. The recommendation to compare the nutritional content of the alternative protein source to the meat and dairy products they would replace could also apply to single-cell proteins produced by SynBioM (Wickramasinghe et al., 2021).

high nucleic acid content that can elevate serum uric acid levels and could, when consumed in high amounts, lead to hyperuricaemia and inferior protein uptake (Sarwar Gilani et al., 2012).

For animal feed, the guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b) indicates that for nutritional additives naturally occurring in plants and animals, no efficacy studies are needed. For analogues of amino acids, new forms of trace elements, and compounds with similar effects on vitamins and urea derivatives, nutritional equivalence can be demonstrated from the existing literature. If this information is not available, bioequivalence studies should be conducted. For novel additives, long-term efficacy studies are necessary.

### Phase 2 evaluation

**Table 12:** Phase 2 summary on the adequacy testing of existing guidance documents for the nutritional assessment. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The product is a food enzyme, therefore nutritional assessment is not required. | Not applicable | |
| **2 and 3** | The presence of XNA and xenoproteins in the product needs to be evaluated for nutritional and/or antinutritional consequences. | Not fully adequate | Updated guidance is recommended for assessing new-to-nature products |
| **4** | The nutritional assessment of the biomass of SynBio *Aspergillus oryzae* in soy sauce can be based on the comparison of the composition with the non-GM counterpart with history of human consumption. | Adequate | No update needed |
| **5** | The nutritional assessment of the biomass of SynBio yeast cannot be based on the comparison of the composition with the non-GM counterpart because it does not have a safe history of human and animal consumption as biomass. | Adequate | No update needed |
| **6** | The nutritional assessment of the biomass of SynBio cyanobacteria cannot be based on the comparison of the composition with the non-GM counterpart because it does not have a safe history of human and animal consumption as biomass. | Adequate | No update needed |
| **7–9** | The engineered strains are designed to improve the nutritional and sensorial properties of food and feed. They are not expected to be nutritionally disadvantageous compared with the non-GM parental strain that can be used as the non-GM counterpart in the nutritional assessment. | Adequate | No update needed |
| **10** | The product is composed of bacteriophage for food decontamination, therefore nutritional assessment is not required. | Not applicable | |
| **11 and 12** | The product is a feed additive, (category zootechnical additives), therefore, nutritional assessment is not required. | Not applicable | |
| **13** | The product is a plant biostimulant, therefore, nutritional assessment is not required. | Not applicable | |
| **14** | The product is a plant virus used as plant protection product, therefore nutritional assessment is not required. | Not applicable | |

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **15** | The product is a gut bacterium used as feed additive, therefore nutritional assessment is not required. | Not applicable | |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy*: The nutritional assessment of the food and feed products classed as Categories 1–4 SynBioMs can be based on existing guidances.

*Need for updates*: Current guidances do not describe in detail the methodologies (e.g. for assessing bioavailability) necessary for the nutritional assessment in general and, in particular, for non-GM, GM or SynBioM biomasses used for food. At present this is done on case-by-case basis. Guidance should be developed for the nutritional assessment of new-to-nature products.

## 3.11. Exposure assessment

The GMM guidance 2011 provides for exposure assessment in general terms:

> 'In particular it is of interest to establish whether the intake of the food or feed consisting of, containing or produced from the GMMs is expected to differ from that of the conventional product which it may replace. In this respect, specific attention will be paid to the GMM and/or derived products aimed at modifying the nutritional quality. Such products may require PMM to confirm the conclusion of the exposure assessment (see Section D.).'

This guidance fully applies for SynBioMs. Details for performing such exposure assessments will vary case by case and will depend on the type of organism, viability status and survival capacity as well as possible secondary routes of exposure depending on its specific use.

Secondary routes can derive, for instance, when a Category 4 SynBioM is used in animals and persists in the faecal matter. The spread of manure may lead to contamination of crops and derived food, with a possible exposure to consumers. Another possible secondary exposure is, for example, due to translocation from plants as a result of possible epiphytic and endophytic colonisation of edible plants by microorganisms used as plant biostimulants or by microbial PPP (mPPP). mPPP e.g. *Bacillus thuringiensis*, were found within and at the surface of plant leaves due to epiphytic and endophytic colonisation at concentrations of $10^2$ (internally in the leaf) and $10^3$ (at the surface of the leaf) CFU/leaf sample (EFSA BIOHAZ Panel, 2016a). Also *Pseudomonas* spp. used as biocontrol strains were found in the roots and the plant edible parts up to concentrations of 5.51–5.79 log CFU/g (Sun et al., 2014; Andreoli et al., 2019; EFSA PPR Panel, 2020 on *Pseudomonas chlororaphis*). In addition to the microorganisms, also the metabolites produced by the microorganisms can be translocated into the edible parts of the plant. This aspect is treated in the PPP regulation, where it shall be stated whetherthe active substance is translocated in plants and whether such translocation is apoplastic, symplastic or both (part A, Section 3.3 Effects on harmful organisms) and how the translocation takes place (part B, 2.2.2 Mode of action).

