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## Guidance on the characterisation of microorganisms used as feed additives or as production organisms

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López Alonso, Secundino López Puente, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace, Pieter Wester, Boet Glandorf, Lieve Herman, Sirpa Kärenlampi, Jaime Aguilera, Montserrat Anguita, Rosella Brozzi and Jaume Galobart

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### Abstract

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**Correspondence:** feedap@efsa.europa.eu

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**Panel members:** Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Secundino López Puente, Marta López-Alonso, Alberto Mantovani, Baltasar Mayo, Fernando Ramos, Guido Rychen, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace and Pieter Wester.

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## Background and terms of Reference as provided by EFSA

Regulation (EC) No 1831/2003<sup>2</sup> establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Regulation (EC) No 429/2008<sup>3</sup> provides detailed rules for the implementation of Regulation (EC) No 1831/2003 as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives.

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) has adopted a series of guidance documents which aim at complementing Regulation (EC) No 429/2008 to support applicants in the preparation and submission of technical dossiers for the authorisation of additives for use in animal nutrition according to Regulation (EC) No 1831/2003.

The European Food Safety Authority (EFSA) asked its FEEDAP Panel to:

1. identify from the current guidance documents, those that need to be updated, taking into consideration the most recent scientific developments and the experience gained in the assessment of feed additives
2. update the guidance documents in need of revision accordingly; this activity can be conducted in different rounds of activities on the basis of the priorities identified and on the feasibility of the revision according the resources available
3. Develop a guidance document for the risk assessment of additives produced with genetically modified microorganisms
4. taking into account the sensitivity and the relevance of some of the guidance documents under revision and the entity of the revision itself (e.g. substantial or not), consider initiatives like preparatory info-sessions or public consultations of the draft guidance documents. The relevant comments received in either step will have to be considered and addressed if appropriate in the final version of the guidance documents

The first of the terms of reference was addressed by a statement of the FEEDAP Panel (EFSA FEEDAP Panel, 2016), in which it was identified the need to update most of the guidance documents that it produced and set priorities for this update.

This output addresses the second, third and fourth terms of reference with regards to the update of the guidance documents dealing with the characterisation and assessment of microorganisms used as feed additives or as production organisms (the last including genetically modified microorganisms).

## Scope

This document provides guidance to assist in the preparation and presentation of applications to market feed additives containing microorganisms or produced with microorganisms by fermentation as foreseen in Article 7.6 of Regulation (EC) No 1831/2003 and as required in Section 2 of Annex II and the relevant sections of Annex III of Regulation (EC) No 429/2008.

For fermentation products, only those aspects directly linked to the production organism, including the safety aspects of the genetic modifications where relevant, are considered.

For the purpose of this guidance document, microorganisms covered include bacteria, yeasts and filamentous fungi. For other taxonomical groups (such as *Archaea* or microalgae), the basic principles also apply on a case-by-case basis.

Products in which viable GMMs are present, intentionally (such as probiotics) or unintentionally, fall under Directive 2001/18/EC on the deliberate release of GMOs to the environment, and are out of the scope of this document.

<sup>2</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29–43.

<sup>3</sup> Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.05.2008, p. 1–65.

For other elements of the assessment of microorganisms or products of microbial origin, applicants are referred to the other relevant FEEDAP guidance documents.<sup>4</sup>

This guidance supersedes the following documents:

- EFSA GMO Panel Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011) with respect to the requirements for feed additives in the former Categories 1, 2 and 3 of that document
- FEEDAP Guidance documents: Technical guidance - Compatibility of zootechnical microbial additives with other additives showing antimicrobial activity (EFSA, 2008a), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel 2012a), Guidance on the safety assessment of *Enterococcus faecium* in animal nutrition (EFSA FEEDAP Panel 2012b), Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel 2014), Technical Guidance: Microbial Studies (EFSA, 2008b)

## 1. Assessment

Based on the nature of the product and on the applicable regulatory requirements, two different types of feed additives are considered in this document:

- Feed additives containing viable microorganisms (active agents)
- Feed additives produced by GM or non-GM microorganisms (production strains)

A summary of the requirements for the characterisation of both types of additives is shown in Table 1.

**Table 1:** Requirements for scientific information according to the type of product

	Section	Feed additives containing viable microorganisms		Fermentation products	
		Bacteria	Fungi - yeasts	Bacteria	Fungi - yeasts
Identification	2.1	✓	✓	✓	✓
Antimicrobial susceptibility	2.2	✓		✓	
Antimicrobial production	2.3	✓	✓	✓	✓
Toxigenicity and pathogenicity	2.4	✓	✓	✓	✓
Genetic modification	2.5.			For GMMs only	For GMMs only
Absence of the production strain	3.1			✓	✓
Presence of DNA from the production strain	3.2.			where relevant	where relevant
Compatibility with other authorised additives	4.2	✓			

A specific approach to safety assessment applies to those species of microorganisms included in the Qualified Presumption of Safety (QPS) list (EFSA 2007, EFSA BIOHAZ Panel, 2017). QPS provides a generic approach to the safety assessment of microorganisms intentionally introduced into the food and feed chain. To justify that a microorganism is suitable for being evaluated according to the QPS approach, its taxonomic status should be unequivocally established, and be a species included in the QPS list. In addition, any qualification set in the most recent QPS statement/opinion should be complied with. Those strains qualifying for the QPS approach are presumed safe for target species, consumer and the environment without the need for specific studies.

The QPS concept applies to the two above categories. In the case of additives produced by GMMs, QPS may apply to the parental strain but not to the production strain.

