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SCIENTIFIC OPINION

New developments in biotechnology applied to microorganisms

EFSA Panel on Genetically Modified Organisms (GMO) | Ewen Mullins | Jean-Louis Bresson | Ian Crawford Dewhurst | Michelle M. Epstein | Leslie George Firbank | Philippe Guerche | Jan Hejatko | Francisco Javier Moreno | Hanspeter Naegeli | Fabien Nogué | Nils Rostoks | Jose Juan Sánchez Serrano | Giovanni Savoini | Eve Veromann | Fabio Veronesi | Pier Sandro Cocconcelli | Debora Glandorf | Lieve Herman | Rodrigo Jimenez Saiz | Lorena Ruiz Garcia | Jaime Aguilera Entrena | Andrea Gennaro | Reinhilde Schoonjans | Dafni Maria Kagkli | Tamas Dalmay

Correspondence: nif@efsa.europa.eu

Abstract

EFSA was requested by the European Commission (in accordance with Article 29 of Regulation (EC) No 178/2002) to provide a scientific opinion on the application of new developments in biotechnology (new genomic techniques, NGTs) to viable microorganisms and products of category 4 to be released into the environment or placed on the market as or in food and feed, and to non-viable products of category 3 to be placed on the market as or in food and feed. A horizon scanning exercise identified a variety of products containing microorganisms obtained with NGTs (NGT-Ms), falling within the remit of EFSA, that are expected to be placed on the (EU) market in the next 10 years. No novel potential hazards/risks from NGT-Ms were identified as compared to those obtained by established genomic techniques (EGTs), or by conventional mutagenesis. Due to the higher efficiency, specificity and predictability of NGTs, the hazards related to the changes in the genome are likely to be less frequent in NGT-Ms than those modified by EGTs and conventional mutagenesis. It is concluded that EFSA guidances are 'partially applicable', therefore on a case-by-case basis for specific NGT-Ms, fewer requirements may be needed. Some of the EFSA guidances are 'not sufficient' and updates are recommended. Because possible hazards relate to genotypic and phenotypic changes introduced and not to the method used for the modification, it is recommended that any new guidance should take a consistent risk assessment approach for strains/products derived from or produced with microorganisms obtained with conventional mutagenesis, EGTs or NGTs.

KEYWORDS

CRISPR, genetically modified microorganism(s), GMM, new genomic techniques, NGT

CONTENTS

SUMMARY

EFSA was requested by the European Commission (in accordance with Article 29 of Regulation (EC) No 178/2002) to provide a scientific opinion on the application of new developments in biotechnology (new genomic techniques, NGTs) to microorganisms and products of category 4 to be released into the environment or placed on the market as or in food and feed, and to products of category 3 to be placed on the market as or in food and feed. Category 3 refers to 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. Category 4 refers to products consisting of or containing GMMs capable of multiplication or of transferring genes.

NGTs are defined as techniques altering the genetic material of an organism developed after the publication of Directive 2001/18/EC. Established genomic techniques (EGTs) refer to those developed prior to this date. Conventional mutagenesis refers to techniques that make use of e.g. physical or chemical mutagens applied to cells; even though conventional mutagenesis was developed prior to the entry into force of the Directive 2001/18/EC, it is excluded from Directive 2001/18/EC (Annex IB).

In particular, EFSA is requested to address the following Terms of Reference:

- Identify novel potential hazards and risks that new developments in biotechnology applied to microorganisms could pose for humans, animals and the environment (ToR1).
- Determine whether the existing guidelines for risk assessment of GMM are applicable, fully or partially, and sufficient to risk assess new development in biotechnology applied to microorganisms (ToR2).
- In case existing guidelines for risk assessment are considered not applicable, partially applicable or not sufficient, to identify on which aspects existing guidelines should be updated, adapted or complemented (ToR3).

ToR1

The most relevant NGT is the CRISPR-Cas mechanism to modify the genomes of microorganisms by introducing either specific or random mutations in targeted genome regions.

The EFSA GMO Panel did not identify any novel potential hazard to humans, animals and the environment of NGTs applied to microorganisms compared to EGTs or compared to conventional mutagenesis, related to the technique itself. Some NGTs can produce mutations in microorganisms that also occur in nature. Due to the higher efficiency, specificity and predictability of NGTs, the hazards related to the changes in the genome are likely to be less frequent in microorganisms developed with NGTs (NGT-Ms) than those modified by EGTs and conventional mutagenesis.

Given that no novel potential hazards of NGT-Ms were identified as compared to those obtained by EGTs, or by conventional mutagenesis, no novel potential risks to humans, animals and the environment are expected even if there is exposure. Potential hazards may be related to new trait(s) regardless of the technique used and a product-based risk assessment is proposed for NGT-Ms and those obtained by EGTs, or by conventional mutagenesis.

ToR2

A horizon scanning exercise and an EFSA call for data informed the GMO Panel about NGT-Ms and their products and confirmed the fact that the majority of the identified products are to be expected on the market in the next 10 years. A total of 35 NGT-Ms and their products were identified by the horizon scanning; 57% of which belonging to category 4 and 43% to category 3 products. A total of 77% of the reported cases made use of CRISPR-based techniques, and the remaining 23% of a combination of EGTs and NGTs. The subject organisms include yeasts (63%), bacteria (31%), fungal endophyte and microalgae (3% each). The results of the horizon scanning and the EFSA call for data, indicated that a variety of products containing NGT-Ms, falling within the remit of EFSA, are expected to be on the market in the next 10 years.

The relevant legislative framework and guidance documents were summarised and then challenged with a selection of 13 case studies in which NGTs were applied for a variety of category 3 and 4 products. Of these guidance documents only some principles and recommendations, or parts of them, can be used for the purpose of the risk assessment of NGT-Ms due to the nature and diversity of the microorganisms (i.e. bacteria, yeasts, microalgae, viruses) and their applications. It is concluded that EFSA guidances are 'partially applicable' therefore, on a case-by-case basis, for specific NGT-Ms, fewer requirements may be needed. Some of the EFSA guidances are 'not sufficient' and updates are recommended.

Because possible hazards relate to genotypic and phenotypic changes introduced and not to the method used for the modification, it is recommended that any new guidance should take a consistent risk assessment approach for strains/products derived from or produced with microorganisms obtained with conventional mutagenesis, EGTs or NGTs.

ToR3

For the risk assessment of GMMs, including NGT-Ms, update of the guidance(s) is recommended on the following topics:

- For comparative approach
  - include microorganisms with no history of safe use

- For microbial characterisation
  - develop phenotypic characterisation and interpretation of WGS data for protists/microalgae and viruses
  - develop methodologies to determine antimycotic resistance of viable yeasts/fungi
- For toxicology
  - reduce the dependence on animal (rodent) studies
- For gut microbiome
  - establish suitable endpoints and validated methodologies to assess effects on the gut microbiome
- For allergenicity
  - consider adjuvanticity in relation to allergenicity
- For exposure
  - address primary and potential secondary exposure for all uses and microorganisms under the remit of EFSA
- For environmental risk assessment (ERA)
  - include all uses and microorganisms under the remit of EFSA
  - detail all areas of risk as per Commission Directive (EU) 2018/350 (Annex II Section D.1)
  - consider cases in which the HGT assessment and PMEM may not be needed
  - include fit-for purpose approaches to monitor for potential adverse environmental effects
  - broaden scope to include all uses under the remit of EFSA

Specific for the risk assessment of NGT-Ms an update is recommended on the analysis of the presence/absence of CRISPR-Cas or similar systems intentionally introduced in the microorganism.

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

In 2011, the EFSA GMO Panel issued its Guidance on the risk assessment of genetically modified microorganisms (GMMs) and their products intended for food and feed use (EFSA GMO Panel, 2011a). In this guidance, the principles of the risk assessment of GMMs were developed, starting with categorising them in four groups depending on the type of product and the degree of purification.[1]

In 2020, in the frame of the European Commission (EC) mandate (mandate M-2018-0205) on synthetic biology, EFSA commissioned a horizon scan of synthetic biology developments for microorganisms with application in the agri-food sector.[2] Cases were listed with the corresponding genetic modification technique used, the status towards commercialisation and the intended application. This work supported the development of two EFSA Opinions regarding risk assessment aspects of synthetic biology applied to microorganisms:

- The 'Evaluation of existing guidelines for their adequacy for the microbial characterisation and environmental risk assessment of microorganisms obtained through synthetic biology' was published in 2020 (EFSA Scientific Committee, 2020).
- The 'Evaluation of existing guidelines for their adequacy for the food and feed risk assessment of microorganisms obtained through synthetic biology' was published in 2022 (EFSA Scientific Committee, 2022).

The EC study[3] on new genomic techniques (NGTs) published in April 2021 covered the use of NGTs in plants, animals and microorganisms, in a broad variety of potential applications, including the agri-food, medicinal and industrial sectors. The study was supported by expert work, including a report of the Commission Joint Research Centre (JRC) on current and future market applications of NGTs. This report indicated that NGTs have potential for microbial strain improvement and are already being used by several companies. NGTs are becoming standard tools, along with established techniques such as classical mutagenesis and recombinant DNA technology. At the same time, the report underlined that collecting data on NGT-derived microorganisms was challenging in terms of identifying data sources. A survey to private companies resulted in a relatively small number of companies participating, compared with those providing information on the plant sector, and generally the answers disclosed less detail (e.g. microbial strain, specific technique used, or specific trait obtained).

The Commission's study on NGTs also revealed that safety data are mainly available for genome editing in plants, making it difficult to draw relevant conclusions on other techniques and on animals or microorganisms. It therefore recognised the importance of generating relevant information in these areas.

Based on the above, the EC requested EFSA to produce an opinion (in accordance with Article 29 of Regulation (EC) No 178/2002) on new developments in biotechnology applied to microorganisms. For the purpose of this opinion, and in accordance with the conclusion of the Commission's study on NGTs, EFSA is requested to perform the following two-step work on (i) microorganisms and products of category 4 to be released into the environment or placed on the market as or in food and feed;[4] (ii) products of category 3 to be placed on the market as or in food and feed.[5]

The mandate of the EC requires two deliverables: (I) a horizon scanning on microorganisms and their products obtained by new developments in biotechnology and (II) an EFSA opinion on potential novel hazards/risks from new developments in biotechnology applied to microorganisms and adequacy of the current EFSA risk assessment guidance.

I) Horizon scanning on microorganisms and their products obtained by new developments in biotechnology. For the purpose of this task, EFSA can use the JRC report mapping current and future market applications of organisms developed by new genomic techniques as a basis to complement and update.

The expected outcomes of this activity are:

1. Identify microorganisms and their products obtained by new developments in biotechnology described since 2001 including their traits and uses.
2. List the techniques and modifications used, including an explanation of relevant terminology.
3. Identify microorganisms and their products developed since 2001 subject to authorisation procedures by international authorities as well as the available risk assessment should they already exist.

[1]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, most 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); Category 4: Products consisting of or containing GMMs capable of multiplication or of transferring genes (e.g. live starter cultures for fermented foods and feed).

[2]van der Vlugt, C. J. B. (2020). Horizon scan of synthetic biology developments for microorganisms with application in the agri-food sector. EFSA Supporting Publication, EN-1664. https://doi:10.2903/sp.efsa.2020.EN-1664.

[3]https://food.ec.europa.eu/plants/genetically-modified-organisms/new-techniques-biotechnology/ec-study-new-genomic-techniques_en.

[4]I.e. microorganisms subject to the provisions of Directive 2001/18/EC or Regulation (EC) No 1829/2003.

[5]I.e. products subject to the provisions of Regulation (EC) No 1829/2003.

4. Information on risk assessment approaches taken by risk assessors and potential available guidances for the risk assessment.

II) EFSA opinion on potential novel hazards/risks from new developments in biotechnology applied to microorganisms and adequacy of the current EFSA risk assessment guidance.

Based on the horizon scanning report, EFSA is requested to produce an opinion on new developments in biotechnology applied to microorganisms. The expected outcomes of this activity are:

1. Identify novel potential hazards and risks that new developments in biotechnology applied to microorganisms could pose for humans, animals and the environment (ToR1).
2. Determine whether the existing guidelines for risk assessment of GMM are applicable, fully or partially, and sufficient[6] to risk assess new development in biotechnology applied to microorganisms (ToR2).
3. In case existing guidelines for risk assessment are considered not applicable, partially applicable or not sufficient, to identify on which aspects existing guidelines should be updated, adapted or complemented (ToR3).

1.2 | Definition of new developments in biotechnology for the Terms of Reference

- With the term new developments in biotechnology, the GMM NGT Working Group refers to **New Genomic Techniques** (NGTs) as techniques which are capable of altering the genetic material of an organism, and which have been developed after the publication of Directive 2001/18/EC (Broothaerts et al., 2021). The techniques taken into consideration in the context of the mandate are described in Section 3.2.1.
- With the term **Established Genomic Techniques** (EGTs), the GMM NGT WG refers to the genomic techniques developed prior to the publication of Directive 2001/18/EC (Broothaerts et al., 2021) excluding conventional mutagenesis.
- With the term **conventional mutagenesis,** the GMM NGT WG refers to the genetic modification technique which makes use of e.g. physical or chemical mutagens applied to cells. The resulting modified microorganisms are excluded from the requirements of Directive 2001/18/EC (see Annex IB of Directive 2001/18/EC for details on the excluded techniques). For the purpose of this mandate, the term 'classical mutagenesis' and 'conventional mutagenesis' are inter-changeably used.

NGTs	Techniques after Directive 2001/18/EC
EGTs	Techniques prior to Directive 2001/18/EC
Conventional (or classical) mutagenesis	Technique excluded from requirements of Directive 2001/18/EC (Annex IB)

1.3 | Interpretation of the Terms of Reference

Because in some cases the same trait can be introduced by EGTs, NGTs or conventional mutagenesis the recommended update for guidance, where relevant, also includes microorganisms obtained by conventional mutagenesis or EGTs.

The products on which this mandate focuses on are category 3 and 4 products. These categories were defined in the EFSA GMM Guidance (EFSA GMO Panel, 2011a, 2011b) and are interpreted for the scope of this mandate as follows:

Category 3: Products containing the DNA of the inactivated GMM and in which no viable cells are present (placed on the market as or in food and feed, e.g. heat-inactivated starter cultures, biomasses, single-cell protein preparations).

Category 4: Products containing viable cells of the production microorganism (e.g. live starter cultures for fermented foods and feed, microbial plant protection products (PPPs)).

In the context of this mandate, and in particular with regard to ToR2 and ToR3, the terminology received by the European Commission was interpreted as follows (see also Figure 1):

- All principles and recommendations included in the selected EFSA Guidance documents apply to the risk assessment of NGT-microorganisms (NGT-Ms). Hence, these selected guidances would be considered 'fully applicable' for the purpose of the risk assessment of NGT-Ms.
- Some existing principles and recommendations do not apply to the risk assessment of NGT-Ms. Hence, these selected EFSA guidance documents would be 'partially applicable'.
- Sufficient means that there is no need for further additions to/modifications of the existing text of the EFSA Guidance documents consulted. Hence, the EFSA Guidance is sufficient for the purpose of completing the risk assessment and does not need to be complemented.

[6]In the context of this mandate, 'applicable' means 'that can be used for the purpose', 'fully applicable' means 'that can be used in full', 'partially applicable' means 'that can be used only in part' and 'sufficient' means 'that does not need to be complemented'.

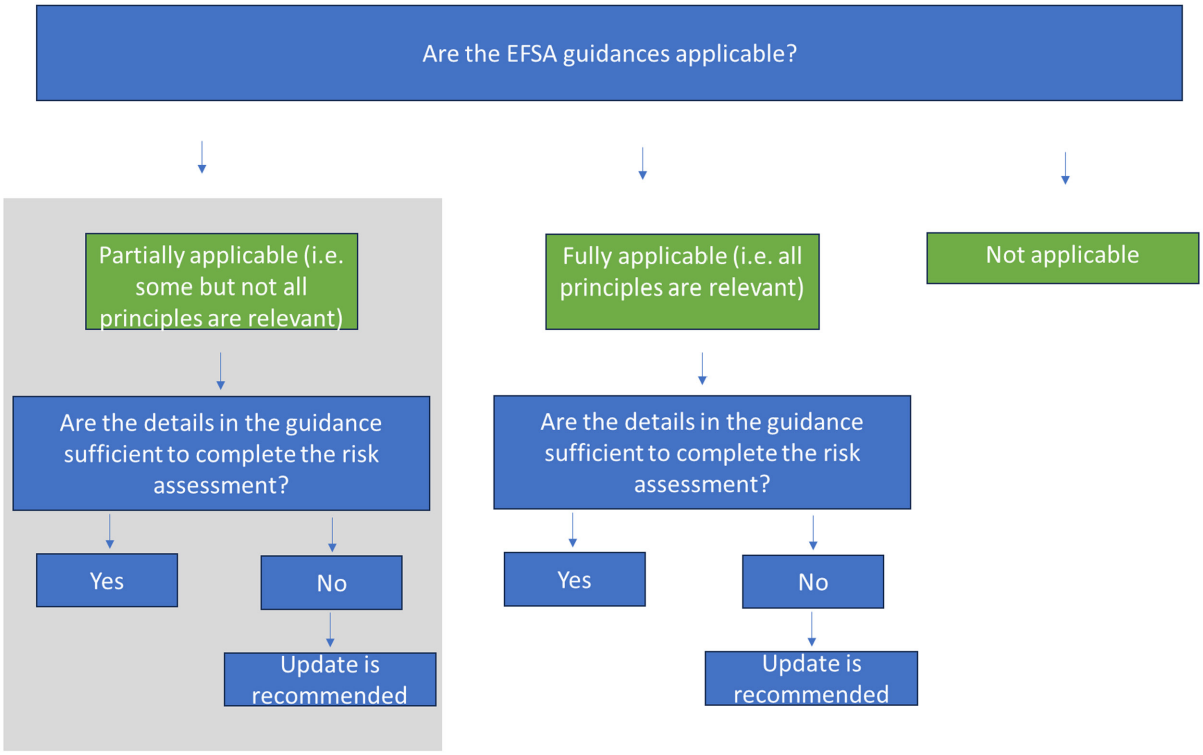

FIGURE 1 Flowchart to assist in the interpretation of the applicability and sufficiency of the relevant guidance documents for the risk assessment of GMMs. The grey box indicates that only partially applicable guidance documents were identified in this opinion, due to the nature and diversity of the microorganisms (i.e. bacteria, yeasts, microalgae, viruses) and their applications.