According to the intended use, various guidance documents and tools from EFSA can be consulted and are applicable for the exposure assessment/characterisation related to food and feed consumption:

- EFSA guidance on the use of the EFSA Comprehensive European Food Consumption Database in exposure assessment (2011).
- EFSA CEP guidance on food enzymes (2021) Section 5 and associated calculation tool.
- EFSA FEEDAP Panel guidance (2017a) on the assessment of the safety of feed additives for the consumer (Section 4.3) and associated calculation tool; based on the Total Organic Solids (TOS) concept that remains applicable.
- EFSA NDA Panel guidance (2016a) on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283 (Section 2.7).
- EFSA ANS guidance for submission for food additive evaluations (2012, updated in 2020) (Section 3).

- EFSA Food Additives Intake Model (FAIM).[10]
- EFSA Food Enzymes Intake Model (FEIM) available for baking,[11] brewing[12], cereal[13], egg[14], modified fats[15] and molasses.[16]
- EFSA Pesticide Residue Intake Model calculator[17] (applicable for Categories 1 and 2 products only).

### Phase 2 evaluation

**Table 13:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for exposure assessment. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The product is a food enzyme and exposure assessment can be determined following the food enzyme guidance. | Adequate | No update needed |
| **2** | The xeno lantibiotics will be consumed; the xeno amino acids supplied in the fermentation medium may be present in the end-product. The exposure depends on the degree of purification of the end-product and on the scope of the application. | Adequate | No update needed |
| **3** | The exposure to XNA and xenonucleotides will depend on the product produced. The exposure depends on the degree of purification of the end-product and on the scope of the application. | Adequate | No update needed |
| **4–6** | The exposure to the SynBioM (non-viable in the biomasses) and their DNA depends on the degree of purification of the end-product and the scope of the application. | Adequate | No update needed |
| **7–12, 15** | Exposure assessment for viable cells and bacteriophages has to be performed on a case-by-case basis. | Adequate | No update needed |
| **13** | Exposure depends on the translocation of the SynBioM used as biostimulant to edible plant parts. | Not fully adequate | Update is needed |
| **14** | Exposure of humans and animals to the SynBio plant virus is expected by plant intake. Exposure to the dsRNA is considered negligible (EFSA, 2014; EFSA, 2018). | Adequate | No update is needed |

### Conclusions on Phase 1 and Phase 2 evaluations

*Conclusions on adequacy*: The existing guidances are adequate for calculating exposure to most SynBioM products derived from fermentation (Categories 1–3) and for Category 4 products. Exposure of consumers and animals (farmed and pets) to viable microbial cells through food and feed or by secondary exposure routes (e.g. plants and water) is to be assessed on a case-by-case basis.

*Need for updates*: An update is needed for oral exposure assessment of non-GM, GM and SynBioM, able to epiphytically or endophytically colonise plants, when used as PPP and biostimulants.

[10] https://zenodo.org/record/154725#.YWAeQbgzbD4
[11] https://zenodo.org/record/4382037#.YWAevrgzbD4
[12] https://zenodo.org/record/4382046#.YWAgxLgzbD4
[13] https://zenodo.org/record/4382057#.YWAg2rgzbD4
[14] https://zenodo.org/record/4353056#.YWAhBbgzbD4
[15] https://zenodo.org/record/4354782#.YWAhHLgzbD4
[16] https://zenodo.org/record/4354558#.YWAhBbgzbD4
[17] https://zenodo.org/record/4447293#.YWAfHLgzbD4

## 3.12. Post-market monitoring

In reference to European Commission Directive 2001/18/EC, post-market environmental monitoring (PMEM) is applied to identify any direct or indirect, immediate and/or delayed adverse effects of GMOs, and their management on human health or the environment, after the GMO has been placed on the market. In line with this legal requirement, the GMM guidance (EFSA GMO Panel, 2011) provides for the assessment of both PMM plans and PMEM plans to be delivered for food and feed derived from GMMs. For feed additives, there is no need for specific requirements for a PMM plan other than those established in the Feed Hygiene Regulation[18] and Good Manufacturing Practice. The PMM plan is required only for nutritional, zootechnical, coccidiostats and histomonostats, and additives derived from GMM. Also in the guidance of the EFSA FEEDAP Panel (2021) for renewals of applications, the assessment of PMM plans is envisaged.

The application of PMEM to SynBioMs has been already addressed in Opinion 1 (EFSA Scientific Committee, 2020) SynBioM ERA, that covers the deliberate release of viable cells (Category 4).

The aim of PMM is to address the following questions:

- Is the product use as predicted/recommended?
- Are known effects and side effects as predicted?
- Does the product induce unexpected side effects?

Therefore, PMM should recognise possible concerns not identified in the pre-market risk assessment. According to the EFSA GMO Panel (2011) guidance, it should be required only in specific cases, such as foods with altered nutritional composition and modified nutritional value and/or with specific health claims. However, no details were provided on the approach to be used.

If the performance of PMM is deemed necessary, the reliability, sensitivity and specificity of the proposed methods should be demonstrated.

The PMM aims to detect potential adverse effects on human health (including allergenicity), animal health and the environment as a consequence of the GMM use, this will apply equally to SynBioMs of all Categories (1–4) that will enter food and feed and, in particular, when the SynBioM product is designed to affect the nutritional characteristics of food and feed, expected to affect the gut environment, or contains xenoproteins or xenonucleic acids.