<sup>4</sup> Under preparation

## 2. Characterisation of the microorganism

### 2.1. Identification

The following taxonomic information needs to be provided for the microorganism: genus, species and strain name or code. For bacteria, taxonomy and nomenclature are maintained at the International Committee on Systematics of Prokaryotes<sup>5</sup> and covered by the International Code of Nomenclature of Prokaryotes (Parker et al., eds. 2015). New taxonomic units or re-assignments to the taxonomy and nomenclature are published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM<sup>6</sup>). The nomenclature and taxonomy of fungi are covered by the International Code of Nomenclature for algae, fungi, and plants (ICN) (McNeill et al., 2012<sup>7</sup>). The currently approved nomenclature for fungi can be found on the MycoBank database.<sup>8</sup>

The organism under assessment should be deposited in an internationally recognised culture collection having acquired the status of International Depositary Authority under the Budapest Treaty (preferably in the EU) and maintained by the culture collection for the authorised period of the additive. A valid certificate of deposition from the collection, which shall specify the accession number under which the strain is held, must be provided.

The organism under assessment should be identified unambiguously at species level based on up-to-date methodologies and current knowledge.

- Bacteria: Data from whole genome sequence (WGS) analysis should be used for identification of the microorganism. This can be achieved by comparing the sequences commonly used for taxonomic identification (e.g., 16S rRNA gene), or other characteristic genes (e.g. housekeeping genes) to relevant databases, or by computational approach for taxonomic assignments (e.g., phylogenomics or average nucleotide identity [ANI])
- Yeasts: Data from whole genome sequence (WGS) analysis should be used for identification of the microorganism. This should be done by phylogenomic analysis (e.g. using a concatenation of several conserved genes to produce a phylogeny against available related genomes)
- Filamentous fungi: When WGS is available, identification should be made by a phylogenomic analysis comparing the genome against available related genomes. If no WGS is available, identification should be made by comparing the 18S rRNA gene and/or ITS regions and other characteristic genes (e.g., tubulin) with sequences deposited in databases

The origin of the organism and history of modifications, including mutagenesis steps performed during the development of the strain, shall be reported. Any genetic modification as defined in Directive 2001/18/EC<sup>9</sup> shall be characterised according to Section 2.5.

#### 2.1.1. Use of whole genome sequence for characterisation of microorganisms

Whole genome sequence analysis (including chromosome(s) and/or extra-chromosomal genetic elements e.g. plasmids) is required for bacterial and yeast strains intended for use either as products or production strains. WGS analysis is also recommended for filamentous fungi. WGS data provide information for the unequivocal taxonomic identification of the strain, as well as for the characterization of the strain regarding their potential functional traits of concern (e.g., virulence factors, production of or resistance to antimicrobials of clinical relevance, production of known toxic metabolites).

The minimum set of information includes:

<sup>5</sup> <http://www.the-icsp.org/>

<sup>6</sup> <http://ijs.microbiologyresearch.org/content/journal/ijsem/about>

<sup>7</sup> <http://www.iapt-taxon.org/nomen/main.php>

<sup>8</sup> <http://www.mycobank.org>

<sup>9</sup> 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. Official Journal of the European Communities L106: 1-38

- the sequencing strategy and instrumentation used
- the assembly method applied (e.g., the bioinformatic approach, *de novo* or re-seq strategy)
- the statistical measure of sequence quality (e.g., number of reads, coverage, N50 and K-mer)
- the number of contigs and scaffolds required to represent the genome, their absolute length and their length relative to the genome size
- the annotation protocol used
- for fungi: information on the quality of the annotations obtained from relevant databases (e.g., BUSCO<sup>10</sup>)

## 2.2. Antimicrobial susceptibility

This section is applicable to bacteria intended for use as viable cells in feed additives and used as production organisms.

Microbial feed additives should not add to the pool of antimicrobial resistance (AMR) genes already present in the gut bacterial population or otherwise increase the risk of transfer of AMR. Antimicrobials considered are those relevant to their use in humans and animals (critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs), last revision WHO, 2016). The possibility of transfer of resistance from viable microorganisms to other microorganisms is related to the genetic basis of the resistance and is considered to be most plausible when the resistance is mediated by added/acquired genes.

For this, two sets of data should be provided:

- Phenotypic testing based on determination of a minimum inhibitory concentration (MIC) for a selected group of antimicrobials
- A search of the WGS for the presence of known AMR genes

### 2.2.1. Phenotypic testing

It is essential that such tests are made in a consistent manner using internationally recognised and standardised methods. As a basic requirement, the MICs (expressed as mg/L or µg/mL) should be determined for the antimicrobials listed in Table 2. These antimicrobials are chosen to detect a wide range of resistance determinants. For those bacteria not listed in Table 2, the antimicrobials tested should be relevant either to Gram + or Gram - organisms according to Table 2. Resulting MIC values should be compared with existing published values for that specific or related species and/or those generated in house.

MICs should be determined using serial two-fold dilution procedures in agar or broth, including relevant quality control strains. The tests should be performed according to internationally recognised standards such as EUCAST,<sup>11</sup> the Clinical and Laboratory Standard Institute (CLSI<sup>12</sup>), ISO standard or similar. After incubation, the MIC is defined as the lowest concentration of the antimicrobial that inhibits bacterial growth. Qualitative or semi-quantitative methods to determine MIC indirectly, such as diffusion methods, are not acceptable.

The culture medium should allow growth of the strain under assessment. Whenever possible, dedicated media to survey antimicrobial resistance/susceptibility profiles (e.g., Mueller-Hinton or IsoSensitest) should be used. However, for specific bacterial species or strains, other formulations (such as MLS for some lactic acid bacteria and bifidobacteria species [Klare et al., 2005]) might be required. Potential interference by medium components (e.g., p-aminobenzoic acid, thymidine, glycine, divalent cations), test type (broth microdilution versus agar dilution), and culture conditions (pH, temperature, time of incubation) on the susceptibility levels to some antimicrobials should be taken into account.