For answering the terms of reference (ToRs) 1, 2 and 3, specific questions were addressed (see protocol in Annex A) as below:

ToR1: Identify novel potential hazards and risks that new developments in biotechnology applied to microorganisms could pose for humans, animals and the environment.

*AQ1. What are the new **techniques/approaches** developed since 2001 (namely, new developments in biotechnology) which could be applied/are applied to microorganisms?*
*AQ2. Are there any novel **hazards** that these new developments in biotechnology applied to microorganisms could pose to humans, animals and the environment, as compared to established genomic techniques and conventional mutagenesis?*
*AQ3. Are there any novel **risks** that these new developments in biotechnology applied to microorganisms could pose to humans, animals and the environment, as compared to established genomic techniques and conventional mutagenesis?*

ToR2: Determine whether the existing guidelines for risk assessment of GMMs are applicable, fully or partially, and sufficient to risk assess new developments in biotechnology applied to microorganisms.

AQ1. What kind of GM microorganisms and GM microbial products within the EFSA remit have been identified that were developed using new developments in biotechnology?
AQ2. What kind of GM microorganisms and GM microbial products within the EFSA remit can be expected in the next 10 years to be developed using new developments in biotechnologies?
AQ3. Which are the existing guidelines to be used for the risk assessment of these GMMs?
AQ4. Are the existing guidelines for risk assessment applicable, fully or partially, and sufficient for the risk assessment of GMMs generated with the use of the new developments in biotechnology?

ToR3: In case existing guidelines for risk assessment are considered not applicable, partially applicable or not sufficient, to identify on which aspects existing guidelines should be updated, adapted or complemented.

AQ1. Which aspect (if any) of existing guidelines should be updated, adapted or complemented?
AQ2. What recommendations can be formulated for future guidance updates?

EFSA evaluated in previous opinions the existing guidelines for their adequacy for risk assessment of microorganisms obtained through synthetic microbiology. These opinions describe the microbiological characterisation, the environmental and the food and feed risk assessment (EFSA Scientific Committee, 2020, 2022). The NGTs considered in the present opinion are techniques that may be used in synthetic biological approaches, already considered in the two opinions related to

synthetic microbiology. The present opinion will update specific sections related to recent technological developments and will further expand on previous opinions.

1.4 | General outline of risk assessment for genetically modified microorganisms

Risk assessment is a scientifically based process consisting of four steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation. In this process, the likelihood and severity of an adverse effect(s) to humans, animals or the environment resulting from exposure to the GMMs is evaluated.

The comparative approach is a key general principle in the risk assessment of GMMs. It consists in the comparison of the characteristics of the GMM against those of a comparator (generally the non-GMM), with the aim to identify potential hazards resulting from the newly introduced trait(s). If a hazard is identified, the associated risk is evaluated considering exposure levels. Whenever an appropriate comparator is available, hazard identification is focused on the identification of differences between the GMM and the comparator. The comparative approach is further discussed in Section 3.2.3.1.

Risk assessment of GMMs is performed on a case-by-case basis to identify and evaluate potential adverse effects resulting from the genetic modification, either direct or indirect, immediate or delayed, on human and animal health and the environment.

In addition to the risk assessment in compliance with the EU specific legislation on GMOs, (Directive 2001/18/EC, Directive (EU) 2018/35, Regulation (EC) No 1829/2003) the specific use of a GMM might need additional assessment under other legal frameworks (e.g. food additives, feed additives, plant protection products). Such risk assessment of GMMs seeking an authorisation in the EU under specific sectoral regulations is described in the relevant EFSA Guidances.

The main elements of the risk assessment of GMMs are:

- Microbial and molecular characterisation: to identify and characterise the microbial strain and to identify related hazards (e.g. antimicrobial resistance (AMR), virulence, pathogenicity, toxin production).
- The food and feed risk assessment: to assess potential adverse effects to human and animal health when used in food or in feed. Herewith, toxicological, nutritional and allergenic effects are assessed together with potential effects on the gut microbiome.
- The environmental risk assessment (ERA): to assess potential adverse effects to the environment resulting from the deliberate release of the GMM. The ERA takes into account all receiving environments where the GMM will be introduced and proliferate resulting from both primary (direct introduction) and secondary (indirect e.g. in manure) routes of exposure (See also Section 3.2.3.9). Humans and animals are considered as part of the environment because they may come into contact with GMMs (e.g. inhalation, skin contact, incidental consumption). An ERA is further complemented with post-market environmental monitoring.

2 | DATA AND METHODOLOGIES

2.1 | Ad hoc expert working group and its methodology

EFSA established an ad hoc expert Working Group (GMM NGT WG) of the EFSA GMO Panel for the development of this opinion. In delivering its Scientific Opinion, the EFSA GMO Panel, together with the GMM NGT WG, considered:

- the current legislation and corresponding (EFSA) guidance documents (see Section 3.2.2 and Table 2)
- results derived from the call for data launched by EFSA (see Section 2.3) and the horizon scanning report on available published information for the identification of relevant case studies (Ballester et al., 2023)

The methodology adopted by the WG to address the ToRs, including the use of the relevant case studies to answer the specific questions of the ToRs, is published on EFSA's website as supporting document of this Scientific Opinion (Annex A sections A.2.1 and A.3- Protocol).

2.2 | Consultations

In line with EFSA's policy on openness and transparency, an online public consultation was done after the endorsement of the opinion by the GMO Panel (February 2024). Stakeholders were invited to submit their comments on the draft Scientific Opinion between February 2024 and April 2024.[7] Following this consultation process, the document was revised by the members of the GMM NGT WG and the EFSA GMO Panel. The comments received were considered and, if appropriate, incorporated into the current Opinion. The outcome of the public consultation is reported in detail and will be published on

[7] https://connect.efsa.europa.eu/RM/s/publicconsultation2/a0ITk000000C3VB/pc0848.

EFSA's website as supporting document together with this Scientific Opinion as adopted by the EFSA GMO Panel (Annex B).

2.3 | Horizon scanning contractor and call for data

In accordance with the ToRs of the mandate, EFSA assigned to an external contractor the horizon scanning on microorganisms and their products obtained by new developments in biotechnology under the framework contract OC/EFSA/MESE/2022/03 – CT 01.

In addition, to capture additional data from public and private technology providers, including confidential data, EFSA Nutrition and Food Innovation (NIF) Unit launched a public call for data from the 7th of March until the 30th of April 2023 on the EFSA website.[8]

The methodology, study selection and appraisal of results of the horizon scanning and the survey are described in the protocol and published on EFSA's website as supporting document together with this Scientific Opinion as adopted by the EFSA GMO Panel (Annex A).

2.4 | Selection and description of the case studies

In search of relevant cases to support the answering of the ToR, the GMM NGT WG considered all the information available from the Horizon scanning, the call for data, the recent scientific literature and applied its expert judgement. From the many possible organisms/uses, 13 case studies represent a variety of needs for testing the existing guidances for this Scientific Opinion (Table 1) and are used by way of non-exhaustive examples only. Table 1 specifies the aim of the genetic modification and the category of the product as it was further used in the development of this opinion.

For selecting the most relevant, non-exhaustive list of case studies, the following criteria were used:

- Different types of NGT-microorganisms (NGT-Ms), virus, bacteria, yeast, filamentous fungi and algae, falling into category 3 or category 4, within the remit of EFSA.
- Development has advanced and the product is likely to reach the market in the next 10 years.
- Different routes of exposure (intended use such as food, feed or agricultural application) and anticipation of hazards and risks for humans, animals and the environment.
- If possible, different techniques based on NGTs to be represented.
- Based on recent developments in the field, the GMM NGT WG created three additional case studies potentially reaching the (EU) market in the next 10 years.

[8] https://www.efsa.europa.eu/en/call/survey-new-biotechnologies-microorganisms.

TABLE 1 Case studies selected that include different microorganisms, different applications and different NGT techniques used. RA: Risk assessment.

Case study number	Case study name	Microorganism	Product	Category	Technique(s) used Short description of the modification(s) and its scope	Short description of RA retrieved
1	Pivot Bio PROVEN (Wen et al., 2021)	*Klebsiella variicola* KV137-1036 ΔnifL::PinfC	Nitrogen fixing bacterium as fertiliser for cereal crops	4	Targeted deletion and endogenous promoter insertion by CRISPR/Cas9; Deletion of negative (*nifL*) regulator and insertion of endogenous constitutive promoter upstream *NifA* with the purpose to increase *nif* expression also under higher nitrogen conditions	The company has done studies based on US EPA test guidelines to determine potential toxicity, pathogenicity, and irritancy
2	Non-toxic endophytic *Epichloë coenophiala* (Florea et al., 2021)	*Epichloë coenophiala* e19 ΔEAS1 ΔEAS2	Endophytic fungus of tall fescue (forage grass) devoid of alkaloid production	4	CRISPR-mediated knock out of 2 alkaloid gene clusters Deletions were introduced in the ergot alkaloid biosynthesis clusters *EAS1* (196 kb) and *EAS2* (79 kb) to make this fungus non-toxic for cattle who feed on tall fescue	No RA retrieved
3	CRISPR-Cas systems for genome editing in *Clostridium butyricum* (Zhou et al., 2021)	*Clostridium butyricum*	Probiotic culture of *Clostridium butyricum* for humans and animals	4	Type II CRISPR-Cas9 system and endogenous Type I-B CRISPR-Cas system Deletion of *spo0A* and *aldH* genes in *C. butyricum*, involved in sporulation and energetic metabolism. Proof of concept of the efficacy of the methodology. Increased butyrate production	No RA retrieved
4	CRISPR/nCas9-assisted genome editing system for exopolysaccharide biosynthesis in *Streptococcus thermophilus* (Kong et al., 2022)	*Streptococcus thermophilus*	Bacterial starter culture for dairy products with modified viscosity	4	Genome editing toolbox (pKLH353) based on CRISPR/nCas9 (Cas9 nickase) Deletions of target genes within the eps biosynthetic cluster: Multi-gene editing toolkit based on CRISPR/nCas9 was developed in *S. thermophilus* and employed to create deletions of either *epsA, epsB, epsC*, *epsE* or *epsG* genes. The impact of the different deletions caused changes in the molecular weight, viscosity, and monosaccharide composition of EPS	No RA retrieved
5	*Saccharomyces cerevisiae var. boulardii* as a probiotic strain (Durmusoglu et al., 2021)	*S. cerevisiae var boulardii*	Vitamin-producing probiotic that can colonise the gut (as demonstrated in germ-free mice)	4	Transformation and *CRISPR-Cas12a* nucleases-mediated gene insertion into *S. boulardii* CRISPR-based integration of genes encoding beta carotene biosynthesis pathway	No RA retrieved

(Continues)

TABLE 1 (Continued)

Case study number	Case study name	Microorganism	Product	Category	Technique(s) used Short description of the modification(s) and its scope	Short description of RA retrieved
6	The Korean Rice Wine-(Makgeolli) (Jung, Kang, Hwang, Baek, & Seo, 2023)	*Saccharomyces cerevisiae* GRL6	Starter culture to brew Makgeolli (the Korean traditional rice wine) with a lower content of ethyl carbamate as compared to the parental strain	4	CRISPR-mediated knock out of the genes CAR1 and GZF3, which regulate urea metabolism and ultimately ethyl carbamate production. CAR1 encodes for arginase, responsible for urea formation. GZF3 encodes the transcription factor that represses DUR1, 2 and 3 gene expression, which are genes that mediate urea absorption and degradation. CAR1 expression was abrogated and expression of DUR1, 2 and 3 was increased in the modified strain. Cas9-Hyg plasmid was used for the Cas9 expression for target-specific double-strand DNA breakage in the genome of *S. cerevisiae*. The pRS42K-gRNA-CAR1 and pRS42K-gRNA-GZF3 plasmids were constructed for the sgRNA expression to guide the CAR1 and GZF3 gene deletion by Cas9, respectively	No RA retrieved
7	Guided Biotics®, a technology platform that uses CRISPR-Cas systems to precisely edit the genes of specified bacteria within a microbiome and to regulate the metabolic functions of microbes in a microbiome One commercial product (BiomElix) is already on the market by Folium Science[9] *Escherichia coli* containing two plasmids one of which is conjugative to *Salmonella enterica* (https://worldwide.espacenet.com/patent/search/family/057217569/publication/US10463049B2?q=pn%3DUS10463049B2)	*Escherichia coli*	*Escherichia coli* containing two plasmids one of which is conjugative to *Salmonella enterica*	4	Existing genetic modifications were used. The effect of the GMM is, however, based on CRISPR/Cas technology. *E. coli* containing two plasmids, one containing a microcin expression cassette and another one containing the CRISPR/Cas3 genes engineered to be constitutively expressed. The plasmids have the machinery to be conjugated/introduced to *Salmonella enterica*. The Cas3 enzyme initiates the nuclease activity on *Salmonella*-specific pathogenicity islands (SPIs) leading to the degradation of *Salmonella enterica*. The microcin (peptide with bactericidal action, naturally present in *E. coli*) has a synergistic function. This case study is designed to drive the transmission of genetic elements in a target population	Approved by CTNBio (Brazil) as non-GMO

[9]https://foliumscience.com/products/ accessed 20/06/2024.

TABLE 1 (Continued)

Case study number	Case study name	Microorganism	Product	Category	Technique(s) used Short description of the modification(s) and its scope	Short description of RA retrieved
8	Lipid production in *Nannochloropsis gaditana* is doubled by decreasing expression of a single transcriptional regulator (Ajjawi et al., 2017)	*Nannochloropsis gaditana* (microalga)	A microalga that produces twice as much lipid than wild type to be used as food and feed	3	CRISPR/Cas9 mediated mutation of transcription factor $Zn(II)_2Cys_6$ Attenuation of $Zn(II)_2Cys_6$ allows higher C partitioning of carbon into lipids and the mutant can grow under reduced N as well as in normal conditions	No RA retrieved
9	Modification of T2 phage infectivity toward *Escherichia coli* O157:H7 using CRISPR/Cas9 (Hoshiga et al., 2019)	T2 bacteriophage	Expand the host-range of *E. coli* O157:H7-specific phages	4	CRISPR/Cas9 system Modification of both long and short phage tail fibres to widen the host range	No RA retrieved
10	*Saccharomyces cerevisiae* producing hop compounds (Denby et al., 2018)	*Saccharomyces cerevisiae* (beer yeast strain WLP001)	*Saccharomyces cerevisiae* beer yeast engineered to over-produce geranyl pyrophosphate (GPP) and convert this further into two hop aroma compounds, linalool and geraniol	3	CRISPR-Cas-based integration (using Cas9) of synthetic DNA. The native *ADE2* gene was first deleted and re-inserted together with the constructs. This allowed discrimination of mutants based on colour. Integration was realised in all 4 *ADE2* chromosomal loci of the tetraploid starting strain. G418 resistance was used to select for the Cas9 plasmid. The plasmid (and marker) was lost after the transformation. Four major modifications were made: 1) Overexpression by the native CCW12 promoter of a truncated native *HMG1* gene lacking its regulatory domain (mutant allele called tHMGR, resulting in over-active HMG-CoA reductase), 2) Expression of a mutated native FPPS-encoding gene *ERG20* driven by the native *TEF1* promoter (reduced FPP synthase activity) 3) Expression of truncated (lacking the plastid targeting sequence) and codon-optimised *Mentha citrate* (mint) linalool synthase (t67-*Mc*LIS) 4) expression of *Ocimum basilicum* (Basil) synthetic, codon-optimised geraniol synthase gene (*Ob*GES)	FDA GRAS clearance for the commercial variant of the strain, yBBS002 to Berkeley Brewing Science (GRAS Notice GRN No. 798)

(Continues)

TABLE 1 (Continued)

Case study number	Case study name	Microorganism	Product	Category	Technique(s) used Short description of the modification(s) and its scope	Short description of RA retrieved
11*	*Hypothetical case study* - Adaptive laboratory evolution using CRISPR-Cas mutagenesis yields probiotic *Saccharomyces cerevisiae* with improved acid tolerance. (Appendix A)	*Saccharomyces cerevisiae* var *boulardii*	Variants with improved tolerance to organic acids. Strain to be used as probiotic (food and feed uses)	4	A targetable Cas3 enzyme fused to a base editor (cytidine deaminase) to introduce random mutations in two genes, *TRT2* and *IRA2*, that have previously been linked to acid resistance in *S. cerevisiae*. WGS sequencing revealed 3 mutations: Two of these were located within the IRA2 gene. A third mutation was found elsewhere in the genome, outside the target region, in a non-coding region 27 nucleotides upstream of the *ATF1* gene, which is involved in the production of volatile acetate esters	No RA retrieved
12*	*Hypothetical case study* - Improving acetic acid tolerance of a *Saccharomyces cerevisiae* probiotic yeast by introducing natural alleles through CRISPR-Cas genome editing (Appendix A)	*Saccharomyces cerevisiae* var *boulardii*	Acid tolerant variants to survive the harsh GI tract conditions (resistance to acid)	4	CRISPR/Cas introduction of three natural alleles of genes TRT2, IRA2 and MET4 in *S. cerevisiae var boulardii*. Apart from the intended mutations, unintended mutations were also reported, including a chromosome duplication, point mutations in a cell-surface protein that confers adhesion to surfaces and tissues, and a deletion of 60 nucleotides	No RA retrieved
13*	*Hypothetical case study* - ***SCRaMbLE*** *Saccharomyces cerevisiae to generate a beer yeast that produces lycopene* (Appendix A)	*Saccharomyces cerevisiae*	Beer that contains lycopene, which gives the beer an attractive red colour and is also a known antioxidant with a potential beneficial health effect	3	Synthetic Chromosome Rearrangement and Modification by LoxP-mediated Evolution (SCRaMbLE 2.0) One synthetic chromosome (chromosome II) was mated to a natural strain (S288c/BY4741) to generate a diploid strain that carries one synthetic and one natural copy of chromosome II in addition to the 15x2 natural chromosomes Next, the lycopene biosynthesis pathway (4 genes derived from tomato, driven by a yeast promoter) was inserted on a plasmid Next, SCRAMBL was induced generating a population of yeasts that show genomic rearrangements, mutants with improved lycopene production were picked	No RA retrieved

Note: Some products can be classified as category 3 or 4 depending on the exact process implementation, for example depending on whether the production process includes a step that kills/inactivates the microorganism (e.g. pasteurisation). The cases were evaluated based on the category indicated in Table 1.