**Phase 2 evaluation**

**Table 14:** Phase 2 evaluation summary on the adequacy testing of existing guidance documents for PMM. Per default the existing guidance refers to the case study Table 3, and if needed an additional guidance is mentioned

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **1** | The products, a food enzyme derive from a SynBioM, is Cat. 1 or 2. No PMM is required. | Not applicable | Not applicable |
| **2 and 3** | Cats. 1–4 products, but containing xeno compounds | Adequate | No update needed |
| **4** | Cat. 3 products for food | Adequate | No update needed |
| **5 and 6** | Feed and food biomasses | Adequate | No update needed |
| **7–9, 13** | Cat. 4 products | Adequate | No update needed |
| **10** | Bacteriophages as decontamination agents may lead to emergence of resistant strains in the target species. | EFSA BIOHAZ Panel (2010) guidance is adequate, although not specifically designed for bacteriophages. | Update recommended based on gained experience |

[18] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

| Case | Specific evaluations of the case, including intended and unintended effects | Conclusions on the adequacy of existing guidance | Updates recommended for future guidance |
|---|---|---|---|
| **11, 12 and 15** | Cat. 4 feed additives | Adequate | No update needed |
| **14** | Cat. 4, plant protection product | Adequate | No update needed |

**Conclusions on Phase 1 and Phase 2 evaluations**

*Conclusions on adequacy:* EFSA guidances provide the principles for the PMM of SynBioMs products.

*Need for updates:*

- Future updates would benefit from including descriptions of fit-for-purpose approaches to monitor for potential adverse effects of microorganisms (non-GM, GM and SynBioM).
- EFSA BIOHAZ guidance (2010) future updates may expand on bacteriophages, based on gained experience.

# 4. Outlook – Phase 3 evaluation

A summary of the conclusions on adequacy and need for updates from Phases 1 and 2 can be found under each section. The final step in the methodology followed by the WG is Phase 3, which aimed at performing an overall gap analysis that could not be captured by the previous Phases 1 and 2.

## 4.1. Tiered approach for risk assessment of living cells ingested by humans/animals

In view of efforts to harness the use of microorganisms in the food and feed chain, the EFSA SC herewith recommends a concerted effort on developing internationally agreed guidance and harmonised frameworks for identifying and addressing living cell intake in the RA process. This would facilitate safety assessment, dietary exposure and related effect assessment of living microbial cells (non-GM, GM or SynBioM) ingested by humans/animals.

## 4.2. Evolving from a technique-driven risk assessment approach towards a strain-driven approach

In the past, RA of GMMs was based on an extensive assessment of the applied genetic modification steps and on the comparison with its non-genetically modified counterpart. With the development in the continuum from GMM to SynBioM, the descriptions of the genetic modifications became more and more complex, making a consistent RA by this technique-driven approach more and more challenging. Moreover, with multiple genetic modification techniques being used at the same time, finding a comparator that is suitable as the non-genetically modified counterpart is also challenging.

Therefore, the RA of GMMs is already being developed towards an approach based on assessing the whole genome of the GMM itself. Such analysis is independent from the genetic modification techniques used. Hypothetically, the same modified organism with exactly the same genetic material could be developed using different technologies that vary from mutagenesis to genome editing.

For the above reason, the most recent EFSA guidances for the risk assessment of GMM are based on the analysis of the WGS. Guidance is provided for production strains (Categories 1 and 2 mainly) applied in the food and feed area (EFSA CEP Panel, 2021; EFSA FEEDAP Panel, 2018a) and an EFSA statement has detailed the requirements for WGS of microorganisms including quality criteria/ thresholds that should be reached and how the data should be assessed related to the taxonomic identification, the characterisation of the genetic modification and the identification of possible genes of concern (EFSA, 2021). Possible genes of concern are those coding for or contributing to virulence, pathogenicity and/or toxigenicity and to resistance to antimicrobials relevant to their use in humans and animals, (EFSA, 2021). When a strain of a typically susceptible species is resistant to a given antimicrobial drug, it is considered to have an 'acquired resistance' for that compound. In contrast, intrinsic resistance to an antimicrobial is understood as inherent to a bacterial species and is typical of all the strains of that species. Intrinsic antimicrobial resistance is generally not considered a safety concern (EFSA, 2021; EFSA FEEDAP Panel, 2012b).

The WGS-based approach is already requested by EFSA for the risk assessment of bacteria and yeast intentionally introduced in the food chain and recommended for fungi, where bioinformatic analyses on the presence of potential genes of concern are requested (EFSA FEEDAP Panel, 2018).

Further, the RA of GMMs is currently being developed towards an approach based on the assessment of the safety for food and feed use, independently of the non-genetically modified counterpart.

Designed new-to-nature sequences and sequences without a non-GM counterpart should follow a food and feed risk assessment per se, based on the molecular characterisation of the strain and on the safety of the product it produces, including e.g. testing the pathogenicity, toxicity, nutrition, allergenicity and gut–environment interactions of the product. A safety assessment per se has been already provided in the EFSA GMO Panel GMM 2011 guidance. For Category 4, see Section 4.1. As the technique-driven risk assessment has its limitations, especially for the assessment of SynBioM, a strain-driven approach can be envisaged for all future SynBioM assessments.