<sup>10</sup> <http://busco.ezlab.org>

<sup>11</sup> <http://www.eucast.org>

<sup>12</sup> <http://www.clsi.org>

- 241 For the purpose of distinguishing resistant from susceptible strains, the FEEDAP Panel has defined  
242 microbiological cut-off values based on published data. On this basis, strains can be categorised as:
- 243 • Susceptible when its growth is inhibited at a concentration of a specific antimicrobial equal to  
244 or lower than the established cut-off value ( $S \leq x$  mg/L)
  - 245 • Resistant when its growth is not inhibited at a concentration of a specific antimicrobial equal  
246 to or lower than the established cut-off value ( $R > x$  mg/L)



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**Table 2:** Microbiological cut-off values (mg/L) for Gram-positive bacteria

	ampicillin	vancomycin	gentamicin	kanamycin	streptomycin	erythromycin	clindamycin	tetracycline	chloramphenicol	tylosine	ciprofloxacin	colistine	fosfomycin
<i>Lactobacillus</i> obligate homofermentative <sup>a</sup>	2	2	16	16	16	1	4	4	4	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus acidophilus</i> group	1	2	16	64	16	1	4	4	4	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus</i> obligate heterofermentative <sup>b</sup>	2	n.r.	16	64	64	1	4	8 <sup>c</sup>	4	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus reuteri</i>	2	n.r.	8	64	64	1	4	32	4	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus</i> facultative heterofermentative <sup>d</sup>	4	n.r.	16	64	64	1	4	8	4	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus plantarum/pentosus</i>	2	n.r.	16	64	n.r.	1	4	32	8	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus rhamnosus</i>	4	n.r.	16	64	32	1	4	8	4	n.r.	n.r.	n.r.	n.r.
<i>Lactobacillus casei /paracasei</i>	4	n.r.	32	64	64	1	4	4	4	n.r.	n.r.	n.r.	n.r.
<i>Bifidobacterium</i>	2	2	64	n.r.	128	1	1	8	4	n.r.	n.r.	n.r.	n.r.
<i>Pediococcus</i>	4	n.r.	16	64	64	1	1	8	4	n.r.	n.r.	n.r.	n.r.
<i>Leuconostoc</i>	2	n.r.	16	16	64	1	1	8	4	n.r.	n.r.	n.r.	n.r.
<i>Lactococcus lactis</i>	2	4	32	64	32	1	1	4	8	n.r.	n.r.	n.r.	n.r.
<i>Streptococcus thermophilus</i>	2	4	32	n.r.	64	2	2	4	4	n.r.	n.r.	n.r.	n.r.
<i>Bacillus</i>	n.r.	4	4	8	8	4	4	8	8	n.r.	n.r.	n.r.	n.r.
<i>Propionibacterium</i>	2	4	64	64	64	0.5	0.25	2	2	n.r.	n.r.	n.r.	n.r.
<i>Enterococcus faecium</i>	2	4	32	1024	128	4	4	4	16	4	n.r.	n.r.	n.r.
<i>Corynebacterium</i> and Other Gram +	1	4	4	16	8	1	4	2	4	n.r.	n.r.	n.r.	n.r.
Enterobacteriaceae	8	n.r.	2	8	16	n.r.	n.r.	8	n.r.	n.r.	0.06	2	8

n.r. not required.

<sup>a</sup> including *L. delbrueckii*, *L. helveticus*<sup>b</sup> including *L. fermentum*<sup>c</sup> for *L. buchneri* the cut-off for tetracycline is 128<sup>d</sup> including the homofermentative species *L. salivarius*

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### 2.2.2. WGS search for AMR genes

WGS should be interrogated for the presence of genes coding for or contributing to resistance to antimicrobials relevant to their use in humans and animals (CIAs or HIAs). For this purpose, a comparison against up-to-date specific databases should be performed (e.g., CARD,<sup>13</sup> ARG-ANNOT,<sup>14</sup> ResFinder<sup>15</sup>). The outcome of the analysis should be presented as a table focusing on complete genes coding for resistance to antimicrobials. The table should include at least the gene identification, function of the encoded protein, percentage of identity and e-value.

### 2.2.3. Interpretation of the results from 2.2.1 and 2.2.2

The detection of the MIC above the cut-off values proposed by the FEEDAP Panel for one or more antimicrobials requires further investigation using genomic data to determine the nature of the resistance:

- If no known AMR gene is identified that can be linked to the phenotype, no further studies are required
- If the phenotypic resistance can be directly related to the presence of a known AMR gene, this is considered as a hazard

If the genetic analysis reveals AMR genes for antimicrobials considered to be CIAs or HIAs (WHO, 2016), the MIC values should be determined and compared with values in the literature:

- If MIC ≤ (reference values), the likelihood of the AMR gene to become active should be assessed (e.g. based on sequence comparison with active genes)
- If MIC > (reference values), this is considered as a hazard

## 2.3. Antimicrobial production

Unless the strain belongs to a species known not to produce antimicrobials relevant to use in humans and animals, tests should be made to assess the inhibitory activity of culture supernatants against reference strains known to be susceptible to a range of antibiotics (e.g., *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633, or other reference strains, EUCAST, 2015; FAO, 2006). If there is a positive outcome in one or more species, the inhibitory substance should be identified.

For those production strains for which antimicrobial activity has been identified, the absence of carry over into the final product should be demonstrated. The exact phase of the manufacturing process from which the samples are taken should be indicated. Samples should be taken from industrial scale process. Samples from pilot scale process may be acceptable if those from industrial process are not yet available.

For ionophoric coccidiostats produced from species known to produce other antimicrobials of clinical relevance (WHO, 2016), the presence of antimicrobial activity not related to the ionophore in the fermentation/final product should be investigated e.g., by comparing the inhibitory spectrum of the pure ionophore with that of the additive. The strains described above can be used for this purpose.

Applicants should declare whether any antimicrobial(s) of clinical relevance are used during the manufacturing of the product.

## 2.4. Toxigenicity and pathogenicity

Information relating to toxigenicity and virulence for humans and target species should be provided for active agents and production strains, including history of use of the strain or any close relative.

<sup>13</sup> <https://card.mcmaster.ca/>

<sup>14</sup> <http://en.mediterranean-infection.com/article.php?leref=283%26titre=arg-annot>

<sup>15</sup> <https://cge.cbs.dtu.dk/services/ResFinder/>

This should be based on updated literature searches (according to the provisions from the guidance on the safety for the target species<sup>16</sup>).