*Details on the hypothetical cases can be found in Appendix A.

3 | ASSESSMENT

3.1 | ToR1: Identify novel potential hazards and risks that new developments in biotechnology applied to microorganisms could pose for humans, animals and the environment

3.1.1 | AQ1. What are the new techniques/approaches developed since 2001 (namely, new developments in biotechnology) which could be applied/are applied to microorganisms?

The most relevant novel technique developed since 2001 in the field of genetic modification of microbes is the exploitation of the CRISPR-Cas mechanism occurring naturally in prokaryotes to modify the genomes of microorganisms by introducing edits in specific genome regions. The technology and some of the main applications are further discussed in Section 3.1.1.1.

A second group of techniques is aimed at introducing random mutations or variations in the genome or in specific parts of the genome, often with the aim of generating large collections (libraries) of modified organisms (or more broadly stretches of DNA) that can subsequently be screened to identify potentially interesting/improved variants. Some, but not all, of these techniques rely on CRISPR-Cas systems to induce mutations in specific regions of a genome.

The development and implementation of advanced, large-scale DNA synthesis is also opening new avenues for genetic engineering by allowing the synthesis of large, complex DNA sequences. This is relevant particularly in a combination with AI- and computational design-based approaches for de novo protein engineering.

Lastly, the development of novel DNA sequencing and analysis technologies allows for the accurate sequencing and analysis of the complete genome of (micro)organisms as well as to map (epigenetic) markers such as methylation patterns and chromatin structure. Whereas these new sequencing technologies are not directly used to modify an organism's genome, they do facilitate genetic engineering. The rapid increase in genome sequences of various organisms is an important source of genetic information that can be used to engineer other organisms. Moreover, the new sequencing technologies also facilitate the analysis of genomes that have been engineered as well as the study of the composition of complex microbial communities (microbiota).

3.1.1.1 | *CRISPR-Cas technology*

CRISPR-Cas systems form a natural bacterial immunity system that protects against recurrent bacteriophage infections. The basic elements were discovered in 1987, and further research uncovered its function (Horvath & Barrangou, 2010; Wiedenheft et al., 2012). CRISPR-Cas received more attention in 2012, after pioneering research by Jennifer Doudna, Emanuelle Charpentier and others, who showed how the system could be adapted to function as a molecular tool for making site-specific changes (or edits) in DNA (Jinek et al., 2012). CRISPR-Cas-based techniques have since become a prime tool for genetic engineering and gene editing in microbes and other organisms. These systems for genetic engineering and gene editing are generally based on a Cas enzyme that can be targeted to specific DNA sequences by introducing so-called guide-RNA molecules (gRNAs), that are complementary to the target sequence. Once the Cas enzyme recognises and binds its target sequence, the target is cut by the Cas enzyme, which enables the introduction of mutations or DNA insertions exactly at the site of the target sequence in the genome.

The CRISPR-Cas systems are a significant technological step because they allow precise, site-specific and very efficient engineering of virtually all organisms that can be genetically transformed. The technique increases the spectrum of targeted genetic engineering to species for which this was previously not easy or possible. In general, they increase the precision and predictability with which genomes can be modified. However, even if the precision of NGTs is higher compared to EGTs and conventional mutagenesis techniques, off-target mutations may still occur. At this point, organisms engineered through CRIPSR-Cas technology represent by far the largest group of NGT applications (see also Broothaerts et al., 2021). Most CRISPR-Cas techniques rely on double-stranded breaks.

Although the applications of CRISPR-Cas systems for genetic modifications are rapidly growing, the techniques can currently be divided into six categories:

A) CRISPR-Cas technique allows making specific changes (such as point mutations) at specific loci (sequences) in existing genomes. This is an example of 'targeted mutagenesis' often referred to as 'gene editing'. It is important to note that these specific changes may occur randomly in nature. These techniques and their applications are evolving fast, and it is now routine to generate multiple edits in one genome, or to generate large collections (so-called libraries) of hundreds, thousands, millions or even more individual microbial cells that each carry a different edit. Category A is represented by case study 12.
B) CRISPR-Cas techniques for complex genetic alterations. The sequence-specificity and efficiency of the various Cas enzymes is exploited to insert and/or delete larger DNA fragments. Inserted fragments can originate from the target or related organism (often referred to as self-cloning or cis–/intra-genesis), from another organism, or made synthetically (man-made DNA). Category B is represented by case studies 1, 2, 3, 4,5, 6, 8, 9 and 10.
C) CRISPR-Cas systems are also exploited as part of more complex modification strategies (see also paragraph D). These techniques again often rely on the targeting of Cas to specific regions in a genome. In some cases, alternative or enzymatically inactive (dCas) enzymes are used to prevent the formation of double-stranded breaks. By combining the Cas

enzyme with other enzymes or functional molecules, the nature of the genetic modification can differ from the original Cas-based modifications described in A and B above. For example, Cas enzymes are combined with error-prone polymerases, recombinases or base editors that insert -more or less randomly- one or multiple mutations around the target site in the target genome. As such, these techniques create a large set (library) of (random) mutants or rearrangements of the genome, with most mutations located in a specific part of the genome. These techniques can therefore be seen as a hybrid between traditional mutagenesis (such as UV- or ethyl methanesulfonate (EMS)-induced mutagenesis) and gene editing approaches. Category C is represented by case studies 11 and 13.

D) In the CRISPR-Cas9-assisted non-homologous end-joining (CA-NHEJ) strategy, the double strand breaks created by the CRISPR-Cas are rejoined independently from homologous recombination. The CA-NHEJ genome editing approach, not requiring a repair template, tends to generate errors resulting in frameshift mutations. This approach has been successfully applied to introduce mutations in bacterial and eukaryotic cells (Cai et al., 2019; Su et al., 2016).

E) A series of Cas-based techniques that are being developed target mRNA formation or stability. These techniques do not rely on double-stranded breaks and typically do not introduce genetic changes (i.e. changes in DNA sequence), but instead target site-specific activators or repressors to specific sites in the genome where they influence transcription. Alternatively, Cas enzymes can also be targeted to RNA to either introduce mutations or interfere with RNA stability or translation. Some of these techniques are still in their infancy but may become more important in the (near) future. The best-known technique in this category is CRISPR interference (CRISPRi), which exploits the CRISPR-Cas targeting mechanism to target a Cas or dCas (de-activated Cas) enzyme to a site in or near a gene. The Cas complex binds to the DNA, which results in steric interference with transcription initiation or elongation. For example, this can be used to (temporarily) modify the expression of certain genes in a microbe without making a permanent change to the genetic material of the microbe (apart from integrating the Cas system itself). Besides CRISPRi, further applications are still in their infancy, and it is therefore difficult to predict if this will become important.

F) A special use of the CRISPR-Cas technology involves inserting a controllable Cas system into a microbe with the aim to employ the system to modify the genetic material of the microbe itself and/or other microbes in the environment. The difference with other applications of CRISPR-Cas is that in this case, the CRISPR-Cas system is not (only) used to make changes in the host genome itself. Instead, the system is activated after the host cells are released into an ecosystem, where it induces genetic modification of other cells. Such systems can be used to construct gene drives (DiCarlo et al., 2015). Other potential applications of these systems could involve targeted genetic modification of microbes in an ecosystem such as the gut microbiome of a consumer. This category of applications of CRISPR-Cas technology is represented by case study 7.

3.1.1.2 | *New technologies for mutagenesis*

The increased use of (microbial) cell factories and enzymes also lead to an increased use of directed evolution to obtain superior variants. Directed evolution has traditionally been used to obtain microbial variants with superior characteristics. The technology has relied so far on natural or induced (physical, chemical) random mutations coupled with selection. Classic mutagenesis methods include both physical mutagens (e.g. UV irradiation, gamma rays) and chemical mutagens (e.g. EMS, nitrous acid) that induce mutations throughout the genome.

Classic mutagenesis protocols are conceptually simple and broadly applicable. However, specific kinds of mutations are favoured and they target the whole genome, which increases the chance of mutants acquiring multiple mutations, including mutations that are unrelated to the property of interest and may even be harmful. Therefore, several alternatives have been developed. The main advantage of genome editing is that the number, type and/or the location of the mutations in the genome can be better controlled, and/or that the process of generating the mutations and selecting superior variants is made more efficient.

Examples of these NGTs include the use of mutator strains that show a higher intrinsic mutation rate, techniques to target mutations to specific regions in the genome (e.g. by fusing base editors with Cas enzymes) or extrachromosomal elements (e.g. specific plasmids that attract plasmid-specific error-prone polymerases), and technologies that evoke specific types of mutations (e.g. the integration of specific recombination sites such as LoxP into genomes that can trigger random structural recombination events when specific recombinases such as Cre are activated). An example of the latter is the so-called SCRaMbLE technology that was first introduced in the Sc2.0 project. The Sc2.0 project aims at building a complete synthetic *S. cerevisiae* genome, where every non-essential gene is flanked downstream by a LoxPsym recombination site. Activation of the Cre recombinase triggers recombination between these LoxPsym sites and thus results in a 'scrambling' of the genome, i.e. massive random genomic rearrangements (deletions, inversions, translocations and duplications) (Swidah et al., 2020- see also case study 13).

3.1.1.3 | *Other site-directed nucleases*

Apart from CRISPR-Cas-based techniques, other endonucleases, such as homing endonucleases, zinc-finger nucleases and transcription-activator-like effector nucleases (TALEN) can also be used for genome editing and engineering. While they are still used for certain applications (e.g. introduction of larger pieces of DNA into certain genomes), these techniques have been largely replaced by CRISPR-Cas.

3.1.1.4 | *Synthetic biology*

Synthetic biology is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms (EFSA Scientific Committee, 2020). It is possible to create novel genetic information, biological networks (including complete genomes) and/or novel biological functions, either completely de novo or by combining and/or modifying existing naturally occurring DNA modules. In microbes, this has most notably led to the integration of complex biosynthetic pathways (EFSA Scientific Committee, 2020, 2022) or de novo protein generation using machine learning-based approaches meeting structural design targets (Ni et al., 2023).

3.1.1.5 | *Genome minimisation and genome design*

Minimal genomes are the result of the removal of non-essential genetic elements and are composed by the minimal set of genes required for basic cell functions (self-maintenance and growth) plus those providing the trait(s) of interest, depending on the application. The reduced complexity of minimised genome bacteria makes them particularly suitable for use in engineering processes aimed to the development of biotechnological products. Minimal genomes may be obtained using a top-down approach by a series of subsequent deletions in a wild-type genome or can be designed in silico by selecting the pool of the necessary essential genes, followed by chemical synthesis of the minimal genome (EFSA Scientific Committee, 2022; Rees-Garbutt et al., 2020; Xu et al., 2023).

3.1.1.6 | *Enabling technologies-DNA sequencing*

The further development of DNA sequencing technologies, including long-read sequencing techniques, makes it possible to routinely and quickly sequence, assemble and analyse the complete genome of most microbes, even if no reference genomes are available (Garg, 2021). This is important in the context of genetic modification because it allows investigating and verifying all the changes that occur in the DNA. Moreover, the technology and associated steep rise in the number of sequenced and analysed microbial genomes also makes it easier to check the phylogeny of a (modified) microbe, to investigate which DNA sequences occur in nature, and to predict their function (including, for example, the production of known toxins, antibiotics, etc.).

In this context, novel machine-learning and deep learning methods are currently being developed to also analyse GMOs before and after their development. In the first case, they are used to optimise the design of gRNAs, so as to maximise on-target activity while minimising off-target effects (Sherkatghanad et al., 2023). In the second case, they support the detection of genetically modified sequences (even unknown), usually obtained with high-throughput sequencing data (Hurel et al., 2020).

3.1.2 | AQ2. Are there any novel hazards that these new developments in biotechnology applied to microorganisms could pose to humans, animals and the environment, as compared to established genomic techniques and conventional mutagenesis?

Following the methodology described in section 2, the EFSA GMO Panel did not identify any novel potential hazard to humans, animals and the environment of NGTs applied to microorganisms compared to EGTs or compared to conventional mutagenesis, related to the technique itself.

Some NGTs can produce mutations in microorganisms that also occur in nature. Off-target modifications and/or unintended effects can occur for EGTs, NGTs and conventional mutagenesis techniques. Due to the higher efficiency, specificity and predictability of NGTs, the hazards related to the changes in the genome are likely to be less frequent in a GMM developed with NGTs than those modified by conventional mutagenesis, or GMMs obtained with EGTs. Apart from some exceptions, like gene-drive like systems, the introduced CRISPR-Cas system should be removed during the process of modification when using NGTs. If present, the potential new modifications need to be assessed on a case-by-case basis.

Potential hazards may be related to new trait(s) regardless of the technique used and a product-based risk assessment of NGT-Ms is proposed and will be addressed in Section 3.3.

3.1.3 | AQ3. Are there any novel risks that these new developments in biotechnology applied to microorganisms could pose to humans, animals and the environment, as compared to established genomic techniques and conventional mutagenesis?

The assessment of risk always takes into consideration the hazard and the exposure. Given that no novel potential hazards of microorganisms obtained by NGTs were identified as compared to those obtained by EGTs, or by conventional mutagenesis, no novel potential risks to humans, animals and the environment are expected even if there is exposure.

3.2 | ToR2: Applicability and sufficiency of the existing guidelines for risk assessment of GMM to risk assess new developments in biotechnology applied to microorganisms

3.2.1 | AQ1 and AQ2. What kind of GM microorganisms and GM microbial products within the EFSA remit have been identified and can be expected in the next 10 years that were developed using new developments in biotechnology?

A horizon scanning exercise was conducted to identify GMMs and GM microbial products within the EFSA remit that were developed using NGTs and can be expected to be developed in the near future (next 10 years). This interval was considered meaningful to capture the most promising NGT-M developments with potential to reach the market in the near future and discriminate them from the ones in earlier developmental stages. In addition to the horizon scanning (Ballester et al., 2023), an online[10] call for data launched by EFSA, complemented the horizon scanning. It further informed the GMO Panel about GMMs and GM microbial products developed with the use of NGTs and confirmed the fact that the majority of the identified products are to be expected on the market in the next 10 years.

Horizon scanning. A total of 35 GMMs and GM microbial products developed with the use of NGTs (NGT-Ms) were identified by the horizon scanning; 57% of which belonging to category 4 and 43% to category 3 products. A total of 77% of the reported cases made use of CRISPR-based techniques, and the remaining 23% of a combination of EGTs and NGTs to develop the final product. Their expected uses are 86% as source of food or food additives, 8% as (or as a source of) feed or feed additives, and 6% for agricultural purposes, to be released in the environment. The horizon scanning also categorised the GMMs based on their phase of development as follows:

- Commercialisation (8 cases): GMMs currently marketed in at least one country.
- Pre-commercial/patent applications (9 cases): GMMs which are patented or part of patent applications.
- Under development (18 cases): GMMs at the proof-of-concept stage (i.e. testing gene targets for traits of commercial interest).