# 5. Conclusions

## 5.1. Identification of newer sectors/advances

ToR1: EFSA was asked to consider whether and which newer sectors/advances should be considered among SynBio developments, in addition to the six identified by the SCs. Previous conclusions on this ToR remain valid (EFSA Scientific Committee, 2020). In the current Opinion, additional literature was searched for new developments for all category SynBioM products for all different food/feed use applications falling under the remit of EFSA. The following conclusions are complementary to the previous ones:

- No other sectors/advances were identified in addition to the six identified by the SCs.
- There are no clear criteria to differentiate between a GMM and a SynBioM. Cases 1 and 4–15 are part of a continuum between classical GMM and SynBioM.
- From a technical point of view, there are SynBioM applications that could be ready for food and feed use in the EU in the next decade (Cases 1 and 4–15). However, xenobionts (Cases 2 and 3), falling within the remit of EFSA, are not expected for practical application in the next decade.
- Information on new SynBioM products may not be made publicly available at early stages of their development. This situation limits the predictive capacity of this Opinion.

## 5.2. New hazards/risks

ToR2: EFSA was requested to identify, if possible, potential risks in terms of impact on humans, animals and the environment that current and near-future SynBio developments could pose; EFSA was also asked to identify potential novel hazards compared with established techniques of genetic modification. For the molecular characterisation and ERA, conclusions have been published in a previous opinion (EFSA Scientific Committee, 2020). This Opinion is focused on the food and feed safety assessment for humans and animals (farmed and pets). The assessment was to identify novel hazard or risk and this should be performed on a case-by-case basis. Following a generic evaluation in this Opinion, it was concluded that SynBioMs may lead to novel hazards compared with microorganisms developed with established genetic modification techniques:

- Unusual and/or new-to-nature components:
  - needs attention for xenobionts containing XNA and/or producing xenoproteins to assess potential concerns regarding their presence, their stability and/or their potential degradation into harmful metabolites;
  - may trigger concern for allergenicity for new-to-nature proteins;
  - may cause imbalanced nutrition, e.g. by altering bioavailability;
  - may cause an adverse effect on the gut environment.

## 5.3. Adequacy of existing guidelines

ToR3: EFSA was requested to determine if the existing guidelines for risk assessment were adequate and sufficient for current and near-future SynBio developments or if there was a need for updated guidance.

The existing guidances relevant for the food and feed risk safety assessment of SynBioM are listed in this Opinion (Table 2). The relevant guidances must be selected according to the product and its intended use.

Concluding remarks on the adequacy of existing guidelines for the food and feed risk safety assessment of SynBioM:

- The categorisation of the food and feed products produced by GMM as proposed in the GMM guidance (EFSA GMO Panel, 2011) is applicable to SynBioMs and their products expected to reach the EU market in the near and wide future. Currently, Categories 1 and 2 are not being distinguished in practice.
- Relying on the QPS status for the safety assessment of building blocks of SynBioM is valid when there is sufficient familiarity with the SynBioM/chassis with the QPS microorganism.
- The existing EFSA guidances are generally adequate for assessing the product, the production process and the product preparation process and are applicable for the different products made from or with SynBioMs.
- Existing guidances are relevant for detecting the different viability stages of bacteria and fungi, including spores, and the presence of DNA from SynBioM in food and feed products.
- The EFSA GMO Panel (2011) guidance describes well the principles of the comparative approach that is also applicable for SynBioM. The use of a comparator in the risk assessment is adequate for those SynBioM (Categories 1–4) with sufficient familiarity with the non-GMM counterpart. SynBioM producing new-to-nature products and xenobionts would require a safety assessment per se for the new-to-nature components, as is provided already in the EFSA GMO Panel 2011 guidance. The GMM guidance of 2011 had foreseen that in cases when the parental organism of the GMM does not have a history of use in the particular application, conventional food products may still be used as comparators to identify possible compositional changes and to assess their safety implications.
- In general terms, existing guidance sufficiently covers genotoxicity and systemic toxicity assessment of SynBioM products, including those produced from and with XNA and/or xeno amino acids. Guidance for assessing SynBioM products for effects on the immune system, including inadvertent immunomodulation can be based on existing guidances from EFSA (EFSA NDA Panel, 2016b) and OECD (OECD, 2018a, TG443 and OECD, 2018b, TG408), respectively.
- The existing guidances define the general framework for the risk assessment of GMM interactions in the gastrointestinal environments. In general terms this is also adequate for SynBioMs. In more detail:
  - The GMM guidance (EFSA GMO Panel, 2011) on the 90-day rodent studies describes the importance of assessing the viability and the residence time of the GMM in the gut ecosystem. It also points out the need to study the interactions of the GMMs with the gut microbiota and their effects on digestive physiology and immune responses. This is also applicable to SynBio of all Categories 1–4. For Categories 1 and 2 products, this will only be relevant when they would have a potential effect on the microbiome.
  - The FEEDAP guidances provide more detailed guidance for assessing the potential effect of feed additives on the growth and persistence of pathogens in the gut environment.
  - Guidance on the use of bioinformatics analysis for measuring the potential of HGT is available.
- For the evaluation of allergenicity, existing guidances for proteins, non-IgE-mediated allergenicity, as well as sensitisation, are also applicable for SynBioM products, with limitations in the predictive tools for allergenic potential of new-to-nature proteins.
- The nutritional assessment of the food and feed products of Categories 1–4 SynBioMs can be based on existing guidances.
- The existing guidances are adequate for calculating exposure to most SynBioM products derived from fermentation (Categories 1–3) and for Category 4 products. Exposure of consumers and animals (farmed and pets) to viable microbial cells through food and feed or by secondary exposure routes (e.g. edible plants and water) are to be assessed on a case-by-case basis.
- EFSA guidances provide the principles for the PMM of SynBioM products.