Any strain development step (including mutagenesis and/or genetic modifications) aimed to reduce the toxigenicity and/or pathogenicity of the strain used should be clearly documented.

#### 2.4.1. Bacteria

For bacterial strains, WGS analysis should be used to identify genes coding for known virulence factors. For this purpose, comparison against specific up-to-date databases (e.g. VFDB,<sup>17</sup> PAI DB,<sup>18</sup> MvirDB<sup>19</sup>) should be performed. The outcome of the analysis should be presented as a table focusing on complete genes encoding recognised virulence factors (e.g. toxins, invasion and adhesion factors) known to exist in the species or related species to which the strain belongs. The table should include at least the gene identification, function of the encoded protein, percentage of identity and e-value. The presence of genes encoding virulence factors may trigger further phenotypic testing (e.g., cytotoxicity tests).

For viable microorganisms in which pathogenicity cannot be excluded by the information from the literature search and by interrogating the WGS, further studies (e.g., tolerance studies according to the guidance on safety for the target species<sup>20</sup>) may be required.

Exceptions to the above requirements are:

- Strains for which safety can be established by specific tests (e.g. *Enterococcus faecium* and *Bacillus species*)
- Other strains which qualify for the QPS approach to safety assessment

##### 2.4.1.1 *Enterococcus faecium*

*E. faecium* consists of two distinct subpopulations or clades. One subpopulation consists predominantly of isolates from the faeces of healthy individuals, and is characterised by susceptibility to ampicillin. The other subpopulation, which contains most of the clinical isolates, shows resistance to ampicillin. The virulence factors and markers *IS16*, *hyEfm*, and *esp* are also considered relevant for the assessment of safety.

The MIC for ampicillin should be determined:

- If the MIC > 2 mg/L, the strain is not considered safe
- If the MIC ≤ 2 mg/L, the absence of the genetic elements *IS16*, *hyEfm*, and *esp* should be investigated by interrogating the genome sequence

If none of the three genetic elements are detected, then the strain is considered safe. If one or more of the three genetic elements are detected, then the strain is considered hazardous.

##### 2.4.1.2 *Bacillus spp.*

For *Bacillus* species other than the *B. cereus* group, a cytotoxicity test should be made to determine whether the strain produces high levels of non-ribosomal synthesised peptides, as one of the qualifications of the QPS approach. In the absence of animal models shown to be able to distinguish hazardous from non-hazardous strains, the FEEDAP Panel relies on the use of *in vitro* cell-based methods to detect evidence of a cytotoxic effect (see Annex). Such tests should be made with culture supernatants since the concentration of cells obtained in a broth culture would always exceed that found in animal food products. In addition, they should be made preferably with Vero cells or other epithelial cell lines using culture supernatant following the protocol described by Lindbäck and Granum (2005). Detection based on <sup>14</sup>C-leucine uptake is described, but other methods

<sup>16</sup> Under preparation

<sup>17</sup> <http://www.mgc.ac.cn/VFs/main.htm>

<sup>18</sup> [http://www.paidb.re.kr/about\\_paidb.php](http://www.paidb.re.kr/about_paidb.php)

<sup>19</sup> <http://mvirdb.llnl.gov>

<sup>20</sup> Under preparation

such as those based on lactate dehydrogenase release or propidium iodide uptake could be used alternatively (Fagerlund et al., 2008).

The selection of strains belonging to the *B. cereus* taxonomic group, for direct use in animal production or as production strains, is considered unadvisable. If, however, they are proposed for use, a bioinformatic analysis should be made of the WGS for genes encoding enterotoxins (*nhe*, *hbl* and *cytK*) and cereulide synthase (*ces*) (Stenfors-Arnesen et al., 2008). If there is evidence for similarity, the non-functionality of the genes should be demonstrated. Strains with toxigenic potential are not considered safe.

## 2.4.2. Eukaryotic microorganisms

For eukaryotic microorganisms, their potential pathogenicity or ability to produce metabolites that could be harmful to humans and/or animals should be assessed. A literature search should be carried out to identify the capacity of the species or a closely related species to produce known toxic compounds (following the principles of Section 4 of the Guidance on the assessment of the safety of feed additives for the target species). Where such compounds are identified, analyses should be made to exclude their presence or demonstrate that their concentration in the additive is not of concern.

## 2.5. Genetic modifications

If the strain is genetically modified according to the definition in Directive 2001/18/EC,<sup>21</sup> the genetic modification should be described.

### 2.5.1. Purpose of the genetic modification

The purpose of the genetic modification should be described. A description of the traits and changes in the phenotype and metabolism of the microorganism resulting from the genetic modification is required.

### 2.5.2. Characteristics of the modified sequences

#### Inserted sequences

The sequences inserted in the GMM can be derived from defined organisms or may be designed. When the inserted DNA is a combination of sequences from different origins, the pertinent information for each of the sequences should be provided.

The following information should be provided:

#### *DNA from defined donor organisms*

The taxonomic affiliation (genus and species) of the donor organism(s) should be provided. The description of the inserted sequence(s) should include:

- nucleotide sequence of all inserted elements including a functional annotation and the physical map of all the functional elements
- structure and function of the inserted elements, including coding and non-coding regions
- name, derived amino acid sequence(s) and function(s) of the encoded protein(s). When available, E.C. number of the encoded enzymes

#### *Designed sequences*

Designed sequences are those not known to occur in nature (e.g. codon-optimised genes, rationally designed chimeric/synthetic genes or genes harbouring chimeric sequences). In such cases, information should be provided on:

- rationale and strategy for the design

<sup>21</sup> Reproduce the definition

- DNA sequence and a physical map of the functional elements
- derived amino acid sequence(s) and function(s) of the encoded protein(s)
- similarity with sequences in up-to-date databases (e.g. ENA,<sup>22</sup> NCBI,<sup>23</sup> UniProt<sup>24</sup>). This should identify the functional domains of the recombinant protein; the best hits should be reported and described

## Deletions

A description of the intentionally deleted sequence(s) should be provided, together with an explanation of the intended effect.