All products currently commercialised belong to category 4 GMMs. Their uses are for food and feed additives, starter cultures and fertilisers in agriculture. The desired traits aim at reducing the use of synthetic fertilisers (e.g. case study 1/GMM-01), boosting immunity/reduction of toxicity (e.g. case study 7/GMM-02), optimising the production process and improving flavour/colour (e.g. case study 10/GMM-16). For three cases (GMM-01, GMM-02, GMM-19 as described in Ballester et al., 2023), information to support a risk assessment was retrieved. The remaining five cases (GMM-16, GMM-17, GMM-18, GMM-20 and GMM-21 as described in Ballester et al., 2023) refer to starters for beer fermentation, but the exact strain was not identified and for this reason neither was their risk assessment. None of these products is commercialised in the EU (Ballester et al., 2023). It is noteworthy that one product has been authorised as non-GMO in Brazil (case study 7/GMM-02) (Ballester et al., 2023).

The vast majority of the pre-commercialised cases (8 out of 9) belong to category 3 products, while only one product described to be used as endophyte in agriculture belongs to category 4 (case study 2/GMM-03). The uses of these products are for food/feed and agriculture, while the desired traits include reduction of toxic compounds, increased production yield, optimisation of the production process and improved food composition.

Eleven out of 18 cases that are currently under development fall under category 4, while the remaining 7 are under category 3. The uses of these products are for food and feed purposes and the desired traits are increased production and improved characteristics of the product with regards to colour, flavour, composition (case study 6/GMM-04, case study 3/GMM-13 and case study 5/GMM-15).

The subject organisms identified through the horizon scanning include yeasts (63%), bacteria (31%), fungal endophyte and microalgae (3% each). The species used are *Klebsiella variicola, Leuconostoc citreum, Bacillus licheniformis, Bacillus subtilis, Escherichia coli, Lactococcus lactis, Lactobacillus casei, Pediococcus acidilactici, Clostridium butyricum, Saccharomyces cerevisiae (var boulardii)* and *Epichloë coenophiala*.

While the majority of GMM assessed refer to a single species, it is worth noting that some patent applications claim the NGTs are to be potentially applied to engineer multiple species. The horizon scanning also pointed out engagement in the development of NGT-Ms by both private and public/academic entities. On the one hand, the private sector holds a larger number of commercial applications, while on the other hand, public institutions play a dominant role in research and development, resulting in a diverse number of traits being developed. Overall, the findings of this horizon scanning indicate a growing implementation of NGTs in producing GMMs and their products.

EFSA online call for data. The results of the online call for data by EFSA were similar to the ones of the horizon scanning and confirmed the fact that the majority of the identified products are to be expected in the next 10 years. The majority of the NGT-Ms developed belong to the same microbial groups previously described, i.e. yeasts (41%), bacteria (36%) and filamentous fungi (20%), while the online call for data also revealed the development of a phage (1%), which was not retrieved during the horizon scanning. Unlike the horizon scanning, during the EFSA online call for data, none of the respondents

[10] https://www.efsa.europa.eu/en/call/survey-new-biotechnologies-microorganisms.

reported the development of microalgae. However, 2% of the respondents attributed the microbial group of the modified organism to 'other'.

Most cases were category 4 products (61%) while the remaining ones belonged to category 3 (39%). It is worth mentioning that 75% of the cases implement the use of both EGTs and NGTs for the development of the final product. The responses indicate that the final use of the products would be food/feed additives/flavourings/ingredients, products for agricultural use (e.g. biopesticides, biostimulants) and biomasses.

The respondents were mainly from industry (64%) and academia (29%), while the remaining 7% could not be classified to any of the above categories, based on their affiliations. They indicated that seven products are already on the market (US, Canada, India and Brazil and other South American countries), while 35 are expected to be on the market in the next 10 years. No response with regard to the time to market was provided for 12 products. The respondents also pointed out the speed with which the NGTs can lead to the desired trait, and the use of the same NGT-M for various uses applicable both in the food and the feed sector.

Taken together, the results of the horizon scanning and the EFSA call for data, it can be deduced that a variety of products containing NGT-Ms, falling within the remit of EFSA, are expected to be on the market in the next 10 years. These would include food and feed additives, flavourings, colourings or ingredients, but also products to be used in the agricultural sector. Most products identified are expected to be category 4 products developed with the use of NGTs or a combination of both NGT and EGT. The preferred traits seem to be improved texture, nutritional composition and/or colour, reduction of toxic compounds, but also optimisation of the production process.

However, according to the respondents of the survey, the time to market would depend on the regulatory framework and requirements applied to these products.

3.2.2 | AQ3. Which are the existing guidelines to be used for the risk assessment of these GMMs?

In delivering its opinion, the GMO Panel together with the GMM NGT Working Group considered the current GMO legislation and EFSA guidelines. The list of selected documents is presented in Table 2.

TABLE 2 Existing legislation and guidelines covering the risk assessment of microorganisms or their products relevant for this mandate.

References	Title
1. Directive 2001/18/EC	Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms
2. Regulation (EC) No 1829/2003	Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed
3. Directive (EU) 2018/350	Commission Directive (EU) 2018/350 of 8 March 2018 amending Directive 2001/18/EC of the European Parliament and of the Council as regards the environmental risk assessment of genetically modified organisms
4. EFSA GMO Panel (2010)	Guidance on the environmental risk assessment of genetically modified plants
5. EFSA GMO Panel (2011a)	Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use
6. EFSA ANS Panel (2012)	Guidance for submission for food additive evaluations
7. EFSA FEEDAP Panel (2023)	Guidance on the assessment of the safety of feed additives for the users
8. EFSA FEEDAP Panel (2017a)	Guidance on the assessment of the safety of feed additives for the target species
9. EFSA FEEDAP Panel (2017b)	Guidance on the safety of feed additives for consumers
10. EFSA FEEDAP Panel (2018a)	Guidance on the characterisation of microorganisms used as feed additives or as production organisms
11. EFSA FEEDAP Panel (2018b)	Guidance on the assessment of the efficacy of feed additives
12. EFSA FEEDAP Panel (2019)	Guidance on the assessment of the safety of feed additives for the environment
13. EFSA CEP Panel (2021)	Scientific Guidance for the submission of dossiers on Food Enzymes
14. EFSA (2021)	EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain
15. EFSA BIOHAZ Panel (2023)	Scientific Opinion on the update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA.
16. EFSA GMO Panel (2017)	Guidance on allergenicity assessment of genetically modified plants
17. EFSA FAF Panel (2021)	Scientific guidance for the preparation of applications on smoke flavouring primary products

3.2.3 | AQ4. Are the existing guidelines for risk assessment applicable, fully or partially, and sufficient for the risk assessment of GMMs generated with the use of the new developments in biotechnology?

The relevant legislative framework and guidance documents relevant for the risk assessment of microorganisms have been summarised in Section 3.2.2. None of the selected guidances (see Table 2) are sufficient for the risk assessment of new development in biotechnology applied to microorganisms as per ToR2.

To address their sufficiency, the selected case studies have been assessed against the designated requirements described in the relevant guidances in the following fields of risk assessment which constitute the below subsections: 3.2.3.1, the use of the comparative approach; 3.2.3.2, microbial characterisation; 3.2.3.3, manufacturing process and product specifications; 3.2.3.4, compositional analysis; 3.2.3.5, toxicology; 3.2.3.6, assessment of the impact on the gut microbiome; 3.2.3.7, allergenicity; 3.2.3.8, nutritional assessment; 3.2.3.9, exposure assessment; 3.2.3.10, environmental impact of the GMM and their products; 3.2.3.11, horizontal gene transfer; 3.2.3.12, post-market environmental monitoring.

3.2.3.1 | *Comparative approach: Use of a comparator*

The comparative approach for risk assessment of GMMs is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 3 describes the evaluation of this guidance based on the selected case studies.

TABLE 3 Sufficiency of guidance for the comparative approach based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1–6; 10–12	For all cases the comparative approach is feasible	Sufficient	None
7	*E. coli* strain, not previously used in the food and feed chain but with a general body of knowledge on safety	Not sufficient	Update definition of comparative approach to include the body of knowledge of a taxonomic unit as basis for safety of the non-modified counterpart
8	Parental strain was not used in the food or feed chain and is a microalga. The parental strain can be used as comparator for risk assessment of the genetic modification. The body of knowledge is not yet sufficient	Not sufficient	The assessment of the genetic modification in a microalga is still to be developed Update definition of comparative approach to include the body of knowledge of a taxonomic unit as basis for safety of the non-modified counterpart
9	Parental strain was not used in the food and feed chain and is a virus. The body of knowledge is feasible	Not sufficient	The assessment of the genetic modification in a virus is still to be developed Update definition of comparative approach to include the body of knowledge of a taxonomic unit as basis for safety of the non-modified counterpart
13	Strain extensively modified. Comparative approach is feasible as there is sufficient body of knowledge on the parental strain (which is a QPS microorganism)	Not sufficient	Update definition of comparative approach to include the body of knowledge of a taxonomic unit as basis for safety of the non-modified counterpart

A comparator is a keystone in microbial risk assessment and is used in many of the risk assessment areas described in this opinion. The comparative approach for risk assessment of GMMs, as described in the GMM guidance (EFSA GMO Panel, 2011a), was based on the Codex Alimentarius concept where the comparator (conventional counterpart) has been defined as 'a microorganism/strain with a known history of safe use in producing and/or processing the food and related to the recombinant-DNA strain' or 'food produced using the traditional food production microorganisms for which there is experience of establishing safety based on common use in food production'. This definition of the comparator is recommended to be expanded for its use in the frame of the microbial risk assessment at EFSA, including the risk assessment of NGT-Ms. This is because for many microbial applications in the food and feed chain (including the environment), a comparator with a history of safe use may not always be available. For example, if the genetic modification is intended to produce a new compound to be used in the food and feed chain, it is possible that the non-modified counterpart (which does not produce such compound) has never been employed for those purposes. Another example would be when a microorganism is modified to eliminate undesirable traits to make it adequate for its use in food and feed chain.

In many cases, the comparator is the 'conventional counterpart' strain as originally conceived in the Regulation (EC) No 1829/2003, meaning the non-modified parental/recipient strain. Although the use of a conventional counterpart as defined above may not be applicable in all cases, the use of a comparator, even when it has no history of safe use, is very useful in the risk assessment of GMMs including NGT-Ms, as it provides information on any possible safety issues related to the biology of the microorganism, and enables direct genome comparison so that the genetic modifications can be well

characterised and assessed. When the recipient/parental strain is not yet used in the food and feed chain or when the strain has been extensively modified, the general biological information available in literature, the so-called body of knowledge, can be used. Also, information on a different strain of the same species or a phylogenetically close relative which is used in food, feed or in the environment may be used. The body of knowledge informs also on the general exposure of humans or animals by food and feed intake without the history of a deliberate introduction of the microorganism in the food and feed chain.

3.2.3.2 | *Microbial characterisation*

Microbial characterisation was originally described in the GMM guidance (EFSA GMO Panel, 2011a) and was further expanded in the newer sectorial guidances to include the use of WGS and the Qualified Presumption of Safety (QPS) concept (EFSA BIOHAZ Panel, 2023; EFSA CEP Panel, 2021; EFSA FEEDAP Panel, 2018a). Table 4 describes the evaluation of these two guidances based on the selected case studies. The currently used sectorial guidances for assessing GMM derived products are focused on the risk assessment of category 1 and 2 products. For category 3 and 4 products, the risk assessment requires some extra information as indicated in the GMM guidance (EFSA GMO Panel, 2011a).

TABLE 4 Sufficiency of guidances for the microbial characterisation based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA CEP Panel, 2021; EFSA FEEDAP Panel, 2018a)	Recommendations for future guidance updates (see Section 3.3)
1*-4, 7	WGS is adequate and necessary (also for fungi as in case 2), for taxonomic identification including QPS/non-QPS confirmation, absence of genes of concern, characterisation of the genetic modification	Sufficient	None
5–6, 10–13	WGS is adequate for taxonomic identification and characterisation of the genetic modification. The antimycotic susceptibility should be assessed as qualification of the QPS status when NGT-M is used as category 4 product	Not sufficient	Develop guidance for testing the possible antimycotic resistance of yeasts/fungi used as category 4 products, including NGT-yeast
8	WGS is preferred for the microbial characterisation of the NGT microalgae	Not sufficient	Develop guidance for microbial characterisation of microalgae, including NGT microalgae
9	WGS is necessary for the microbial characterisation of the NGT-viruses	Not sufficient	Develop guidance for microbial characterisation of viruses, including NGT viruses
1–8, 10–12	Heterologous CRISPR-Cas system was intentionally introduced in the microorganism	Not sufficient	Develop guidance for confirming presence/absence of CRISPR-Cas or similar systems, intentionally introduced in microorganisms

*When a case study is mentioned more than once, it is because different aspects are being assessed.

In the currently used sectorial guidances (EFSA CEP Panel, 2021; EFSA FEEDAP Panel, 2018a), it is requested that the purpose of the development of the GMM must be explained in all cases. Whole genome sequencing (WGS) is the preferred approach for the characterisation of the microorganism under assessment. It is mandatory for bacteria and yeasts, and optional for filamentous fungi. The WGS approach should be used preferentially to identify the microorganism and to document the genetic modification. The WGS will also be used for searching for the presence of genes of concern, such as AMR genes and virulence/toxigenic factors (EFSA, 2021).

The whole genome of the GMM is compared with the appropriate comparator (often the parental/recipient strain) (see Section 3.2.3.1 Comparative approach). Such analysis is independent from the genetic modification techniques used and is therefore also useful for assessing NGT-Ms. In the case where the analysis of the WGS is limited due to technical constraints (e.g. highly repetitive sequence regions), the microbial characterisation approach can be based on the assessment of the parental organism and the applied genetic modification steps.

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 belonging to taxonomic units (species) qualifying for the QPS approach are presumed safe for target species, consumer and the environment, encompassing possible effects on human and animal health. Some aspects of the microbial risk assessment are not covered by the QPS status and have to be separately assessed, when applicable: (1) hazards for users handling the product (e.g. dermal contact, inhalation, ingestion); (2) potential allergenicity of the microbe or its residual components or produced metabolites; (3) environmental hazards of plant protection products; and (4) hazards linked to the formulation and/or the production and product purification process. Meeting the criteria for a QPS approach includes the confirmation at strain

level that it belongs to a taxonomic unit on the QPS list, and that it meets the qualifications formulated for this taxonomic unit.

The QPS status is also applicable to GMMs used as production organisms, biomasses or active agents for which the species of the parental/recipient strain qualifies for the QPS status, and for which the genetic modification does not give rise to safety concerns (EFSA BIOHAZ Panel, 2024). The QPS approach is also a fundamental tool for the risk assessment of NGT-Ms. For microbial 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 process).

3.2.3.3 | *Information relating to the manufacturing process and product specifications*

Information on the manufacturing process and product specifications was originally described in the GMM guidance (EFSA GMO Panel, 2011a) and was further expanded in the newer sectorial guidances (EFSA ANS Panel, 2012; EFSA CEP Panel, 2021; EFSA FAF Panel, 2021; EFSA FEEDAP Panel, 2018a). Scientific concepts laid down in the Novel Food Guidance (EFSA NDA Panel, 2021) may also constitute a basis for the assessment of the product if needed. Table 5 describes the evaluation of these guidances based on the selected case studies.

TABLE 5 Sufficiency of guidances for the manufacturing process and product specification assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA ANS Panel, 2012; EFSA CEP Panel, 2021; EFSA FAF Panel, 2021; EFSA FEEDAP Panel, 2018a; EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1–3; 5–7; 10–13	Bacteria, fungi, yeast are produced following the typical fermentation conditions; produced as viable organisms	Sufficient	None
4	Yeast with modified EPS production increasing the viscosity requiring adaptations of the current fermentation process: produced as viable organism	Sufficient	None
8	The production of microalgae is not covered by current guidances	Not sufficient	Update is recommended for protists/microalgae
9	The production of viruses is not covered by current guidances	Not sufficient	Update is recommended for viruses

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. The detection of microorganisms (viable and/or inactivated) and/or their genetic material are key elements for the safety evaluation of NGT-Ms. In category 3 products, methodologies to inactivate/lyse the microbial cells should be reported.

The available EFSA guidances (EFSA ANS Panel, 2012; EFSA CEP Panel, 2021; EFSA FAF Panel, 2021; EFSA FEEDAP Panel, 2018a; EFSA GMO Panel, 2011a) are in general adequate for assessing the product, its production and purification process, the presence/absence of DNA and/or viable bacterial, yeast and fungal cells.

3.2.3.4 | *Compositional analysis*

The compositional analysis for risk assessment of GMMs is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 6 describes the evaluation of this guidance based on the selected case studies.

TABLE 6 Sufficiency of guidances for the compositional analysis based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidance (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1–3, 5, 7*, 9, 11–12	Not applicable as there is no biomass for food and feed use		
4, 6, 10, 13	Given the QPS status only changes in the composition of the final product may need toxicologic/nutritional assessment.	Sufficient	None
8	No history of use of lipids produced by *Nannochloropsis gaditana* in food or feed. Biomass composition may need toxicologic/nutritional assessment	Sufficient	None

TABLE 6 (Continued)

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidance (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
7–9, 13	No comparator is available	Not sufficient	The strategy of the Novel Food Guidance (EFSA NDA Panel, 2021) may be informative

*When a case study is mentioned more than once, it is because different aspects are being assessed.