## 5.4. Need for Updates of guidance or lack of methodologies

ToR4: In the latter case, EFSA was requested to identify the specific areas for which such updated guidance is needed. For food and feed safety assessment of SynBioMs, as well as for GMMs, the development of guidance and risk assessment tools are recommended as follows.

Updated guidance is recommended:

- For categorisation: Categories 1 and 2 products could be merged into one category, resulting in only three categories: a first category not containing recombinant DNA and viable cells, a second category containing recombinant DNA but no viable cells, and a third category containing viable cells (including recombinant DNA).
- For production processes:
  - Fermentations of xenobionts containing XNA and/or producing xenoproteins to assess potential concern regarding their presence, their stability and/or their potential degradation into harmful metabolites.
  - The specificities of the manufacturing and purification processes for non-GM, GM and SynBio protists/microalgae and bacteriophage fermentation, and for bacteriophages, also the possible formation of phages with transducing properties of genes coding for virulence factors and toxins.
- For detection:
  - of non-GM, GM and SynBio bacteriophages, protists and microalgae, in the final product;
  - of SynBioM with XNA and/or xeno amino acids on adapted culturing conditions and culture-independent detection;
  - of XNA, which would not be able to be amplified by conventional PCR.
- For the toxicological safety assessment: guidance is recommended for non-GM, GM and SynBioM bacteriophages; especially for those propagated in pathogens. This can be based on gained experience of already evaluated non-GM bacteriophages. Specific indications for tolerance and efficacy in insects as target species of feed additives should be developed for non-GMM, GMM and SynBioM.
- For the assessment of the effect of bacteriophages on the gut microbiome. This could be based on gained experience. This applies to non-GM, GMM and SynBioM.
- For oral exposure assessment of non-GM, GM and SynBioM used as PPP and biostimulants.
- For PMM:
  - Future updates would benefit from including descriptions of fit-for-purpose approaches to monitor for potential adverse effects of microorganisms (non-GMM, GMM and SynBioM).
- EFSA BIOHAZ (2010) guidance future updates may expand on bacteriophages, based on gained experience.

Development of risk assessment tools is recommended:

- For the gut microbiome:
  - No guidances are available for **methodologies** other than those for 90-day rodent studies for Category 4 GMM and other than for feed additives, nor for the **interpretation** of the results. Limited reference is made to the use of next generation sequencing techniques or other 'omic approaches to study the gut microbiome. No consensus exist as to what constitutes a healthy baseline in the analysis of gut microbiota.
  - Considering the complexity of the gut microbiome, general **methodology** is missing for **any product** (non-GM, GMM and SynBioM, new-to-nature compounds) to determine the effect on the microbiome structure and metabolism, as well as potential adverse effects derived from microbiome perturbations on gut functions (including metabolic, barrier defence and immune function). Methods to measure endpoints (e.g. potential persistence and colonisation, effects on the gut microbiome) should be explored to allow the correct interpretation of the impact of the product on the host in which it is used. The suitability of such methods depends on the type of microorganism, survival capacity and level of exposure, as well as on the host. It could be envisaged that the guidance would be

updated with uniform methods for certain endpoints when they become available and experience would be gained from the RA of such products. This would facilitate the interpretation of the results on microbial persistence and colonisation, as well as the potential overgrowth/disturbance of the microbiome balance (structure and functionality) and gut function.
  - Regarding the interpretation of observed effects on microbiome, internationally agreed criteria are needed to establish causality of the experimental observation and their relevance for risk assessment of a given substance.

- For assessing the HGT potential of sequences of concern present in non-GM, GM and SynBioM products and the impact of environmental conditions on transfer rates and possible adverse effects in the main receiving environment (e.g. the human and animal gut) and beyond.
- For assessing the allergenic potential of new-to-nature proteins.
- For nutrition:
  - Methodology for assessing bioavailability necessary for the nutritional assessment in general (i.e. for non-GMM, GMM or SynBioM) biomasses used for food. At present this is done on a case-by-case basis.
  - The nutritional assessment of new-to-nature products.

## 6. Recommendations

- In view of international efforts to harness the use of microorganisms in the food and feed chains, the EFSA SC recommends a concerted international effort towards developing internationally agreed guidance and harmonised frameworks for identifying and addressing living cell intake in the RA process.
- It is recommended that further research on testing methods for risk assessment is continued, including 'omics' high-throughput experimental studies, and the application of bioinformatics tools.
- As the technique-driven risk assessment has his limitations, especially for the assessment of SynBioM, a strain-driven approach can be envisaged for all future SynBioM assessments. Bioinformatics analysis of WGS will form the basis of this assessment.
- As a way to reduce the amount of data and studies required for the RA of SynBioM and their products, applicants should be encouraged to include food and feed safety aspects throughout the SynBio design.