## Base pair substitutions and frameshift mutations

Intentionally introduced base pair substitutions and/or frameshift mutations should be indicated, together with an explanation of their expected effect.

### 2.5.3. Structure of the genetic modification

The characterisation of the structure of the genetic modification depends on whether WGS data are used or not.

#### 2.5.3.1 Structure of the genetic modification using WGS data

Detailed information should be provided, including a map or graphic presentation of all genomic regions (chromosome, contig or plasmid) harbouring genetic modifications, indicating:

- the open reading frames (ORF) actually inserted, modified or deleted. For each ORF, the gene products should be described in detail (at least the amino acid sequence, the function, metabolic role). Introduced genes of concern should be highlighted. Genes of concern are those known to contribute to the production of toxic metabolites and antimicrobials of clinical relevance, or to AMR
- the non-coding sequence(s) inserted/deleted/modified. The role and function of these sequences (e.g. promoters, terminators) should be indicated

This can be done e.g. by comparing the WGS of the GMM with that of the non-modified parental or recipient strain.

#### 2.5.3.2 Structure of the genetic modification without WGS data

When WGS is not available, all the steps to obtain the genetic modification should be described. The information provided should allow for the identification of all genetic material potentially introduced into the recipient/parental microorganism.

#### *Characteristics of the vector*

The description of the vector(s) used for the development of the GMM should include:

- the source and type (plasmid, phage, virus, transposon) of the vector. When helper plasmids are used, they should also be described
- a map detailing the position of all functional elements and other vector components
- the map should accompany a table identifying each component, properly annotated, such as coding and non-coding sequences, origin(s) of replication and transfer, regulatory elements, AMR genes, their size, origin and role

<sup>22</sup> <http://www.ebi.ac.uk/ena>

<sup>23</sup> <https://www.ncbi.nlm.nih.gov/>

<sup>24</sup> <http://www.uniprot.org/>

#### Information relating to the genetic modification process

The genetic modification process should be described in detail. This should include:

- methods used to introduce, delete, replace or modify the DNA into the recipient/parental, and methods for selection of the GMM
- it should be indicated whether the introduced DNA remains in the vector or is inserted into the chromosome(s) and/or, for eukaryotic microorganisms, into DNA of organelles (e.g. mitochondria) if appropriate

#### Structure of any vector and/or donor nucleic acid remaining in the GMM

- a map detailing the position of the sequences actually inserted, replaced or modified
- in the case of deletion(s), the size and function of the deleted region(s) must be provided

#### Genes of concern

Any genes of concern as defined in Section 6.3. (such as genes encoding AMR, toxins and virulence factors) inserted in the GMM shall be clearly indicated.

The absence of any sequence of concern (such as AMR genes) not intended to be present in the GMM should be tested experimentally. This includes:

- sequences used transiently during the genetic modification process including vectors and helper plasmids
- Sequences in plasmids/replicons from which a fragment was derived and used for transformation

This should be analysed by using appropriate methods, such as Southern analysis or PCR.

- Southern blots shall include appropriate positive and negative controls. The length and location of the probe(s) used should be indicated. The amount of DNA loaded in the agarose gel should be provided, together with an image of the gel before blotting. Positive control shall be loaded in a concentration corresponding to 1-10 copies of the target fragment per genome of the production strain. If several probes are used, they shall be tested in separate experiments.
- PCR experiments shall include a positive control containing the same gene as that used during strain development, together with proper positive controls to exclude PCR inhibition and to ensure sufficient sensitivity. A negative control should also be included.

### 3. Fermentation products

This section refers to the characterisation of the feed additives obtained by fermentation of a production strain and covers the safety aspects directly linked to the production strain. For products for which more than one production strain is involved, data should be provided for each of them. For other aspects of the product characterisation, the applicant should follow the relevant guidance.<sup>25</sup>

#### 3.1. Absence of the production strain

The absence of viable cells of the production strain should be investigated using a well-described method for the detection. The techniques used to remove/inactivate microbial cells in the course of the downstream processing should be described in detail:

- the absence of viable cells should be verified by means of a culture-based method targeted to the detection of the viable cell. Cultivation-independent methods are not acceptable

<sup>25</sup> Under preparation

- the procedure should enable the recovery of stressed cells by cultivation in or onto media with a minimal selective pressure and/or by providing a longer (at least 2 times) incubation time compared to the normal culturing time
- the detection should also consider specificity against contaminating microbiota possibly occurring in the sample in case it interferes with the detection of the production strain
- if the strain is able to form endospores, their possible presence should be analysed by using germination procedures (e.g. thermal treatment for bacteria) adapted to the organisms, and subsequent culturing
- absence should be demonstrated in a volume corresponding to at least 1 g or mL of product, obtained from a sample of at least 10 g or mL of product (e.g. 10 g of product diluted in 90 mL, 10 mL analysed)
- at least nine samples obtained from a minimum of three independent batches should be analysed. The exact phase of the manufacturing process from which the samples are taken should be indicated. Samples should be taken from industrial scale process. Samples from pilot scale process are acceptable if it can be justified that those from industrial process are not available. In this case it should be documented that the pilot scale process (fermentation and downstream) is representative of the industrial scale process
- a positive control with samples spiked with low counts (e.g. 10-1000 cells per plate) of viable cells of the production strain should be included to prove that the medium and cultivation conditions enable growth of any possible viable cells remaining in the product
- when the additive has several formulations obtained in the same production scheme, at least the intermediate product obtained upstream in the process should be analysed. For different production schemes, each of the formulations/products of the additive should be tested

### 3.2. Presence of DNA from the production strain

This section applies to:

- products obtained using genetically modified production strains. The possible presence of DNA from the production strain in the product should be verified in compliance with regulatory requirements
- products not highly purified (e.g., by crystallization, ultrafiltration, membrane dialysis, chromatography) obtained using non-genetically modified production strains carrying AMR genes

The presence of DNA from the production strain should be tested in the product by PCR, targeting a fragment specific for this strain. Detailed information should be provided on the specific target sequence, primers and polymerase used and amplification conditions:

- in case the production strain contains AMR genes, whether GMM or not, primers should be designed to amplify a fragment not exceeding the size of the smallest antimicrobial resistance gene. If the production strain is a GMM not containing AMR genes, the targeted sequence should cover maximum 1 Kb
- DNA from at least 1 g or 1 ml of product shall be extracted. Upstream intermediate products can be used as long as they are equally or more concentrated than the final product. For products with different formulations, the most concentrated one should be tested. For different production schemes, each of the formulations/products should be tested.
- at least three independent batches of product should be sampled, each analysed in triplicate. The exact phase of the manufacturing process from which the samples are taken should be indicated. Samples should be taken from industrial scale process. Samples from pilot scale process are acceptable if it can be demonstrated that those from industrial process are not available. In this case it should be documented that the pilot scale process (fermentation and downstream) is representative of the industrial scale process



- to recover DNA from non-viable cells potentially remaining in the product, the DNA should be extracted using a methodology suitable for all cellular forms of the production strain (e.g. vegetative cells, spores)
- the following controls and sensitivity tests should be included:
  - a) total DNA from the production strain, as a positive control for the PCR
  - b) total DNA from the production strain, added to the product sample before the DNA extraction process, starting with a known quantity and in different dilutions until DNA extinction
  - c) a positive control with total DNA from the production strain, added to the DNA extracted from each of the three batches of the product tested, to check for any factors causing PCR failure
  - d) A negative control without sample
- if PCR failure is encountered, the causes should be investigated (e.g. PCR inhibition, presence of nucleases)

For the purpose of this assessment, the applicant should demonstrate that the target DNA is not detected in analyses having detection threshold of 10 ng of DNA per gram or mL of product or lower.

## 4. *In vivo* studies

### 4.1. Impact on gut microbiota

For the purpose of this guidance, the impact on gut microbiota is assessed by examining whether the use of the additive results in an overgrowth or shedding of potentially pathogenic microorganisms. This is required for those additives:

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

The impact of an additive on zoonotic agents can be studied in target animals naturally colonised with the enteropathogen(s) under investigation or with animals deliberately inoculated. In the latter case, consideration should be given to the pathogenic strain(s) selected (e.g., strains/serotypes specific to the target animal, use of multiple strains/serotypes, challenge dose). Shedding should be monitored with methods sensitive enough to identify the target pathogen(s).

### 4.2. Compatibility with other additives showing antimicrobial activity

The combination of a microbial additive with a second additive should not adversely affect the viability of the microbial cells.

In dry feed, it is generally assumed that no interaction between the two additives occurs and therefore no effects on compatibility are expected.

When interaction is possible (e.g. both additives administered in water or in wet or liquid feeding) then viability (i.e., microbial counts at time zero and after the expected time for which additives are in contact) should be investigated reflecting the conditions of practical use, particularly the duration for which the additives remain in contact.

To demonstrate compatibility under those circumstances, two treatments should be used – the microbial additive and the microbial additive plus the product with antimicrobial activity – and microbial cell numbers measured. Studies should be designed using the lowest proposed dose of the microbial additive and the maximum proposed dose of the product showing antimicrobial activity.



For products containing multiple microbial strains, the viability should be separately assessed for each strain.

### ***In vitro studies***

The purpose of the *in vitro* studies is to establish whether the viability of the microbial additive is likely to be affected at the probable concentration of the antimicrobial additive in the digestive tract, and consequently whether *in vivo* studies are necessary. This is done by determining the MIC of the antimicrobial additive.

The MIC should be determined according to Section 3.2.1. In case of microorganisms producing spores, the MIC should be calculated with vegetative cells.

For products composed by multiple microbial strains, the MIC should be determined for each individual strain and the results interpreted in terms of the most sensitive component.

If the MIC is greater than four times the maximum concentration of the antimicrobial in feed/water, compatibility is assumed and no *in vivo* tests are required.

If the MIC is equal to or below four times the maximum concentration of the antimicrobial in feed/water, incompatibility cannot be excluded and should be assessed *in vivo*.

### ***In vivo compatibility studies in target species***

To demonstrate compatibility *in vivo*, one short-term experiment comparing two treatments (microbial additive and microbial additive plus product with antimicrobial activity) should be performed. Studies should be designed using the lowest proposed dose of the microbial additive and the maximum proposed dose of the product showing antimicrobial activity. The trials should be conducted ensuring that the health of animals and the husbandry conditions (e.g., veterinary intervention) do not adversely affect the interpretation of the results. Care should be taken to avoid cross-contamination of feed, and this should be demonstrated experimentally. The experimental design should have adequate statistical power.

Compatibility should be determined by analysing viable cell numbers of the strain(s) under assessment in gastrointestinal contents (faecal, ileal or caecal). Cultivation-independent methods are not acceptable. To avoid the possible interference of the animal's gut microbiota in the determination of cell counts, recognition of the active agent at strain level should be achieved. For products composed of multiple microbial strains, each strain should be individually enumerated.

In the case of *Bacillus* and other spore formers, both vegetative cells and spores should be enumerated.

Compatibility is demonstrated between the two groups if the gastrointestinal counts of vegetative cells (and spores when present) are similar (within 0.5 log order). The variability of the experimental set up should be taken into account.

In the absence of estimates of microbial numbers, performance data alone are not considered sufficient to establish compatibility.

## **5. Outcomes**

The following sections refer to the outcome of the assessment of those elements referring to the microorganism (active agent or production strain) only. Further aspects of the safety of the product for target species, consumers, users and the environment should be separately considered, as appropriate (according to the guidance on consumer safety, safety for the target species, for the user and the environment<sup>26</sup>).