A comparative compositional analysis may be necessary for category 3 and 4 products (see Section 1.3). The GMM Guidance (EFSA GMO Panel, 2011a) also applies to the comparative compositional analysis of NGT-Ms and products obtained from NGT-Ms. Qualitative and, when appropriate, quantitative compositional analyses of the NGT-Ms or their product and the respective conventional comparators, where available, should be provided. This analysis should include the relevant nutrients, antinutrients, other metabolites typical of the NGT-Ms, their products and the respective conventional comparators. If applicable, specific impurities need to be included in the analysis. Intended and unintended changes of composition should be subjected to a toxicologic and nutritional assessment taking into account the anticipated intake levels. The possible impact on allergenicity of the NGT-Ms or their products should also be evaluated.

As indicated in Section 3.2.3.1, the comparative approach is not feasible in the case that the composition of the NGT-Ms and their products is modified substantially (by quantitative and/or qualitative criteria) or when the parental/recipient strain has not been used in the food and feed chain. For these cases, a safety assessment should be carried out without comparator. Relevant elements of the strategy laid down in the Novel Food Guidance may be informative for this assessment (EFSA NDA Panel, 2021).

3.2.3.5 | *Toxicology*

The toxicological analysis for risk assessment of GMMs is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 7 describes the evaluation of this guidance based on the selected case studies.

TABLE 7 Sufficiency of guidances for the toxicological assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidance (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1	Increased expression of an endogenous protein. The body of knowledge proves safety of the nitrogen fixing bacterium in cereal crops and transfer to the food and feed chain is assumed to be negligible	Sufficient	None
2	No newly expressed proteins to be assessed. The presence of new metabolites or altered levels of constituents other than proteins would need to be assessed by a comparative approach	Sufficient	None
3	Parental organism already assessed to be safe and no newly expressed proteins to be assessed	Sufficient	None
4	No newly expressed proteins to be assessed	Sufficient	None
5	Multiple newly expressed proteins to be assessed	Sufficient. Multiple animal (rodent) studies may be required according to current guidances	Develop guidance for the use of alternative protein safety analyses (e.g. in silico, in vitro) to replace animal studies
6	No newly expressed proteins to be assessed	Sufficient	None
7	No newly expressed proteins to be assessed, but increased expression of microcin (a peptide) with potential effects on the microbiome: the parental *E. coli* needs to be assessed as it is not declared as safe	Sufficient	None
8	No newly expressed proteins but the comparative assessment is not feasible as there is no history of safe use of the non-GM parental organism	Sufficient	None
9	This case is a virus	Not sufficient	Include viruses in future guidance

(Continues)

TABLE 7 (Continued)

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidance (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
10	Multiple newly expressed proteins to be assessed	Sufficient. Multiple animal studies may be required	Develop guidance for the use of alternative protein safety analyses (e.g. in silico, in vitro) to replace animal studies
11	Mutated proteins to be assessed	Sufficient. The assessment may require animal toxicity studies if the mutations have no history of safe use/familiarity	Develop guidance for the use of alternative protein safety analyses (e.g. in silico, in vitro) to replace animal studies
12	No newly expressed proteins, but intended mutations need assessment	Sufficient. The assessment may require animal toxicity studies if the mutations have no history of safe use/familiarity	None
13	Newly expressed proteins with a history of safe consumption (tomato). Therefore, no need for toxicological studies. The presence of new metabolites or altered levels of constituents other than proteins would need to be assessed	Sufficient	None

The EFSA GMO Panel (2011a) guidance on GMMs, which is in place to cover the toxicological assessment, also applies to NGT-Ms and products obtained from NGT-Ms. In addition to the GMM guidance, other guidances listed in Section 3.2.2 may be consulted in certain instances (e.g. case 5, 10 or 11), particularly the guidances of the EFSA FEEDAP Panel (2017a, 2017b) and the guidance for the submission of food enzyme applications of the EFSA CEP Panel (2021).

In addition to newly expressed proteins, new constituents other than proteins, as well as any anticipated changes in metabolic pathways due to the modification, are to be evaluated. The toxicological assessment should also take into account the history of use and the body of knowledge related to the parental microorganism. Depending on this assessment, the toxicological analysis could be waived in certain cases (e.g. when the NGT-M qualifies for QPS). The need for a toxicological assessment should also consider the production process, including any relevant change resulting from the use of the NGT-Ms. The toxicological assessment should include the possible production of harmful compounds by the NGT-M, including the impact of any changes in the NGT-Ms and/or their products resulting from the genetic modification.

Evaluation of new or altered constituents other than proteins

New constituents, other than proteins, including any anticipated changes in metabolic pathways due to genetic modification, should be assessed on a case-by-case basis. If, due to alterations in metabolic pathways, the levels of naturally occurring metabolites have been changed (intentionally or unintentionally), an evaluation based on the knowledge of the biochemical function and/or toxic properties of these constituents, as well as the anticipated changes in intake levels should be carried out. The result of this assessment will determine if, and to what extent, further toxicological tests are required.

Assessment of the product (including cases when the NGT-M itself is the product)

The hazard identification of an NGT-M is based on the molecular characterisation of the genetic modification, a comparative compositional analysis in relation to appropriate comparators, and the assessment of any identified intended and unintended effects. If these analyses indicate a need to perform an animal study to test whether the NGT-M or its intended products (cell extracts, biomasses, etc.) are as safe as the comparators, a 90-day rodent feeding study with the NGT-M biomass or its product should be performed. Due to the wide variety of possible products, the requirements for toxicological testing should be established individually for each case and justified. The need for such an animal study applies in particular when no conventional counterpart exists (e.g. when the composition of the NGT-M is substantially modified in comparison to existing food and feed).

3.2.3.6 | *Gut microbiome*

Microbiota encompasses the living microorganisms present within a specific environment. Meanwhile, the microbiome includes the entirety of genomes derived from all microorganisms in that environment but also their structural components, metabolites and the prevailing environmental conditions. Therefore, the term microbiome is broader than microbiota (Hou et al., 2022). No EFSA guidance addresses the impact of NGT-Ms on the gut microbiome (Moreno et al., 2024). However, the term gut microbiota and the requirements to assess the impact on the human and animal gut are mentioned in the GMM guidance (EFSA GMO Panel, 2011a). Subsequent sectorial guidance describes the requirements to assess the impact on animal gut of microorganisms for certain feed applications (EFSA FEEDAP Panel, 2018a). Table 8 describes the evaluation of these guidances based on the selected case studies.

TABLE 8 Sufficiency of guidances for the gut microbiome assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA FEEDAP Panel, 2018a; EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
2, 4, 6–7	NGT-Ms to be incorporated in food or feed as viable cells (Category 4 products). Viability, and residence time in the gut, and any impact on the microbiome of the human or animal gut needs to be described	Not sufficient	Existing guidances (EFSA GMO Panel, 2011a, 2011b) do not specify what is considered an impact on the microbiome, neither provide recommendations for their determination. Validated endpoints and standardised test guidelines are still missing
3, 5	The NGT-M strain produces higher amount of butyrate, or vitamin A expected to have a beneficial effect on host health	Not sufficient for food/ sufficient for feed	Develop a guidance for food
1	NGT-M to be used as fertiliser. Potential transfer to edible parts and hence impact on gut microbiome may need to be assessed	Not sufficient	Develop a guidance for food and feed for secondary exposure
9	Existing guidance are not applicable to microalgae and viruses for their impact on gut microbiome	Not sufficient	Develop new guidance
8, 10, 13	No viable cells are present in the food/feed obtained by NGT-Ms but DNA is present (category 3 products). Therefore, no impact on gut microbiome is expected other than those resulting from HGT. The safety of the products derived by NGT-Ms is assessed under sectorial guidance for food and feed	Not sufficient	Update is recommended to explain that assessment of HGT for biomasses is only needed if they contain genes of concern which could offer a selective advantage (e.g. AMR genes)

Microorganisms in category 4 products may be modified by conventional mutagenesis, EGTs and NGTs to deliver specific functions and successfully compete in the gastrointestinal tract but, conversely, this competitive advantage can also have unexpected adverse effects on gut microbiome (e.g. by outcompeting commensal resident microorganisms and/or affecting the metabolic end products of the gut microbiome). Strains modified to improve their technological performance during food and feed production could still result in unintended effects on the gut microbiome upon consumption. Moreover, GMMs including NGT-Ms may be specifically developed to exert actions in the gut, such as probiotic activities or reduction of enteric pathogens (cases 3, 5, 7 and 9).

Existing guidances (EFSA FEEDAP Panel, 2018a, EFSA GMO Panel, 2011a) provide limited information about the risk assessment of microorganisms or their products present in food and feed in relation to the gut environment. Available guidance includes testing the viability and residence time of the GMM in the gut ecosystem in the 90-day rodent studies (EFSA GMO Panel, 2011a) and the potential effect of feed additives on the growth and persistence of pathogens in the gut (EFSA FEEDAP Panel, 2018a).

The GMM guidance (EFSA GMO Panel, 2011a) also mentions the necessity to inform about the viability and residence time of viable GMM (category 4 products) in the animal or human gut, and to describe any impact that the GMM may have on the animal or human gastrointestinal microbiota.

Regardless of this general recommendation, there are still critical limitations to justify incorporating the effects on gut microbiome on risk assessment schemes soon, including:

- Limited causal relationships between changes in the gut microbiome and health, hampering the evaluation of the potential (adverse) effects derived from microbiome perturbations on gut functions (including metabolic, barrier defence and immune function) and host health.
- No generally accepted endpoints (e.g. potential persistence and colonisation, effects on the gut microbiome) and validated methodologies (e.g. in vitro methodologies translating to human/animal situation) for assessing the impact of GMMs including NGT-Ms on the microbiome structure and metabolism in the short and long term.
- No consensus exists as to what is a healthy baseline in the analysis of gut microbiota and underlying factors responsible of inter-individual variation in microbiome responses to dietary factors are for the most unknown.

Guidance for the assessment of the effect of viruses or microalgae on the gut microbiome is missing.

3.2.3.7 | *Allergenicity*

Allergenicity is mentioned in the GMM guidance (EFSA GMO Panel, 2011a). Table 9 describes the evaluation of this guidance based on the selected case studies.

TABLE 9 Sufficiency of guidances for the allergenicity assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA GMO panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1	The allergenicity of certain metabolites produced by bacteria intended to be used as fertiliser in view of possible secondary exposure	Not sufficient	Update is recommended for adjuvanticity
2	The allergenicity associated with the functional properties/or biomolecules produced after gene deletion by the fungus, which is intended to be in feed, in view of possible secondary exposure when the product is used in food producing animals	Not sufficient	Update is recommended for adjuvanticity
3, 7	The allergenicity associated with the functional properties and/or biomolecules produced after gene deletion or insertion by bacteria intended to be used in food and/or feed (secondary exposure possible in case of food producing animals)	Not sufficient	Update is recommended for adjuvanticity
4	The allergenicity associated with the functional properties and/or biomolecules produced after gene deletion by bacteria, which are intended to be used as starter	Not sufficient	Update is recommended for adjuvanticity
5, 11, 12	The allergenicity associated with the functional properties and/or biomolecules produced following gene insertion or editing by yeasts intended to be used as probiotic	Not sufficient	Update is recommended for adjuvanticity
6, 10, 13	The allergenicity associated with the functional properties and/or biomolecules produced after gene deletion, gene deletion and insertion of mutated genes, or chromosome rearrangement, by yeasts intended to be used as starter	Not sufficient	Update is recommended for adjuvanticity
8	The allergenicity of the biomolecules produced after gene editing by a microalga that could be potentially used in food and feed	Not sufficient	Update is recommended for adjuvanticity
9	The allergenicity associated with the functional properties following gene editing of viruses intended to be used in food and feed	Not sufficient	Update is recommended for adjuvanticity

Allergenicity is defined as the ability to trigger an abnormal immune response that leads to an allergic reaction. Allergic reactions to foods are usually, but not exclusively, mediated by immunoglobulin (Ig)E that binds to proteins. The allergenic potential of a given food or ingredient without protein content, or hydrolysed proteins (peptides) that go unnoticed for the immune system, is considered low. Another aspect of the allergenicity risk assessment is adjuvanticity, which is the capacity of a substance to augment, or divert, the body's immune response to an antigen (EFSA GMO Panel, 2011a, 2022).

The guidance documents addressing allergenicity cover GMOs, skin and respiratory occupational risks, and non-IgE adverse immune reactions (EFSA FEEDAP Panel, 2023; EFSA GMO Panel, 2011a; EFSA GMO Panel, 2017). These documents have facilitated the assessment of allergenicity of newly expressed proteins. However, there is a need to improve the allergenicity assessment overall (EFSA GMO Panel, 2022). In this regard, experience gained and new developments in the field call for a modernisation of some elements of the GMM allergenicity assessment.

Possible issues for the GMM risk assessment are related to: (i) allergenicity and adjuvanticity of proteins; (ii) carbohydrates; (iii) other substances produced by GMMs; (iv) altered protein secretion by GMMs, or those derived from their metabolism; (v) GMM functionality (e.g. ability to induce allergic sensitisation or conditions prone to it); and (vi) non-IgE-mediated allergy to proteins such as eosinophilic esophagitis or other gastrointestinal disorders (Akdis, 2021; Caminero et al., 2023; EFSA GMO Panel, 2017; Hang et al., 2019; Le Gall et al., 2011; Lee et al., 2022; Levan et al., 2019; Lloyd-Price et al., 2019; Platts-Mills et al., 2021; Plum et al., 2023; Sharma & Karim, 2021; Sozener et al., 2022). In allergic disease, adjuvants alter tissue homeostasis to establish conditions that result in non-specific, innate type 2 (allergic) priming to bystander allergens, thus subverting the development of tolerance (Akdis, 2021; Bruton et al., 2020; Ellenbogen et al., 2018; Kopp et al., 2023). In relation to non-IgE-mediated adverse immune reactions to food, detailed risk assessment considerations are provided for the safety profile of the protein or peptide and its potential to cause coeliac disease (EFSA GMO Panel, 2017).

To address these issues, it would be necessary to update the guidance documents considering the functional features of GMMs and GMM-derived metabolites, the ability of adjuvants or GMMs to impair/damage the mucosal/epithelial barrier (Akdis, 2021; Ellenbogen et al., 2018), and the optimisation and standardisation of functional assays such as basophil or mast cell activation tests (Bahri et al., 2018; Elst et al., 2021; Santos et al., 2021). Given that adjuvanticity is currently assessed based on evidence provided by the scientific literature, further research and validation of experimental methodologies are needed to better address adjuvanticity in a regulatory environment.

3.2.3.8 | *Nutritional assessment*

Nutritional assessment is foreseen in the GMM guidance (EFSA GMO Panel, 2011a, EFSA FEEDAP Panel 2018b). Table 10 describes the evaluation of these guidances based on the selected case studies.

TABLE 10 Sufficiency of guidances for the nutritional assessment based on the selected case studies.

Case	Specific evaluations of the case, including intended and unintended effects	Conclusions on the sufficiency of existing guidance (EFSA FEEDAP Panel, 2018b; EFSA GMO panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1–3, 5, 7,9, 11–12	Not applicable as no compositional analyses needed		
4, 6, 10, 13	Compositional changes of the final product involving nutrients, if they occur, need to be assessed	Sufficient	None
8	Composition of biomass will need assessment	Sufficient	None

Approaches for the nutritional assessment of NGT-M-derived food for human consumption are laid down in the EFSA GMM guidance (EFSA GMO Panel, 2011a) and further supported by a newer guidance (EFSA FEEDAP Panel, 2018b). If a corresponding conventional product or biomass exists, the assessment of NGT-Ms is based on the intended and/or unintended compositional changes observed between the NGT-M-derived product or biomass and the conventional counterpart taking into consideration intake levels. If there is no corresponding conventional product with a sufficient history of familiarity, the overall composition of the NGT-M-derived product or biomass in view of the anticipated intake levels must be assessed. In such cases, the guidance on Novel Foods Applications (EFSA NDA Panel, 2021) can be consulted. A possible replacement of the existing food and the associated nutritional consequences should be assessed to determine whether nutrient intakes are likely to be altered by the introduction of such products into the food supply. For animal nutrition, the EFSA FEEDAP Panel guidance (EFSA FEEDAP Panel, 2018b) needs to be considered. In all cases, the NGT-M-derived product or biomass should be demonstrated not to have adverse nutritional consequences compared to the conventional food or feed.

3.2.3.9 | *Exposure assessment*

Exposure assessment is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 11 describes the evaluation of this guidance based on the selected case studies.