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## Glossary

Referenceis made to the glossary of EFSA Scientific Committee, 2020. Key terminology used in this Opinion is repeated and specific terminology added in the list below:

| | |
|---|---|
| **Chassis** | A naturally derived or highly engineered organism repurposed to build, maintain and amplify the components necessary for deployment of synthetic biological systems and their applications. For this Opinion the meaning of the term deals with live cells containing an editable genome. It is noted that cell-free systems, reconstructed vesicles and nucleoid-dissolved cells (i.e. with no DNA) have also been occasionally described as a chassis. |
| **Cisgenesis** | Cisgenesis is the genetic modification of a recipient organism with a gene from a crossable - sexually compatible – organism (same species or closely related species). This gene includes its introns and is flanked by its native promoter and terminator in the normal sense orientation (EFSA GMO Panel, 2012). |
| **Comparative approach** | Analysis of potential adverse effects resulting from a GMM when compared with a counterpart with familiarity. |
| **Deliberate release** | Any intentional introduction into the environment of a GMM or a combination of GMMs for which no specific containment measures are used to limit their contact with and to provide a high level of safety for the general population and the environment. |
| **Design–Build–Test–Learn (DBTL)** | A workflow for synthetic biology applications that entails an iterative cycle of designing the system, building it, testing it and learning from the results of testing, often with the help of machine learning and artificial intelligence. This workflow mirrors those for engineering and computer sciences. |
| **Environmental risk assessment** | is defined as the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMMs may pose and carried out in accordance with Annex II of Directive 2001/18/EC. |
| **Familiarity** | The concept of 'familiarity' refers to the fact that most GMMs to be used for food or feed purposes belong to well characterised microbial species. This 'familiarity' allows the risk assessor to draw on previous knowledge and experience with the introduction of similar microorganisms into food and the environment. 'Familiarity' will also derive from the knowledge and experience available from the risk/safety analysis conducted before the scale-up of the microorganism in a particular environment (EFSA GMO Panel, 2006; OECD, 1993a). |
| **Genome Editing** | Technology in which DNA is inserted, deleted, modified or replaced in the genome of a viable organism. Genome editing targets the modifications to site-specific locations. As explanted in the Scientific Advice Mechanism (SAM) Explanatory note of April 2017 (SAM, 2017), genome editing aims to achieve a precise alteration of a DNA sequence in a cell or to achieve random changes at precise locations. |
| **Hazard** | A biological, chemical or physical agent in, or condition of, food or feed with the potential to cause an adverse health effect [from Regulation (EC) No 178/2002]. |
| **Intended effects** | Changes that are meant to occur due to the genetic modification and that fulfil the objectives of the genetic modification. |
| **Intragenesis** | Intragenesis is a genetic modification of a recipient organism that leads to a combination of different gene fragments from donor organism(s) of the same or a sexually compatible species as the recipient. These may be arranged in a sense or antisense orientation compared to their orientation in the donor organism. Intragenesis involves the insertion of a reorganised, full or partial coding region of a gene frequently combined with another promoter and/or terminator from a gene of the same species or a crossable species (EFSA GMO Panel, 2012). |
| **Metabolic engineering** | Metabolic engineering is generally defined as the re-direction of one or more enzymatic reactions to produce new compounds in an organism, improve the production of existing compounds or mediate the degradation of compounds. Metabolic engineering can also be used to expand the eco-physiology of SynBioM. |

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| **Microalgae** | A polyphyletic group of unicellular photosynthetic eukaryotes, typically found in freshwater and marine systems. |
| **Microbiome** | Microbiome refers collectively to communities of microorganisms and their combined genomes in a defined environment. |
| **Microorganism** | A definition of microorganism is provided in Article 2 of EC Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified microorganisms (European Commission, 2009): 'microorganism' means any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, including viruses, viroids, and animal and plant cells in culture. |
| **Minimal cells** | A cell whose genome only encodes the minimal set of genes necessary for the cell to survive and autonomous growth under specified conditions. |
| **Post-market environmental monitoring** | A risk management tool that provides a mechanism to monitor possible adverse environmental consequences of the GM product included in the risk assessment. In accordance with Annex VII of the Directive 2001/18/EC. |
| **Post-market monitoring** | A risk management tool that provides a mechanism to monitor possible untoward consequences of the GM product included in the risk assessment (EFSA GMO Panel, 2011). |
| **Probiotic** | The WHO definition on probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (WHO/FAO, 2006). |
| **Problem formulation** | The process including the identification of characteristics of the GMM capable of causing potential adverse effects on the environment (hazards), of the nature of these effects, and of pathways of exposure through which the GMM may adversely affect the environment (hazard identification). It also includes defining the assessment endpoints and setting specific hypotheses to guide the generation and evaluation of data in the next risk assessment steps (hazard and exposure characterisation). |
| **Protocells** | An approach to engineering novel biological systems working strictly from the 'bottom up' and attempting to construct new simple forms of living systems, using chemical and physical processes and using as raw ingredients only materials that were never alive. Currently, the systems constructed by bottom-up approaches are not viable organisms, but are chemical vesicles, called 'protocells'. |
| **Qualitative Presumption of Safety (QPS)** | This is a harmonised generic pre-assessment approach applied by EFSA for the safety of biological agents used in food and/or feed. This approach is based on extensive reiterative scientific literature review and absence of reported hazards or risks. |
| **Risk** | A function of the probability of an adverse health or environmental effect and the severity of that effect, consequential to a hazard [from Regulation (EC) No 178/2002]. According to the EC Council Decision of 2002,[19] risk is defined as the combination of the magnitude of the consequences of a hazard, if it occurs, and the likelihood that the consequences occur. |
| **Safe-by-design** | A principle aimed to develop safe new products (e.g. SynBioMs) by taking into account all aspects of the product, as well as of the process, from the initial ideas of the project, up to the well characterised final product. |
| **Synthetic Biology** | An interdisciplinary field at the interface of engineering and biology aimed to develop new biological systems and impart new functions to viable cells with potential applications (for this Opinion) in food and feed, and environment systems. |
| **Systems approaches** | The systems approach principle places individual system items in their environment and observes the relationship between them. This approach relies on large-scale mathematical and statistical models, as well as on semantic technologies, big data analytics and artificial intelligence. |

[19] European Commission, 2002. Council Decision of 3 October 2002 establishing guidance notes supplementing Annex VII to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L, pp. 27–36.