<sup>26</sup> Under preparation

## 5.1. Feed additives containing viable microorganisms

For those strains qualifying for the QPS approach to safety assessment, no hazards and therefore no risks are identified for target species, consumers and the environment. User safety should be assessed in all cases according to the relevant guidance.<sup>27</sup>

For other microbial strains:

- bacterial strains carrying genes that confer resistance to relevant antimicrobial(s) are considered to represent a risk for target species and those exposed to the additive
- pathogenic, virulent or toxigenic strains and those capable of producing relevant antimicrobials according to section 2.3 are considered to represent a risk for susceptible target species and/or those exposed to the additive
- for bacterial strains free from antibiotic resistance determinants and shown to be non-pathogenic/toxigenic, no hazards and therefore no risks are identified for target species, consumers and the environment. User safety should be assessed according to the relevant guidance.<sup>28</sup>
- for yeasts and filamentous fungal strains shown to be non-pathogenic/toxigenic, no hazards and therefore no risks are identified for target species, consumers and the environment. User safety should be assessed according to the relevant guidance.<sup>29</sup>

## 5.2. Feed additives produced by non-GM microorganisms

For those strains:

- qualifying for the QPS approach to safety assessment or
- free from antibiotic resistance determinants and shown to be non-pathogenic/toxigenic

no hazards and therefore no risks are expected to arise from the metabolism of the production strain itself. Further aspects of the safety of the product for target species, consumers, users and the environment should be separately considered as appropriate (according to the overarching guidance<sup>30</sup>).

For other microbial strains:

- bacterial strains carrying genes that confer resistance to relevant antimicrobial(s) are considered to represent a hazard. If the production strain carries AMR genes, and if DNA fragments long enough to cover the corresponding complete genes are detected in the product, the product is considered to represent a risk for target species and those exposed to the additive. However, if the absence of DNA from the production strain can be shown in the additive, or the additive is highly purified (e.g., by crystallization, ultrafiltration, membrane dialysis, chromatography), this is not considered a risk
- products obtained by fermentation using strains which are toxigenic and/or able to produce antimicrobials of clinical relevance are considered to represent a risk for susceptible target species and/or those exposed to the additive, unless absence of the relevant toxins and/or antimicrobials can be demonstrated in the final additive

Further aspects of the safety of the product for target species, consumers, users and the environment should be separately considered as appropriate (according to the overarching guidance).

## 5.3. Feed additives produced by GM microorganisms

The key components of the assessment of GM organisms used as production strains are summarised in Figure 1.

<sup>27</sup> Under preparation

<sup>28</sup> Under preparation

<sup>29</sup> Under preparation

<sup>30</sup> Under preparation

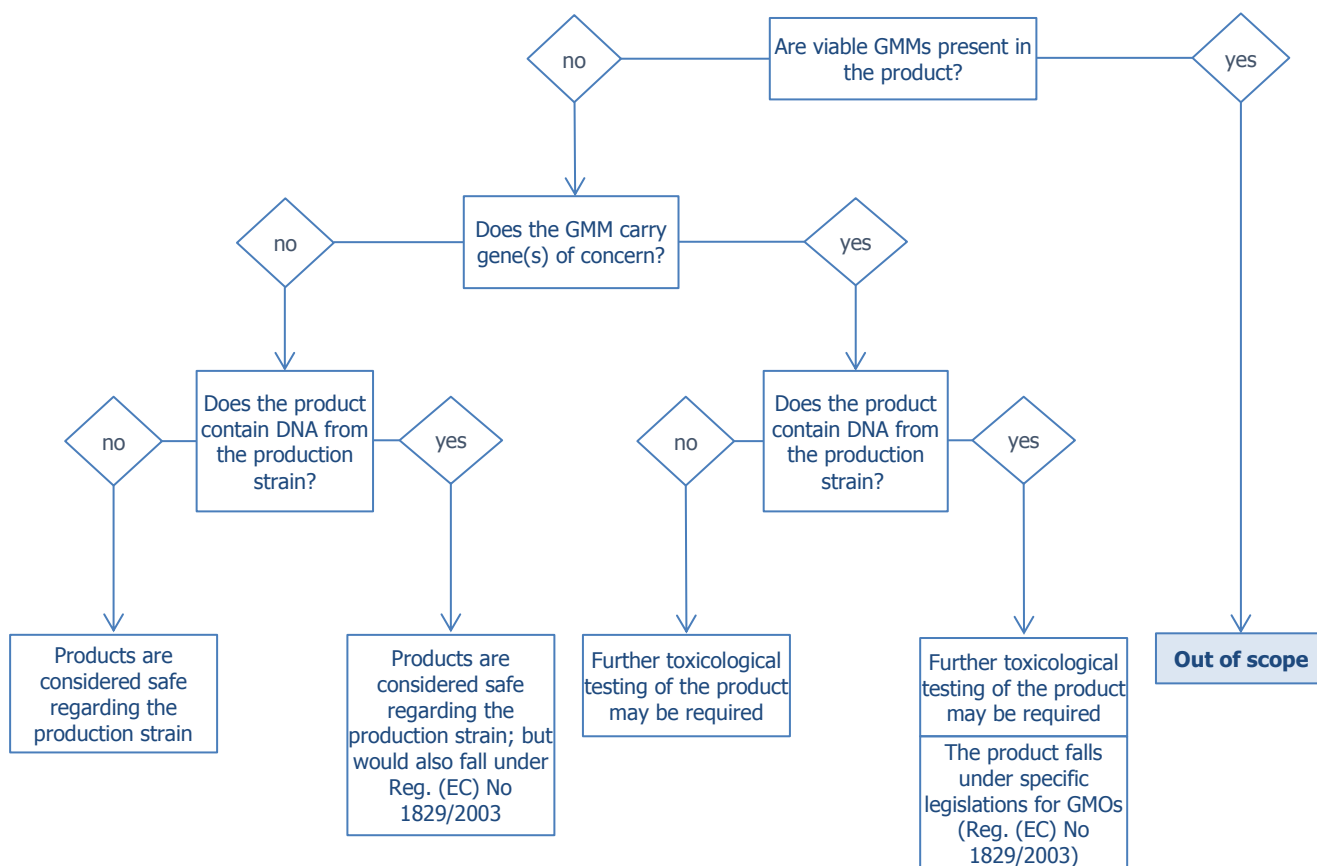
For genetically modified strains, the outcomes stated in Section 5.2 apply.