TABLE 11 Sufficiency of guidances for the exposure assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1	Primary exposure is to environment (nitrogen fertiliser), potential secondary exposure to human, animal gut (consumption of food/feed treated with fertiliser)	Not sufficient	Address primary and secondary exposure routes for all uses incl. biostimulants (such as nitrogen fertiliser)
2	Primary exposure is to environment (endophyte of grass) and to animal gut (via consumption silage), potential secondary exposure to environment and to consumers (via faeces)	Not sufficient	Address primary and secondary exposure routes for all uses, incl. fungal endophytes for use in silage
3*, 5, 7, 11, 12	Primary exposure is animal gut (e.g. probiotics), potential secondary exposure is to consumer and environment (via faeces)	Not sufficient	Address primary and secondary exposure routes
3–6, 9, 11, 12	Primary exposure is to human gut (e.g. wine, beer, probiotics), potential secondary exposure is to environment	Not sufficient	Address primary and secondary exposure routes
8, 10, 13	Primary exposure of human and/or animal gut to genetic material derived from NGT-Ms (Category 3). Potential secondary exposure to environment is considered to be very low	Sufficient	None

*When a case study is mentioned more than once, it is because different aspects are being assessed.

According to the intended use, various guidance documents and tools from EFSA can be consulted and are applicable for the exposure assessment/characterisation of NGT-Ms related to food and feed consumption in the different EFSA risk assessment areas (summarised in SynBio Opinion FF, section 3.11, EFSA Scientific Committee, 2022). Details for performing exposure assessments for NGT-Ms vary case by case and depend on the type of organism, viability status and survival capacity as well as possible secondary routes of exposure depending on its specific use. There is no guidance that addresses primary and potential secondary exposure routes of GMMs, including NGT-Ms for all uses under the remit of EFSA (including for example microbial biostimulants, plant protection products (PPPs), fungal endophytes in silage, microorganisms used for bioremediation) and all types of microorganisms (including for example viruses, microalgae).

Secondary routes of exposure (see also Section 1.4) can derive, for instance, when a viable GMM 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 and to the environment (soil). Another possible secondary route could be exposure of the human or animal gut by consumption of plant material that harbours NGT-Ms used as biostimulants, PPPs or fungal silaging agents.

3.2.3.10 | *Potential environmental impact of GMMs and their products*

Environmental risk assessment (ERA) is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 12 describes the evaluation of this guidance based on the selected case studies.

TABLE 12 Sufficiency of guidances for the environmental risk assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
All category 4 cases (1–7, 9, 11, 12)	ERA is needed for all category 4 cases as or in food and feed	Not sufficient	Update is recommended to elaborate on the relevant areas of risk as per Commission Directive (EU) 2018/350 for the ERA of NGT-Ms
1*, 2, 9	ERA is needed for uses beyond food and feed and for all types of living NGT-Ms	Not sufficient	Update is recommended ERA should address all uses under the remit of EFSA (incl. biostimulants and silaging agents) and all types of microorganisms (incl. viruses)
8, 10, 13	For category 3 products only ERA of horizontal gene transfer (HGT) is necessary	Sufficient	None

*When a case study is mentioned more than once, it is because different aspects are being assessed.

For Category 3 products, the focus of the ERA is on adverse environmental effects resulting from HGT in all environments (see Section 3.2.3.11 on HGT). For Category 4 products, the ERA takes into account all interactions of the GMM/NGT-M with the environment (including effects of HGT) such as effects on plants, animals, humans, soil microbiome and food and feed microbiomes. Potential risks resulting from these interactions are stipulated by Commission Directive (EU) 2018/350 (Annex II, section D) and need to be addressed in ERA of all GMOs (including GMMs, see Annex II, section D.1). EFSA has further specified these risks (or 'areas of risk') and how to address these areas of risk in their opinion on the ERA for GM plants (EFSA GMO Panel, 2010). This further specification has not been complemented yet for ERA of GMMs/NGT-Ms.

In the GMM Guidance (EFSA GMO Panel, 2011a), the general principles of the ERA of GMMs of category 4 and its products are described according to the requirements of Directive 2001/18/EC. This part is equally applicable to NGT-Ms.

The focus of the GMM Guidance is on GMMs for food and feed use and not on other uses of GMMs, such as biostimulants, silaging agents, plant protection agents or GMMs used for bioremediation, and does not refer to other microorganisms such as viruses. Moreover, the ERA in the GMM Guidance does not specify the areas of risk that need to be addressed for GMMs, as has been done for GM plants (EFSA GMO Panel, 2010). A first step in addressing specific areas of risk for GMMs, using a problem formulation approach, has been taken in the frame of the SynBioM ERA Opinion of 2020 (EFSA Scientific Committee, 2020) and is equally applicable to NGT-Ms. Although in the selection of cases no category 4 products other than for agri-food uses were identified, the same principles of ERA will apply to those products.

3.2.3.11 | *Horizontal gene transfer*

Horizontal gene transfer is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 13 describes the evaluation of this guidance based on the selected case studies.

TABLE 13 Sufficiency of guidances for the horizontal gene transfer assessment based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
8, 10, 13	Non-living microorganisms (Category 3)	Not sufficient	Update is recommended to explain that assessment of HGT for biomasses is only needed if they contain genes of concern (e.g. AMR genes)
5, 7	The NGT-M contains newly inserted sequences that are non-endogenous	Sufficient	None
1	The NGT-M contains newly inserted sequences that are endogenous	Not sufficient	Update is recommended to describe the cases in which HGT needs to be assessed, depending on the nature of the modification and the resulting new trait
9, 11, 12	The NGT-M is modified by gene editing		
2–4, 6	The NGT-M contains (targeted) deletions of endogenous sequences	Not applicable, no newly inserted genetic information can be transferred	Update is recommended to explain that no HGT assessment is needed in case of deletions that result in removal or inactivation of genes

The transfer of newly introduced genetic information from a NGT-M (Category 4) or its DNA (Category 3) into other microorganisms may have consequences for human and animal health, and the environment. Genes encoding harmful traits may spread in the microbiota and may provide a selective advantage to one or some of their members, thereby reducing or displacing other microorganisms with beneficial properties.

The process by which this transfer would occur is fundamentally different for both category products. Viable cells have the potential to proliferate in the receiving environment(s) and therefore may potentially increase the probability of transfer. Moreover, genetic exchange between viable cells (e.g. by conjugation and transduction) is far more efficient compared to what can be expected from inactivated biomass which would mainly occur by natural transformation (EFSA GMO Panel, 2004).

Potential risks resulting from HGT of sequences introduced as a consequence of the genetic modification are specifically addressed in the GMM Guidance (EFSA GMO Panel, 2011a) for microorganisms and are considered to be applicable for GMMs, including NGT-Ms.

Some updates are recommended. Although the GMM Guidance (EFSA GMO Panel, 2011a) is applicable to all NGT-Ms in which sequences are introduced, updated guidance could consider the cases in which assessing HGT may not be needed depending on the nature of the modification and the resulting new trait (e.g. NGT-Ms modified with endogenous sequences (self-cloning), NGT-Ms that are only edited). For NGT-Ms in which sequences have been deleted in order to remove or inactivate genes, HGT does not need to be assessed since no new genetic information can be transferred to other microorganisms. For non-living NGT-Ms (Category 3), considering the difference in transfer rates, it is considered that assessment of HGT is only needed in case genes of concern are present due to potential environmental selection of the encoded trait(s) after transfer.

3.2.3.12 | *Post-market environmental monitoring*

Post-market environmental monitoring is described in the GMM guidance (EFSA GMO Panel, 2011a). Table 14 describes the evaluation of this guidance based on the selected case studies.

TABLE 14 Sufficiency of guidances for the post-market environmental monitoring based on the selected case studies.

Case study number	Specific evaluation of the case, intended and unintended effects	Conclusions on the sufficiency of existing guidances (EFSA GMO Panel, 2011a)	Recommendations for future guidance updates (see Section 3.3)
1–7*, 9, 11–12	PMEM is needed for all living GMMs (Category 4)	Not sufficient	Include fit-for- purpose approaches to monitor for potential adverse environmental effects
8, 10, 13	PMEM is not needed for non-living GMMs (Category 3)	Sufficient	None
1–4, 6, 9, 11, 12	The NGT-M contains (targeted) deletions, edits or endogenous sequences	Not sufficient	Clarify that for NGT-Ms that are modified with endogenous sequences (self-cloning), are edited or contain only deletions, the need for PMEM (general surveillance) may be waived, based on the ERA
1, 2, 9	PMEM is needed for all uses and for all types of living NGT-Ms	Not sufficient	Broaden the scope of PMEM to include all types of microorganisms (incl. viruses) and all uses in the remit of EFSA of NGT-Ms, beyond food/feed uses

*When a case study is mentioned more than once, it is because different aspects are being assessed.

In the GMM guidance (EFSA GMO Panel, 2011a), the principles of a post-market environmental monitoring (PMEM) plan are described, which are equally applicable to NGT-Ms. A PMEM plan is obligatory for products consisting or containing

viable GMMs (Category 4). PMEM is meant to identify any direct or indirect, immediate and/or delayed adverse effects of GMMs on human health and the environment after the GMM has been placed on the market. Two types of monitoring are described (e.g. case-specific monitoring and general surveillance), the ways to perform this monitoring and to report on the results. This guidance also applies to NGT-Ms. However, no specific guidance is given on how to perform monitoring.

In order to come to fit-for-purpose approaches for general surveillance of GMMs, existing networks that are already in place for environmental and agricultural monitoring may be used. This approach is in line with approaches taken for general surveillance for GM plants (EFSA GMO Panel, 2011b).

As mentioned above, PMEM is meant to detect any adverse effect on human health and the environment after the GMM has been placed on the market. It can be foreseen on a case-by-case basis for certain NGT-Ms, especially those that are modified with endogenous sequences (self-cloning), deletions or only containing edits, the need for PMEM (general surveillance) may be waived, based on the ERA. This is not exclusive to NGT-Ms but applies equally to GMMs.

3.3 | ToR3: In case existing guidelines for risk assessment are considered not applicable, partially applicable or not sufficient, to identify on which aspects existing guidelines should be updated, adapted or complemented

3.3.1 | AQ1. Which aspect (if any) of existing guidelines should be updated, adapted or complemented?

The EFSA GMO Panel together with the GMM NGT WG concluded that the existing guidelines are not sufficient and updates are recommended as follows (Table 15).

TABLE 15 Applicability and recommended updates of the existing guidance documents in the case the existing guidelines are not sufficient.

Area of risk assessment	Applicable guidance exists (see Section 3)	Recommended updates	NGT specific update
Comparative assessment	EFSA GMO Panel (2011a)	Expand the definition of comparator: inclusion as comparator of microorganisms not previously used in the food and feed chain (no history of safe use)	None
Microbial characterisation	EFSA GMO Panel (2011a), EFSA FEEDAP Panel (2018a), EFSA CEP Panel (2021)	Inclusion of protists/microalgae/viruses Antimycotic resistance of viable yeasts and fungi	Assessment of the presence/absence of the CRISPR-Cas system intentionally introduced
Production process	EFSA GMO Panel (2011a), EFSA FEEDAP Panel (2018a), EFSA CEP Panel (2021), EFSA ANS Panel (2012), EFSA FAF Panel (2021)	Inclusion of protists/microalgae/viruses	None
Compositional analysis	EFSA GMO Panel (2011a)	None	None
Toxicological assessment	EFSA GMO Panel (2011a)	Inclusion of in silico and in vitro methods to replace animal studies	None
Gut microbiome	EFSA GMO Panel (2011a), EFSA FEEDAP Panel (2018a)	The setting of suitable endpoints and the development of validated methodologies are recommended to assess effects on the gut microbiome	None
Allergenicity	EFSA GMO Panel (2011a)	Expand on adjuvanticity and potential methodologies (when available) to assess it	None
Nutritional assessment	EFSA GMO Panel (2011a), EFSA FEEDAP Panel (2018b)	None	None
Exposure assessment	EFSA GMO Panel (2011a)	Address primary and potential secondary exposure for all uses and microorganisms under the remit of EFSA	None
ERA	EFSA GMO Panel (2011a)	Inclusion of all uses and microorganisms under the remit of EFSA Detail all areas of risk as per Commission Directive (EU) 2018/350 (Annex II Section D.1)	None
HGT	EFSA GMO Panel (2011a)	Consideration of cases in which the HGT assessment may not be needed	None
PMEM	EFSA GMO Panel (2011a)	Include fit for purpose approaches to monitor for potential adverse environmental effects Broaden scope to include all uses under the remit of EFSA Considerations of cases in which PMEM may not be needed based on the ERA	None

3.3.2 | AQ2. What recommendations can be formulated for future guidance updates?

It is concluded that none of the EFSA guidances are 'fully applicable' but they are 'partially applicable' and 'not sufficient'. On a case-by-case basis for specific NGT-Ms fewer requirements may be needed.

The EFSA GMO Panel notes that microorganisms are increasingly used in the agri/food chain and are developed for this purpose by using conventional mutagenesis, EGTs and NGTs. Although the techniques differ in the efficiency and the precision by which the modifications are introduced, the risk assessment concentrates on the possible hazards related to the trait. The same mutations and the related hazards can often be introduced by different techniques. Conventional mutagenesis will generate random mutations and relies on selection for traits of interest (e.g. an increased enzyme production, a decreased protease activity). EGTs and NGTs can also be used to generate both random and precise mutations in the microbial genome.

Possible hazards relate to the genotypic and phenotypic changes introduced in the microorganism, not to the method used for the modification. The microbiological risk assessment approach should therefore be based on the strain/product itself, independently of the method used to alter genotypic or phenotypic characteristics. It is therefore recommended that any new guidance should take a consistent risk assessment approach for strains/products derived from or produced with microorganisms obtained with conventional mutagenesis, EGTs or NGTs.

The following aspects of the existing guidelines are recommended to be updated/adapted or complemented.

3.3.2.1 | *Comparative approach*

The definition of the comparator as described in the GMM guidance (EFSA GMO Panel, 2011a) needs to be expanded for its use in the frame of the microbial risk assessment at EFSA, including the risk assessment of NGT-Ms.

It is recommended that:

- Guidance for the comparative approach is updated to include microorganisms not previously used in food and feed.

For this, the body of knowledge available for the species under assessment is taken into consideration as it provides information on any possible safety issue related to the biology of the microorganism, including the genetic information.

This recommendation applies to GMMs, including NGT-Ms.

3.3.2.2 | *Microbial characterisation*

The WGS is essential for the characterisation of all microorganisms. However, as already discussed above (Section 3.2.3.2), there are currently some limitations in the analysis of the WGS data for some microalgae because of the lack of complete information on reference genomes.

It is recommended that guidance considers:

- phenotypic characterisation and, for the interpretation of the WGS data for GM protists/microalgae and viruses guidance is developed.
- antimycotic resistance of viable yeasts/fungi.

These recommendations apply to non-GMMs and GMMs including NGT-Ms.

- evaluation of presence/absence of the CRISPR-Cas or similar systems intentionally introduced in the microorganism.

This recommendation is specific for the NGT-Ms.

3.3.2.3 | *Toxicology*

The existing guidances cover the toxicological assessment of NGT-Ms and their products.

It is recommended that:

- guidance for the toxicological safety assessment is updated, in line with the 3R principles, to reduce the dependence on animal (rodent) studies.

This could be done for example, by making use of artificial intelligence-based tools for the prediction and comparison of three-dimensional protein to assess the safety of newly expressed proteins or the safety of newly introduced amino acid

substitutions (more information is expected under the pending mandate of the GMO Panel on protein safety assessment EFSA-Q-2023-00664[11]).

This recommendation applies to non-GMMs and GMMs including NGT-Ms.

3.3.2.4 | *Gut microbiome*

The existing GMM guidance (EFSA GMO Panel, 2011a) is not fully applicable and sufficient for the risk assessment of micro-organisms because it does not specify what is considered an impact on the microbiota, neither provides recommendations for the determination of the impact and, in addition, does not consider the microbiome.

It is recommended that:

- guidance is updated with uniform methods for certain endpoints when they become available, and with the experience gained during the risk assessment of such products.

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.

This recommendation applies to non-GMMs and GMMs including NGT-Ms.

3.3.2.5 | *Allergenicity*

The current guidance (EFSA GMO Panel, 2011a) is applicable but not sufficient because it does not describe comprehensively the concept of adjuvanticity and potential methodologies to assess it.

It is recommended that:

- guidance for all microorganisms is developed to consider adjuvanticity referred to the functional properties of microbes, including microbial-derived metabolites or biomolecules of different chemical nature.

This new guidance should define the characteristics of adjuvants together with the functional features of GMMs that are associated to adjuvanticity. In doing so, reaching an international scientific consensus on which of the metabolites derived from microorganisms hold adjuvanticity potential will be essential. Furthermore, the techniques to measure adjuvanticity need to be defined and the methodologies established.

This recommendation applies to non-GMMs and GMMs including NGT-Ms.

3.3.2.6 | *Exposure*

The current guidance (EFSA GMO Panel, 2011a) is not sufficient.

It is recommended that:

- future updates address primary and potential secondary routes of exposure of all uses under the remit of EFSA and all types of microorganisms.

This recommendation applies to GMMs including NGT-Ms.

3.3.2.7 | *Environmental risk assessment (ERA)*

The current guidance (EFSA GMO Panel, 2011a) is not sufficient.

It is recommended that:

- an updated guidance covers all uses under the remit of EFSA, all types of micro-organisms, their relevant exposure routes and receiving environments and addresses all 'specific areas of risk' as per Directive 2001/18/EC. These areas of risk in the GMM guidance (EFSA GMO Panel, 2011a) need to be elaborated on for ERA of GM microorganisms including NGT-Ms.