**Unintended effects** changes other than the intended changes in the GMM resulting from its genetic modification. Unintended effects are addressed in the safety and nutritional assessment of the GMM and/or their products under the existing GMM guidance document. Some can be predicted based on bioinformatics analysis.

**Xenobiology** A branch of SynBio that starts to design alternative biochemical components for bioengineering other than DNA or the 20 canonical amino acids.

## Abbreviations

| | |
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| ADME | absorption, distribution, metabolism, and excretion |
| AMR | antimicrobial resistance |
| CD | cluster of differentiation (protein) |
| CFU | colony forming unit |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| CRP | C-reactive protein |
| dsRNA | double-stranded RNA |
| ERA | Environmental Risk Assessment |
| FF | Food and Feed |
| GIT | gastrointestinal tract |
| GM | genetically modified |
| GMM | genetically modified microorganisms |
| GMO | genetically modified organisms |
| GMP | genetically modified plant |
| GSH | glutathione |
| HGT | horizontal gene transfer |
| IBDV | infectious bursal disease virus |
| MC | MICROBIAL Characterisation |
| mPPP | microbial plant protection product |
| ncAA | non-canonical amino acid |
| OECD | Organisation for Economic Cooperation and Development |
| OECD TG | OECD Technical Guideline |
| PMM | post market monitoring |
| PPP | plant protection products |
| PSA | Phage Scott A (from *Listeria monocytogenes*) |
| PTS | peroxisome-targeting signal |
| QPS | Qualified Presumption of Safety |
| RA | risk assessment |
| RCK | Resistance to Complement Killing |
| SAM | *S*-adenosylmethionine |
| SCENHIR | Scientific Committee on Emerging and Newly Identified Health Risks |
| SCHER | Scientific Committee on Health and Environmental Risks |
| SCCS | Scientific Committee on Consumer Safety |
| SCS | stop-codon suppression |
| SynBio | synthetic biology |
| SynBioM | synthetic biology of microorganisms |
| ToR | Term of Reference |
| TOS | total organic solids |
| TU | Taxonomic Unit |
| tRNA | transfer RNA |
| VICH | Veterinary International Cooperation and Harmonisation |
| VP | Virion protein |
| WGS | whole genome sequencing |
| XAA | xenobiotic amino acid |
| XNA | xenobiotic nucleic acid |

## Appendix A – Toxicological tests

Toxicological studies required will usually consist of *in vitro* tests for genotoxicity and *in vivo* studies for systemic toxicity. For **genotoxicity**, the following two *in vitro* tests are recommended as the first step (EFSA Scientific Committee, 2011): bacterial reverse mutation assay (OECD, 2020a, TG471), and *in vitro* mammalian cell micronucleus test (OECD, 2016a, TG487). If the Ames test is not applicable, alternatively a test for induction of gene mutations in mammalian cells, preferably the mouse lymphoma *tk* assay (OECD, 2016c, TG476), could be performed, but it needs to be justified. Following one or more positive *in vitro* tests, further testing may be required to determine whether the hazard is expressed *in vivo*, unless it can be adequately demonstrated that the positive *in vitro* findings are not relevant for the *in vivo* situation. In line with the recommendation of the EFSA Scientific Committee (2011 and 2017), the following *in vivo* tests are considered as a suitable follow-up for substances positive in the *in vitro* basic battery: the *in vivo* mammalian erythrocyte micronucleus assay for *in vitro* clastogens and aneugens (OECD, 2016b, TG474); the *in vivo* mammalian alkaline comet assay for substances that cause gene mutations and/or structural chromosomal aberrations (OECD, 2016d, TG489); the transgenic rodent gene mutation assay to follow-up *in vitro* positive compounds for gene mutations (OECD, 2020b, TG488); or a combination of an *in vivo* micronucleus assay and a comet assay following a positive *in vitro* micronucleus assay. For further guidance on the *in vivo* follow-up of substances positive in the *in vitro* basic battery, the Scientific Committee statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019) should be consulted.

For **systemic toxicity**, a subchronic oral toxicity study should be provided. The protocol according to the OECD (OECD, 2018b, TG408) is recommended. The 90-day study should allow for the identification of substances with the potential to cause neurotoxic, immunological, reproductive organ effects or endocrine-mediated effects. When kinetics testing indicates a lack of systemic availability, studies should at least investigate both pathological and physiological effects in the gastrointestinal tract. The effects of unabsorbed materials on gastrointestinal function and tolerance also need to be investigated. For 'whole foods', the testing requirements should be determined using a case-by-case approach, as special considerations are required for dose selection and the avoidance of possible nutritional imbalances. Relevant historical control data should be provided to enable the judgement of the validity of the study as proposed in the OECD guideline and in EC Regulation (EU) No 283/2013. Decisions on whether additional studies are needed will be taken by EFSA on a case-by-case basis, following the identification of an adverse effect.