In addition, for those GM strains:

- for which the recipient strain qualifies for the QPS approach to safety assessment
- which are free from AMR determinants and shown to be non-pathogenic/toxigenic

and whose genetic modification does not introduce genes/changes of concern, no hazards and therefore no risks are expected to arise from the metabolism of the production strain itself. Further aspects of the safety of the product for target species, consumers, users and the environment should be separately considered as appropriate (according to the overarching guidance<sup>31</sup>).

If DNA from the production strain is present in the product, applicants should follow the requirements of specific legislation regarding GMOs<sup>32</sup> in addition to those for feed additives<sup>33</sup>.



**Figure 1:** Steps for hazard identification of GMM-derived products

<sup>31</sup> Under preparation

<sup>32</sup> Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1-23

<sup>33</sup> Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29-43.

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## 703 Glossary and Abbreviations

704 **Active agent:** any microorganism intended to be used as a feed additive or in the manufacture of a  
 705 feed and that provides the intended effect

706 **Antimicrobial:** An active substance of synthetic or natural origin which destroys microorganisms,  
 707 suppresses their growth or their ability to reproduce in animals or humans, excluding antivirals and  
 708 antiparasitic agents. For the purposes of this guidance, antimicrobials are those relevant to their use  
 709 in humans and animals defined by the WHO as critically important antimicrobials (CIAs) or highly  
 710 important antimicrobials (HIAs).

711 **Gene of concern:** gene known to contribute to the production of toxic metabolites and antimicrobials  
 712 of clinical relevance, or to AMR. For products with viable cells, other virulence factors are also included  
 713 in this definition.

714 **Genetically modified organisms:** (Micro)organisms in which the genetic material has been altered  
 715 in a way that does not occur naturally by mating and/or natural recombination.

716 **Microorganism:** Any microbiological entity, cellular or non-cellular, capable of multiplication or of  
 717 transferring genetic material, including viruses, viroids, animal and plant cells in culture. For the  
 718 purpose of this guidance document, microorganisms cover bacteria, yeasts and filamentous fungi.

719 **Parental strain:** A non-genetically modified microorganism with direct genealogical link to the GMM.

720 **Recipient strain:** The strain that is subjected to genetic modifications which are subject of the  
 721 application. The recipient strain can be the parental or its derivative, mutagenized or genetically  
 722 modified. The recipient strain gives rise to the GMM.

723 **Recombinant DNA:** A form of DNA that is created by combining two or more sequences that would  
 724 not normally occur together.

725 **Recombinant gene:** A gene that is constructed from two or more sequences that would not  
 726 normally occur together.

727 **Vector:** is understood as the agent containing the introduced DNA sequence used as a vehicle to  
 728 transfer such sequence into the transformed cell.

729

AMR	Antimicrobial Resistance
EC	European Commission
EFSA	European Food Safety Authority
GM	Genetically Modified
GMM	Genetically Modified Microorganism
GMO	Genetically Modified Organism
PCR	Polymerase Chain Reaction
QPS	Qualified Presumption of Safety
RT-PCR	Reverse-transcription PCR



## **Annex A – Recommended procedure for the detection of cytotoxicity using epithelial cell lines**

### **Preparation of test substance**

Bacterial cells should be grown in brain heart infusion broth (BHI) at 30 °C and harvested after 6 h when it is anticipated that cells will have reached a density of at least 108 CFU/mL. Cells should be removed by centrifugation at room temperature. Toxicity is determined using 100 µL of supernatant in the Vero cells assay.

### **Cell assay**

Vero cells should be grown in MEM medium supplemented with 5 % foetal calf serum. Cells should be seeded into 24-well plates two-three days before testing. Before use, it should be verified that growth of the Vero cells is confluent and if so, the medium should be removed and the cells washed once with 1 mL preheated (37°C) MEM medium. Then the following steps should be followed:

- Add 1 mL preheated (37°C) low-leucine medium to each well and then add the toxin to be tested (100 µL of non-concentrated supernatant), incubate the cells for 2 hours at 37°C
- Remove the low-leucine medium with the toxin, wash each well once with 1 mL preheated (37°C) low-leucine medium. Mix 8 mL preheated low-leucine with 16 µL <sup>14</sup>C-leucine and add 300 µL of this mixture to each well, incubate the cells for 1 hour at 37°C
- Remove the radioactive medium and add 1 mL 5 % trichloroacetic acid (TCA) to each well, incubate at room temperature for 10 minutes. Remove the TCA, and wash the wells twice with 1 mL 5 % TCA
- After removing the TCA, add 300 µL 0.1 M KOH and incubate at room temperature for 10 minutes. Transfer the content of each well to liquid scintillation tubes with 2 mL liquid scintillation cocktail. Vortex the tubes, and count the radioactivity in a scintillation counter for 1 minute

Percentage inhibition of protein synthesis is calculated using the following formula: ((Neg. ctrl – sample)/Neg. ctrl) × 100; the negative control is Vero cells from wells without addition of sample. Above 20 % inhibition is considered to indicate cytotoxicity.

An alternative method is to measure propidium iodide (PI) uptake in Vero cell suspensions using a spectrofluorimeter. Two day old confluent monolayers of Vero cells should be used as described above. Cell suspensions contained a final concentration of about 10<sup>6</sup> cells in 2 mL EC buffer containing PI (5 µg/mL) should be held in a thermostatically controlled (37 °C) 1 cm quartz cuvette to which the toxin is then added. Cells should be continuously mixed by the use of a magnetic stirrer and 'flea'. Fluorescence should be monitored every 30 seconds using excitation/emission wavelengths of 575/615 nm and 5 nm slits for both. Results are used without subtraction of background fluorescence. For this alternative method with propidium iodide uptake or lactate dehydrogenase, values above 20 % of the fluorescence/absorbance obtained from the positive control (usually detergent treated cells) are considered to indicate cytotoxicity.