This recommendation applies to GMMs including NGT-Ms.

[11] https://open.efsa.europa.eu/questions/EFSA-Q-2023-00664.

3.3.2.8 | *HGT*

The current GMM guidance (EFSA GMO Panel, 2011a) is sufficient to assess potential adverse effects resulting from HGT of GMMs that are genetically modified with new sequences. It does not address GMMs that contain only deletions, edits or are modified with endogenous sequences.

It is recommended that updated guidance takes aboard the following recommendations:

- a clarification that for NGT-Ms modified with endogenous sequences (self-cloning) or containing edits assessment of potential risks resulting from HGT may be waived on a case-by-case basis, depending on the nature of the modification and the resulting new trait.
- for NGT-Ms in which sequences have been deleted in order to remove or inactivate genes assessment of HGT is not considered applicable because there is no new genetic information that can be transferred to other microorganisms.
- for non-living NGT-Ms (Category 3) assessment of HGT is only needed for genes of concern, due to potential environmental selection after transfer.

These recommendations apply to GMMs including NGT-Ms.

3.3.2.9 | *PMEM*

The current GMM guidance (EFSA GMO Panel, 2011a) is not sufficient.

Future updates of the guidance are recommended to:

- include descriptions of fit-for-purpose approaches to monitor for potential adverse effects resulting from the deliberate environmental release.
- broaden the scope of PMEM to include all uses under the remit of EFSA and all types of microorganisms.
- state that for certain GMMs, especially those that are modified with endogenous sequences (self-cloning), only containing edits or deletions, the need for PMEM (general surveillance) may be waived, based on the ERA.

These recommendations apply to GMMs including NGT-Ms.

3.3.3 | Recommendations for additional guidance

In the case of GMMs, including the NGT-Ms, developed to contain engineered gene drive or similar technologies designed to bias, and therefore speed up, the transmission of certain genetic elements in a target population, additional guidance is recommended to be developed.

4 | CONCLUSIVE REMARKS OF THE GMO PANEL

- No novel potential hazards and risks were identified that NGTs applied to microorganisms could pose for humans, animals and the environment as compared to EGTs and conventional mutagenesis. Possible hazards relate to the genotypic and phenotypic changes introduced in the microorganism, regardless of the method (EGTs, NGTs, conventional mutagenesis) used.
- It is concluded that EFSA guidances are 'partially applicable' therefore on a case-by-case basis for specific NGT-Ms fewer requirements may be needed.
- Some of the EFSA guidances are 'not sufficient' and updates are recommended.
- Recommended updates are not exclusive to the assessment of NGT-Ms, apart from one (the evaluation of presence/absence of the CRISPR-Cas or similar systems intentionally introduced in the microorganism).
- The risk assessment approach of microorganisms should be based on the strain/product itself, independently of the method used to alter genotypic or phenotypic characteristics.
- It is recommended that any new guidance should take a consistent risk assessment approach for strains/products derived from or produced with microorganisms obtained with conventional mutagenesis, EGTs or NGTs.

GLOSSARY

Adjuvanticity	the capacity of a substance to augment, or divert, the body's immune response to an antigen.
Allergenicity	the ability of a substance to trigger an abnormal immune response that leads to an allergic reaction.
Antigen	molecule that can induce antibody generation towards itself.

Conventional mutagenesis	genetic modification technique that makes use of e.g. physical or chemical mutagens applied to cells.
Comparative approach	Analysis of potential adverse effects resulting from a GMM when compared with a counterpart with familiarity.
Gene of concern	gene known to contribute to the production of toxic metabolites or antimicrobials of clinical relevance, or to antimicrobial resistance. For products with viable cells, other virulence factors are also included in this definition.
Genetically modified microorganisms (GMMs)	Microorganisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.
History of use	Documented information on the microbial strain regarding its previous deliberate introduction or use in the food chain.
IgE	Immunoglobulin E, key effector antibody in type I hypersensitivity reactions.
Intended effects	Changes that are meant to occur due to the genetic modification and that fulfil the objectives of the genetic modification.
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.
Quantitative 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.
NGT-M	microorganism developed with the use of New Genomic Techniques (NGTs).
Gut microbiota	collection of living microorganisms inhabiting the animal or human gut.
Gut microbiome	Entirety of genomes derived from all microorganisms present in the animal or human gut environment including also their structural components, metabolites, and the prevailing environmental conditions.
Recombinant DNA	A form of DNA that is created by combining two or more sequences that would not normally occur together.
Problem formulation	Process the includes the identification of characteristics of the GM organism capable of causing potential adverse effects to the environment (hazards), of the nature of these effects, and of pathways of exposure through which the GM organism may adversely affect the environment (hazard identification). It also includes the definition of the assessment endpoints and the setting of specific hypothesis to guide the generation and evaluation of data in the next risk assessment steps (hazard and exposure characterisation).
NGT	New Genomic Technique. These are techniques which are capable of altering the genetic material of an organism, and which have been developed after the publication of Directive 2001/18/EC (Broothaerts et al., 2021).
EGT	Established Genomic Technique. These are techniques capable of altering the genetic material of an organism, and which have been developed prior to the publication of Directive 2001/18/EC (Broothaerts et al., 2021).
CRISPR-Cas	Clustered Regularly-Interspaced Short Palindromic Repeats- CRISPR-associated protein.

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CONFLICT OF INTEREST

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REFERENCES

Ajjawi, I., Verruto, J., Aqui, M., Soriage, L. B., Coppersmith, J., Kwok, K., Peach, L., Orchard, E., Kalb, R., Xu, W., Carlson, T. J., Francis, K., Konigsfeld, K., Bertalis, J., Schultz, A., Lambert, W., Schwartz, A. S., Brown, R., & Moellering, E. R. (2017). Lipid production in Nannochloropsis gaditana is doubled by decreasing expression of a single transcriptional regulator. *Nature Biotechnology*, *35*, 647–652. https://doi.org/10.1038/nbt.3865

Akdis, C. A. (2021). Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? *Nature Reviews Immunology*, *21*, 739–751. https://doi.org/10.1038/s41577-021-00538-7

Bahri, R., Custovic, A., Korosec, P., Tsoumani, M., Barron, M., Wu, J., Sayers, R., Weimann, A., Ruiz-Garcia, M., Patel, N., Robb, A., Shamji, M. H., Fontanella, S., Silar, M., Mills, E. N. C., Simpson, A., Turner, P. J., & Bulfone-Paus, S. (2018). Mast cell activation test in the diagnosis of allergic disease and anaphylaxis. *The Journal of Allergy and Clinical Immunology*, *142*, 485. https://doi.org/10.1016/j.jaci.2018.01.043

Ballester, A. R., Roqué, M., Ricci-Cabello, I., Rotger, A., & Malih, N. (2023). Horizon scanning on microorganisms and their products obtained by new developments in biotechnology. *EFSA Supporting Publication*, *20*, 8503. https://doi.org/10.2903/sp.efsa.2023.EN-8503

Broothaerts, W., Jacchia, S., Angers, A., Petrillo, M., Querci, M., Savini, C., Van den Eede, G., & Emons, H. (2021). New genomic techniques: State-of-the-art review, EUR 30430 EN, Publications Office of the European Union, Luxembourg, 2021, ISBN 978-92-76-24696-1. https://doi.org/10.2760/710056

Bruton, K., Koenig, J. F. E., Phelps, A., & Jordana, M. (2020). Perturbations to homeostasis in experimental models revealed innate pathways driving food allergy. *Frontiers in Immunology*, *10*(11), 603272. https://doi.org/10.3389/fimmu.2020.603272

Cai, P., Gao, J., & Zhou, Y. (2019). CRISPR-mediated genome editing in non-conventional yeasts for biotechnological applications. *Microbial Cell Factories*, *18*, 63. https://doi.org/10.1186/s12934-019-1112-2

Caminero, A., Guzman, M., Libertucci, J., & Lomax, A. E. (2023). The emerging roles of bacterial proteases in intestinal diseases. *Gut Microbes*, *15*, 2181922. https://doi.org/10.1080/19490976.2023.2181922

COMMISSION DIRECTIVE (EU) 2018/350 of 8 March 2018 amending Directive 2001/18/EC of the European Parliament and of the Council as regards the environmental risk assessment of genetically modified organisms- OJ L 067, 09.03.2018, p. 30–45.

Denby, C. M., Li, R. A., Vu, V. T., Costello, Z., Lin, W., Chan, L. J. G., Williams, J., Donaldson, B., Bamforth, C. W., Petzold, C. J., Scheller, H. V., Martin, H. G., & Keasling, J. D. (2018). Industrial brewing yeast engineered for the production of primary flavor determinants in hopped beer. *Nature Communications*, *9*, 965. https://doi.org/10.1038/s41467-018-03293-x

DiCarlo, J. E., Chavez, A., Dietz, S., Esvelt, K. M., & Church, G. M. (2015). Safeguarding CRISPR-Cas9 gene drives in yeast. *Nature Biotechnology*, *33*, 1250–1255. https://doi.org/10.1038/nbt.3412

Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration. OJ L 106, 17.4.2001, p. 1–39.

Durmusoglu, D., Al'Abri, I., Collins, S., Cheng, J., Eroglu, A., Beisel, C., & Crook, N. (2021). In situ biomanufacturing of small molecules in the mammalian gut by probiotic saccharomyces boulardii. *ACS Synthetic Biology*, *10*, 1039–1052. https://doi.org/10.1021/acssynbio.0c00562

EFSA (European Food Safety Authority). (2021). EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. *EFSA Journal*, *19*(7), 6506. https://doi.org/10.2903/j.efsa.2021.6506

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources Added to Food). (2012). Guidance for submission for food additive evaluations. *EFSA Journal*, *10*(7), 2760. https://doi.org/10.2903/j.efsa.2012.2760

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Koutsoumanis, K., Allende, A., Álvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Fernández Escámez, P. S., Prieto Maradona, M., … Herman, L. (2023). Scientific opinion on the update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA. *EFSA Journal*, *21*(1), 7747. https://doi.org/10.2903/j.efsa.2023.7747

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Nonno, R., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Fernández Escámez, P. S., … Herman, L. (2024). Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 19: Suitability of taxonomic units notified to EFSA until September 2023. *EFSA Journal*, *22*(1), e8517. https://doi.org/10.2903/j.efsa.2024.8517

EFSA CEP Panel (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids). (2021). Scientific Guidance for the submission of dossiers on food enzymes. *EFSA Journal*, *19*(10), 6851. https://doi.org/10.2903/j.efsa.2021.6851

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings). (2021). Scientific guidance for the preparation of applications on smoke flavouring primary products. *EFSA Journal*, *19*(3), 6435. https://doi.org/10.2903/j.efsa.2021.6435

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). (2017a). Guidance on the assessment of the safety of feed additives for the target species. *EFSA Journal*, *15*(10), 5021. https://doi.org/10.2903/j.efsa.2017.5021

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). (2017b). Guidance on the assessment of the safety of feed additives for the consumer. *EFSA Journal*, *15*(10), 5022. https://doi.org/10.2903/j.efsa.2017.5022

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). (2018a). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal*, *16*(3), 5206. https://doi.org/10.2903/j.efsa.2018.5206

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. L., Bories, G., Chesson, A., Cocconcelli, P. S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., … Martino, L. (2018b). Guidance on the assessment of the efficacy of feed additives. *EFSA Journal*, *16*(5), 5274. https://doi.org/10.2903/j.efsa.2018.5274

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). (2019). Guidance on the assessment of the safety of feed additives for the environment. *EFSA Journal*, *17*(4), 5648. https://doi.org/10.2903/j.efsa.2019.5648

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed). (2023). Guidance on the assessment of the safety of feed additives for the users. *EFSA Journal*, *21*(12), e8469. https://doi.org/10.2903/j.efsa.2023.8469

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2004). Opinion of the Scientific Panel on Genetically Modified Organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. *EFSA Journal*, *2*(4), 48. https://doi.org/10.2903/j.efsa.2004.48

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2010). Guidance on the environmental risk assessment of genetically modified plants. *EFSA Journal*, *8*(11), 1879. https://doi.org/10.2903/j.efsa.2010.1879

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2011a). Scientific opinion on guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. *EFSA Journal*, *9*(6), 2193. https://doi.org/10.2903/j.efsa.2011.2193

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2011b). Scientific opinion on guidance on the post-market environmental monitoring (PMEM) of genetically modified plants. *EFSA Journal*, *9*(8), 2316. https://doi.org/10.2903/j.efsa.2011.2316

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms). (2017). Guidance on allergenicity assessment of genetically modified plants. *EFSA Journal*, *15*(5), 4862. https://doi.org/10.2903/j.efsa.2017.4862

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), Mullins, E., Bresson, J.-L., Dalmay, T., Dewhurst, I. C., Epstein, M. M., George Firbank, L., Guerche, P., Hejatko, J., Naegeli, H., Nogue, F., Rostoks, N., Sanchez Serrano, J. J., Savoini, G., Veromann, E., Veronesi, F., Fernandez Dumont, A., & Moreno, F. J. (2022). Scientific opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology. *EFSA Journal*, *20*(1), e07044. https://doi.org/10.2903/j.efsa.2022.7044

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies). (2021). Guidance on the preparation and submission of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283 (revision 1). *EFSA Journal*, *19*(3), 6555. https://doi.org/10.2903/j.efsa.2021.6555

EFSA Scientific Committee, More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernandez-Jerez, A., Hougaard Bennekou, S., Koutsoumanis, K., Machera, K., Naegeli, H., Nielsen, S. S., Schlatter, J., Schrenk, D., Silano, V., Turck, D., Younes, M., Glandorf, B., Herman, L., … Cocconcelli, P. S. (2020). Scientific opinion on the evaluation of existing guidelines for their adequacy for the microbial characterisation and environmental risk assessment of microorganisms obtained through synthetic biology. *EFSA Journal*, *18*(10), 6263. https://doi.org/10.2903/j.efsa.2020.6263

EFSA Scientific Committee, More, S., Bampidis, V., Benford, D., Bragard, C., Halldorsson, T., Hernández-Jerez, A., Bennekou, S. H., Koutsoumanis, K., Lambré, C., Machera, K., Mullins, E., Nielsen, S. S., Schlatter, J., Schrenk, D., Turck, D., Younes, M., Herman, L., Pelaez, C., … Cocconcelli, P. S. (2022). Scientific opinion on the evaluation of existing guidelines for their adequacy for the food and feed risk assessment of microorganisms obtained through synthetic biology. *EFSA Journal*, *20*(8), 7479. https://doi.org/10.2903/j.efsa.2022.s7479

Ellenbogen Y, Jiménez-Saiz R, Spill P, Chu DK, Waserman S and, Jordana M, 2018. The initiation of Th2 immunity towards food allergens. *International Journal of Molecular Sciences*. *19*(5), 1447. https://doi.org/10.3390/ijms19051447

Elst, J., van der Poorten, M. M., Van Gasse, A. L., De Puysseleyr, L., Hagendorens, M. M., Faber, M. A., Van Houdt, M., Passante, E., Bahri, R., Walschot, M., Mertens, C., Bridts, C. H., Sabato, V., & Ebo, D. G. (2021). Mast cell activation tests by flow cytometry: A new diagnostic asset? Clin Exp allergy. *Novum*, *51*(11), 1482–1500. https://doi.org/10.1111/cea.13984

Florea, S., Jaromczyk, J., & Schardl, C. L. (2021). Non-transgenic CRISPR-mediated knockout of entire ergot alkaloid gene clusters in slow-growing asexual Polyploid fungi. *Toxins*, *13*, 153. https://doi.org/10.3390/toxins13020153

Garg, S. (2021). Computational methods for chromosome-scale haplotype reconstruction. *Genome Biology*, *22*, 101. https://doi.org/10.1186/s13059-021-02328-9

Hang, S., Paik, D., Yao, L., Kim, E., Trinath, J., Lu, J., Ha, S., Nelson, B. N., Kelly, S. P., Wu, L., Zheng, Y., Longman, R. S., Rastinejad, F., Devlin, A. S., Krout, M. R., Fischbach, M. A., Littman, D. R., & Huh, J. R. (2019). Bile acid metabolites control TH17 and Treg cell differentiation. *Nature*, *576*(7785), 143–148. https://doi.org/10.1038/s41586-019-1785-z Erratum in: Nature 2020 Mar;579(7798):E7.