A special case is the assessment of a potential adverse **effect on the immune system**. Outcome variable(s) can be measured *in vivo* in humans by generally accepted methods to substantiate a benefit of the food on specific functions to form a basis for a scientific substantiation of a health claim (EFSA NDA Panel, 2016b). These include: changes in numbers of various lymphoid subpopulations in the circulation, proliferative responses of lymphocytes, phagocytic activity of phagocytes, lytic activity of natural killer cells and cytolytic T cells, production of cellular mediators, serum and secretory immunoglobulin levels, delayed-type hypersensitivity responses and changes in markers of inflammation (including markers of chronic, subclinical inflammation), such as interleukins or C-reactive protein (EFSA NDA Panel, 2016b). Effects of foods in humans, even if designed to indicate beneficial effects on the immune system, may in principle also reveal adverse effects. It should be noted that generally the range of effects that can be measured in humans is hampered, while there are more possibilities to test in experimental animals. This is especially true for the immune system, as the functionality of the immune system can be more easily challenged in animals than in humans, while the most informative way to test for adverse effects on the immune system is to probe the functionality.

In the **extended one-generation reproduction toxicity study** (OECD, 2018a, TG443), cohort 3 is included, in which the functionality of the immune system is assessed by sensitising animals to a T-cell-dependent antigen, and investigating the effects of exposure to the potentially immunotoxic agent by measuring changes in specific antibody responses. In the recently adopted 'Scientific guidance for the preparation of applications on smoke flavouring primary products' (EFSA FAF Panel, 2021), it was decided to add the following parameters in a 90-day oral toxicity study to parameters already included (OECD, 2018b, TG408), if an extended one-generation reproduction toxicity study would not be provided: weighing lymphoid organs, histopathology of the lymphatic organs, including bone marrow cellularity. In blood: immunoglobulin isotypes; complement assays: total serum haemolytic activity or individual components; C-reactive protein (CRP). In the spleen: total and differential white blood cell count; phenotypic analysis of spleen cells [CD4 and CD8 T cells, regulatory

T cells, B cells, natural killer (NK) cells, macrophages]; mitogen stimulation assays for B and T cells, natural killer cell functional analysis, phagocytic activity.

The term **adjuvanticity** is most often used in the context of vaccination, in which it is judged as a beneficial activity aimed at boosting the immune response after vaccination. In the EFSA GMM 2011 guidance (2011), it is dealt with under the heading of allergenicity, in which the term is used as the inadvertent stimulation of immune responses to allergens, i.e. boosting of specific IgE responses and resulting increased risk of allergic reactions to common allergens. However, inadvertent stimulation of immune responses may not only lead to enhanced allergen-specific responses but may also lead to other undesired conditions such as inflammation or autoimmunity. In the context of toxicology, the term would indicate the inadvertent immunostimulation as a result of the food intake. Immunostimulation can be identified using the array of immunological measures indicated above. Judgement of the potential adversity of such effects should be done in the context of all information available, on a case-by-case basis. It is noted that currently there are no validated decisive *in vitro* methodologies for immunomodulators on which one could base a risk assessment.

## Appendix B – Examples of introduced modifications that can influence the gut microbiome

Several risks specifically linked to microorganisms, non-GM, GMM or SynBioM, and their products can be foreseen (Tyagi et al., 2016; Dou and Bennet, 2018). For example, the design of viable GMM/SynBio strains of microorganisms (Category 4) to increase their adhesion abilities to colonise the gut more effectively can displace other microbiota or disrupt the microbial balance, having an adverse long-term effect on the gut epithelial integrity. In a worst-case scenario it could lead to mucus invasion by undesirable microorganisms resulting in gut inflammation (Litvak and Bäumler, 2019). GMM/SynBioMs can also be designed to produce specific metabolites to be delivered in the gut that can be preferentially used by some dominant taxonomic units, therefore breaking microbial balance and metabolism.

Another example is when GMM/SynBioM strains, either viable (Category 4) or inactivated (Category 3), might be designed to boost the immune system and enhance resistance to pathogens. Heat-inactivated bacteria have been demonstrated to also affect the immunological functions of the exposed humans and animals (Akter et al., 2020), by perturbing gut homeostasis and host–microbiome interactions.

As another example, phages may be designed to inhibit enteropathogens. For phages, safety assessment of the interaction with the microbiome, has been based on: (1) the taxonomic identification of the bacteriophage, including host range of the bacteriophage; (2) a bioinformatics analysis of the available WGS of the bacteriophages belonging to the same family for toxins and virulence factors in the light of potential transduction; and (3) the property that the pertinent bacteriophages are strictly lytic with a one unit-length genome packaging mechanism with precise DNA termini recognition, preventing the formation of transducing bacteriophages. This approach was followed for the risk assessment and safety of bacteriophage Listex P100 (EFSA BIOHAZ Panel, 2016b) and the feed additive Bafasal® (EFSA FEEDAP Panel, 2021b).

# Appendix C – Public consultation on the draft scientific opinion on the evaluation of existing guidelines for their adequacy for the food and feed risk assessment of microorganisms obtained through synthetic biology

Appendix C can be found in the online version of this output ('Supporting information' section): https://doi.org/10.2903/j.efsa.2022.7479