Horvath, P., & Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. *Science*, *327*, 167–170. https://doi.org/10.1126/science.1179555

Hoshiga, F., Yoshizaki, K., Takao, N., Miyanaga, K., & Tanji, Y. (2019). Modification of T2 phage infectivity toward Escherichia coli O157:H7 via using CRISPR/Cas9. *FEMS Microbiology Letters*, *366*(4), fnz041. https://doi.org/10.1093/femsle/fnz041

Hou, K., Wu, Z. X., Chen, X. Y., Wang, J.-Q., Zhang, D., Xiao, C., Zhu, D., Koya, J. B., Wei, L., Li, J., & Chen, Z.-S. (2022). Microbiota in health and diseases. *Signal Transduction and Targeted Theraphy*, *7*, 135. https://doi.org/10.1038/s41392-022-00974-4

Hurel, J., Schbath, S., Bougeard, S., Rolland, M., Petrillo, M., & Touzain, F. (2020). DUGMO: Tool for the detection of unknown genetically modified organisms with high-throughput sequencing data for pure bacterial samples. *BMC Bioinformatics*, *21*(1), 284. https://doi.org/10.1186/s12859-020-03611-5

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*, 816–821. https://doi.org/10.1126/science.1225829

Jung, J.-Y., Kang, M.-J., Hwang, H.-S., Baek, K.-R., & Seo, S.-O. (2023). Reduction of ethyl carbamate in an alcoholic beverage by CRISPR/Cas9-based genome editing of the wild yeast. *Food*, *12*, 102. https://doi.org/10.3390/foods12010102

Kong, L., Song, X., Xia, Y., Ai, L., & Xiong, Z. (2022). Construction of a CRISPR/nCas9-assisted genome editing system for exopolysaccharide biosynthesis in Streptococcus thermophilus, Food Research International, 158,2022,111550. ISSN 0963-9969. https://doi.org/10.1016/j.foodres.2022.111550

Kopp EB, Agaronyan K, Licona-Limón I, Nish SA, & Medzhitov R, 2023. Modes of type 2 immune response initiation. *Immunity*, *56*(4), 687–694. doi:https://doi.org/10.1016/j.immuni.2023.03.015

Le Gall, G., Noor, S. O., Ridgway, K., Scovell, L., Jamieson, C., Johnson, I. T., Colquhoun, I. J., Kemsley, E. K., & Narbad, A. (2011). Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. *Journal of Proteome Research*, *10*(9), 4208–4218. https://doi.org/10.1021/pr2003598

Lee, L. Y. G. N., Leow, S. Y., Wen, H., Soh, J. Y., Chiang, W. C., Zhong, Y., Tham, E. H., Loh, W., Delsing, D. J., Lee, B. W., & Huang, C. H. (2022). An evaluation of the mechanisms of Galacto-oligosaccharide (GOS)-induced IgE cross-linking on basophils in GOS allergy. *Frontiers in Allergy*, *28*(3), 840454. https://doi.org/10.3389/falgy.2022.840454

Levan, S. R., Stamnes, K. A., Lin, D. L., Panzer, A. R., Fukui, E., McCauley, K., Fujimura, K. E., McKean, M., Ownby, D. R., Zoratti, E. M., Boushey, H. A., Cabana, M. D., Johnson, C. C., & Lynch, S. V. (2019). Elevated faecal 12,13-diHOME concentration in neonates at high risk for asthma is produced by gut bacteria and impedes immune tolerance. *Nature Microbiology*, *11*, 1851–1861. https://doi.org/10.1038/s41564-019-0498-2 Erratum in: Nature Microbiology 2019 Sep 6.

Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., Andrews, E., Ajami, N. J., Bonham, K. S., Brislawn, C. J., Casero, D., Courtney, H., Gonzalez, A., Graeber, T. G., Hall, A. B., Lake, K., Landers, C. J., Mallick, H., Plichta, D. R., … Huttenhower, C. (2019). Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature*, *569*(7758), 655–662. https://doi.org/10.1038/s41586-019-1237-9

Moreno, F. J., Pazos, F., Garrido-Romero, M., Payen, C., Borrego-Yaniz, G., Chagoyen, M., Corzo, N., Denis, M., Fablet, C., Fernández, M., Granja, A., Guinebretière, M., Guyard, M., Jiménez-Saiz, R., Keita, A., Kerouanton, A., Márquez, A., Martín, J., Montilla, A., … Chemaly, M. (2024). Roadmap for the integration of gastro-intestinal (GI) tract microbiomes (human and domestic animal) in risk assessments under EFSA's remit. *EFSA Supporting Publication*, *21*(2), EN-8597. https://doi.org/10.2903/sp.efsa.2024.EN-8597

Ni, B., Kaplan, D. L., & Buehler, M. J. (2023). Generative design of de novo proteins based on secondary-structure constraints using an attention-based diffusion model. *Chem*, *9*, 1828–1849. https://doi.org/10.1016/j.chempr.2023.03.020

Platts-Mills, T. A., Hilger, C., Jappe, U., van Hage, M., Gadermaier, G., Spillner, E., Lidholm, J., Keshavarz, B., Aalberse, R. C., van Ree, R., Goodman, R. E., & Pomés, A. (2021). Carbohydrate epitopes currently recognized as targets for IgE antibodies. *Allergy*, *76*(8), 2383–2394. https://doi.org/10.1111/all.14802

Plum, M., Tjerrild, L., Raiber, T., Bantleon, F., Bantleon, S., Miehe, M., Jabs, F., Seismann, H., Möbs, C., Pfützner, W., Jakob, T., Andersen, G. R., & Spillner, E. (2023). Structural and functional analyses of antibodies specific for modified core N-glycans suggest a role in TH 2 responses. *Allergy*, *78*(1), 121–130. https://doi.org/10.1111/all.15417

Rees-Garbutt, J., Chalkley, O., Landon, S., Purcell, O., Marucci, L., & Grierson, C. (2020). Designing minimal genomes using whole-cell models. *Nature Communications*, *11*, 836. https://doi.org/10.1038/s41467-020-14545-0

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed.

Santos, A. F., Alpan, O., & Hoffmann, H. J. (2021). Basophil activation test: Mechanisms and considerations for use in clinical trials and clinical practice. *Allergy*, *76*(8), 2420–2432. https://doi.org/10.1111/all.14747

Sharma, S. R., & Karim, S. (2021). Tick saliva and the alpha-gal syndrome: Finding a needle in a haystack. *Frontiers in Cellular and Infection Microbiology*, *20*(11), 680264. https://doi.org/10.3389/fcimb.2021.680264

Sherkatghanad, Z., Abdar, M., Charlier, J., & Makarenkov, V. (2023). Using traditional machine learning and deep learning methods for on- and off-target prediction in CRISPR/Cas9: A review. *Briefings in Bioinformatics*, *24*(3), bbad131. https://doi.org/10.1093/bib/bbad131

Sozener, Z. C., Ozdel Ozturk, B., Cerci, P., Turk, M., Gorgulu Akin, B., Akdis, M., Altiner, S., Ozbey, U., Ogulur, I., Mitamura, Y., Yilmaz, I., Nadeau, K., Ozdemir, C., Mungan, D., & Akdis, C. A. (2022). Epithelial barrier hypothesis: Effect of the external exposome on the microbiome and epithelial barriers in allergic disease. *Allergy*, *77*(5), 1418–1449. https://doi.org/10.1111/all.15240

Su, T., Liu, F., Gu, P., Jin, H., Chang, Y., Wang, Q., Liang, Q., & Qi, Q. (2016). A CRISPR-Cas9 assisted non-homologous end-joining strategy for one-step engineering of bacterial genome. *Scientific Reports*, *6*, 37895. https://doi.org/10.1038/srep37895

Swidah, R., Auxillos, J., Liu, W., Jones, S., Chan, T. F., Dai, J., & Cai, Y. (2020). SCRaMbLE-in: A fast and efficient method to diversify and improve the yields of heterologous pathways in synthetic yeast. *Methods in Molecular Biology*, *2205*, 305–327. https://doi.org/10.1007/978-1-0716-0908-8_17

Wen, A., Havens, K., Bloch, S., Shah, N., Higgins, D., Davis-Richardson, A., Sharon, J., Rezaei, F., Mohiti-Asli, M., Johnson, A., Abud, G., Ane, J.-M., Maeda, J., Infante, V., Gottlieb, S., Lorigan, J., Williams, L., Horton, A., McKellar, M., … Temme, K. (2021). Enabling biological nitrogen fixation for cereal crops in fertilized fields. *ACS Synthetic Biology*, *10*(12), 3264–3277. https://doi.org/10.1021/acssynbio.1c00049

Wiedenheft, B., Sternberg, S. H., & Doudna, J. A. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature*, *482*(7385), 331–338. https://doi.org/10.1038/nature10886

Xu, X., Meier, F., Blount, B. A., Pretorius, I. S., Ellis, T., Paulsen, I. T., & Williams, T. C. (2023). Trimming the genomic fat: minimising and re-functionalising genomes using synthetic biology. *Nature Communications*, *14*, 1984. https://doi.org/10.1038/s41467-023-37748-7

Zhou, X., Wang, X., Luo, H., Wang, Y., Wang, Y., Tu, T., Qin, X., Su, X., Bai, Y., Yao, B., Huang, H., & Zhang, J. (2021). Exploiting heterologous and endogenous CRISPR-Cas systems for genome editing in the probiotic *clostridium butyricum*. *Biotechnology and Bioengineering*, *118*, 2448–2459. https://doi.org/10.1002/bit.27753

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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APPENDIX A

Hypothetical cases

CASE STUDY 11

Adaptive laboratory evolution using CRISPR-CAS mutagenesis yields probiotic *Saccharomyces cerevisiae* with improved acid tolerance.

Starting from the existing and approved probiotic yeast *Saccharomyces cerevisiae var boulardii*, improved variants tolerant to organic acids were generated.

In short, a targetable Cas3 enzyme fused to a base editor (cytidine deaminase) to introduce random mutations in two genes linked to acid resistance (*TRT2* and *IRA2*), was used. The Cas3 enzyme makes it possible to target the deaminase to which it is fused to specific regions in the cell by expressing a gRNA that has the same sequence as the genomic target region. After it finds it target region in the genome, the Cas3 enzyme unwinds the DNA (helicase activity), which exposes the singe-stranded genomic DNA to the activity of the deaminase.

A population of 100 million cells was subjected to the treatment. The population was subsequently exposed to high acid concentrations and recovered the cells that survived a 1-hour exposure by plating the population on rich YPD medium. Five viable colonies were retrieved. Each of these were tested for their resistance to organic acids. One colony showed a 5-fold increased survival rate compared to the original wild-type progenitor *S. cerevisiae* var *boulardii* yeast. Whole-genome sequencing revealed three mutations in the genome. Two of these were located within the IRA2 gene and included a deletion of 6 nucleotides between position 4 and 9 in the open reading frame, as well as one point mutation (C to T) at position 20, which is predicted to cause an amino acid change (proline to leucine). A third mutation was found elsewhere in the genome, in a non-coding region 27 nucleotides upstream of the *ATF1* gene, which is involved in the production of volatile acetate esters.

To investigate which mutations contribute to the desired phenotype, each of the mutations was introduced by CRISPR-CAS into the wild-type *S. cerevisiae* var *boulardii* yeast strain. This analysis revealed that all three mutations contribute to the desirable phenotype, and that all three mutations need to be present at the same time to reach the full resistance. Further sequence analysis of the 2000 sequenced *S. cerevisiae* genomes revealed that the 6-bp deletion has been found in some natural yeasts. The two other mutations have not yet been found in natural isolates.

The objective is to introduce the improved strain with the three mutations to the market as a probiotic. The applicant argues that all of these are small mutations that, although obtained through CRISPR-Cas-based methods, could also occur naturally, and given the QPS status of *S. cerevisiae*, it is argued that none of these mutations are any cause of concern.

CASE STUDY 12

Improving acetic acid tolerance of a *Saccharomyces cerevisiae* probiotic yeast by introducing natural alleles through CRISPR-Cas genome editing.

In this study, information from quantitative trait loci (QTL) studies in Saccharomyces was used to introduce beneficial naturally occurring alleles into the background of an existing probiotic strain to improve its tolerance to acids.

S. cerevisiae has been used as a probiotic for several years. In some cases, the yeast is referred to as *Saccharomyces boulardii*, even though whole genome sequencing indicated that this is not a separate species but rather a natural stress-resistant *S. cerevisiae* strain (https://doi.org/10.3748/wjg.v22.i48.10532; https://doi.org/10.1016/j.ygeno.2020.11.034).

Further improving the acid tolerance of *S. cerevisiae var boulardii* would help it to survive harsh conditions encountered in the gastrointestinal tract. Hence, the objective was to introduce specific point mutations in the genome of the wild-type *S. cerevisiae var boulardii* yeast. Importantly, the alleles introduced occur in other natural *S. cerevisiae* yeast strains. CRISPR-Cas genome editing technique was used to introduce these mutations in the genome of *S. cerevisiae var boulardii* without introducing any marker genes or scars.

Over the past years, several studies have uncovered the natural genetic variation across different natural *S. cerevisiae* strains, and identified specific alleles that contribute to particular phenotypes found in particular natural strains (reviewed in https://doi.org/10.1371/journal.pgen.1002912; https://doi.org/10.1111/j.1567-1364.2011.00777.x; https://doi.org/10.1016/j.gde.2022.101979). For example, Stojiljkovic and co-workers (REF: https://doi.org/10.1186/s13068-020-01761-5) identified alleles that contribute to acetic acid tolerance in yeast. Acetic acid tolerance is a complex trait that depends on several genes. Some of these, like the gene *HAA1* that encodes a transcriptional activator involved in the response to acid stress, have a clear link to the phenotype. However, in many cases, the link between a specific natural allele or mutation and acid tolerance is not yet understood. For example, specific alleles of the genes *TRT2* (2 point mutations G-to-T at position 28 and G to A at position 30), *MET4* (point mutation T to C at position 126), and *IRA2* (deletion of 6 nucleotides between position 4 and 9), were found to contribute to acetic acid stress, but the exact underlying molecular mechanisms are as-yet unknown, even if it is hypothesized that the alleles can for example help stabilise mRNAs or be involved in the general stress response pathway. Importantly, alle of the identified alleles have been identified from QTL studies with natural (non-GM) *S. cerevisiae* strains. This implies that all alleles occur in nature. Note, however, that some alleles have only been found in one of about

2000 sequenced strains of *S. cerevisiae (TRT2)*, whereas the others have been found in a larger fraction (5% for *IRA2* and 99% for *MET4*) of available natural yeast genomes.

All three naturally occurring point mutations in *S. cerevisiae var boulardii* through CRISPR-Cas- engineering. This allowed to change both alleles of the wild-type *S. cerevisiae var boulardii*, without leaving any scars or marker genes in the genome. The mutations were confirmed, first through Sanger sequencing of PCR products of 1 kb around the target regions and next by whole-genome sequencing. The whole-genome sequencing confirmed that all intended mutations had been introduced as planned. However, unintended mutations were noticed. Firstly, the chromosome on which IRA2 resides gained one extra copy that still contained the original IRA2 allele. Second, six other mutations across the genome were identified, including five point mutations and one deletion. Two of the point mutations were in coding regions. First, a mutation at position 6 (glutamine to arginine) in gene FLO1 that encodes a cell surface protein (adhesin) that helps cells adhere to abiotic and biotic materials, including epithelial cells. Second, another non-synonymous mutation was found in gene YLR108C (unknown function). The three other point mutations were located outside of coding regions, one of which was close to the start codon of gene HAP4, which regulates respiration. The deletion was a 60-bp deletion in the promoter of SNF1, a gene involved in carbohydrate signalling.

CASE STUDY 13

SCRAMBLE technology to generate a beer yeast that produces lycopene.

'SCRAMBLE design Science' describes the Sc2.0 project and the embedded scramble technology. In short, Sc 2.0 aims at generating a complete synthetic yeast genome. The synthetic genome is completely produced in a factory. Its sequence is identical to the natural S. cerevisiae genome, with a few modifications rearrangements of tRNA genes, replacement of all TAG STOP codons, deletion of some repetitive regions and, most importantly, insertion of a LoxPsym recombination site downstream of every non-essential gene. These recombination sites can be activated by expressing the Cre recombinase, which leads to random recombination events between all LoxPSym sites, thus generating a 'scrambled' genome in which genes and gene clusters are randomly inversed, duplicated, deleted or translocated. Inducing the Cre recombinase in a population of (millions of) yeast cells thus generates a new population of (millions of) variant cells, each differing in the genome rearrangements they underwent. The idea is that this technology allows investigating the effects and constraints of genome rearrangements. On the applied side, some of these variants may display improved/interesting phenotypes.

The Sc2.0 project is not yet completely finished; but several complete chromosomes have been made. These yeasts contain 15 completely natural chromosomes, and one synthetic one with the LoxPsym sites etc. In addition, some strains now already carry multiple synthetic chromosomes (and thus fewer natural chromosomes). As of today, there is not yet a strain that only contains 16 synthetic chromosomes (likely because of technical issues). However, the existing strains are already used to test the SCRAMBLE technology.

In a second paper, an Sc2.0 intermediate yeast bearing one synthetic chromosome (chromosome II) was mated to a natural strain (S288c/BY4741) to generate a diploid strain that carries one synthetic and one natural copy of chromosome II (plus 15×2 natural chromosomes, of course). Next, the lycopene biosynthesis pathway (consisting of 4 genes derived from tomato, driven by a yeast promoter) was inserted on one plasmid. This yielded a strain that produces a small amount of lycopene (0.32 mg/L). Next, SCRAMBL was induced (by activating the Cre recombinase), generating a population of scrambled yeasts. The authors then selected a variant that produced much more lycopene (5 mg/L) by plating the scrambled population and selecting colonies that show a deeper red colour (lycopene is red). The authors further enhance lycopene production by making variants that contain different promoters driving the four lycopene synthesis genes (tuning of expression levels).

The final strain produced relatively high lycopene levels and could thus be used to produce beer that contains lycopene, which gives the beer an attractive red colour and is also a known antioxidant with a suspected beneficial health effect.