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

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Abstract

This guidance document is intended to assist the applicant in the preparation and the presentation of an application, as foreseen in Article 7.6 of Regulation (EC) No 1831/2003, for the authorisation of additives for use in animal nutrition. It specifically covers the characterisation of microorganisms used as feed additives or as production organisms.

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Background and Terms of Reference as provided by EFSA


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itself (e.g. substantial or not), consider initiatives like preparatory info-sessions or public consultations of the draft guidance documents.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 regard 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).

This guidance document underwent a public consultation (EFSA, 2018).

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.

This guidance is also applicable to feed additives produced from genetically modified microorganisms (GMMs) for which an authorisation under Regulation (EC) No 1829/2003 is required.

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 phages, Archaea or microalgae), the basic principles also apply on a case-by-case basis.

In case of applications for feed materials (biomass) made from GMMs, the basic principles described herein apply to the characterisation of the microorganism(s).

Feed additives in which viable GMMs are present, intentionally or unintentionally, are out of the scope of this document.

This guidance covers the characterisation of microbial strains which are the subject of applications for authorisation of feed additives containing or produced with microorganisms. For other elements of the assessment, applicants should refer to the other relevant FEEDAP guidance documents, e.g. Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a),

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Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b) and Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c).  

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 feed additive

<table>
<thead>
<tr>
<th>Section</th>
<th>Feed additives containing viable microorganisms</th>
<th>Fermentation products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi – yeasts</td>
</tr>
<tr>
<td>Identification</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial susceptibility</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Antimicrobial production</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Toxigenicity and pathogenicity</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Genetic modification</td>
<td>2.5</td>
<td>For GMMs only</td>
</tr>
<tr>
<td>Absence of the production strain</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Presence of DNA from the production strain</td>
<td>3.2</td>
<td>Where relevant</td>
</tr>
<tr>
<td>Compatibility with other authorised additives</td>
<td>4.2</td>
<td>Where relevant</td>
</tr>
</tbody>
</table>

GMM: genetically modified microorganism.

A specific approach to safety assessment applies to those species of microorganisms included in the list of Qualified Presumption of Safety (QPS) status recommended biological agents (EFSA, 2007). 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 for which the parental/recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment, and for which the molecular characterisation of the event does not give rise to concern, the QPS concept can be extended to the GM production strain. Notwithstanding this, presence of DNA from the production strain must be assessed in all products made with GMMs (Section 3.2).

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 and covered by the International Code of Nomenclature of Prokaryotes (Parker et al., 2015). The nomenclature and taxonomy of fungi are covered by the International Code of Nomenclature for algae, fungi, and plants (ICN) (McNeill et al., 2012). The currently approved nomenclature for fungi can be found on the MycoBank database.

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 European Union (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:** Whole genome sequence (WGS) analysis is required for the characterisation of bacteria (Section 2.1.1). Therefore, data from WGS should be used for identification of the microorganism. This can be achieved by computational approach for taxonomic assignments (e.g. phylogenomics or average nucleotide identity (ANI)), or 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.

- **Yeasts:** As for bacteria, WGS is also required for the characterisation of yeasts (Section 2.1.1). Therefore, data from 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.

In the case that the data do not allow the assignment of the strain under assessment to a known microbial species, its phylogenetic position with respect to the closest relatives should be provided.

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 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 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 characterisation of the strains 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:

- the DNA extraction method;
- 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. average Phred score, number of reads, coverage, N50 and K-mer);
- the FASTA file(s) of the WGS;
- the total length of contigs relative to the expected genome size;
- the annotation protocol used;
- for fungi: information on the quality of the annotations obtained from relevant databases (e.g. BUSCO).

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5 [http://www.the-icsp.org/](http://www.the-icsp.org/)
7 [http://www.mycobank.org](http://www.mycobank.org)
8 [http://busco.ezlab.org](http://busco.ezlab.org)
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 spread 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).

When resistance to an antimicrobial is inherent to a bacterial species, it is generally referred to as ‘intrinsic resistance’ and is typical of all the strains of that species. Intrinsic AMR is not considered a safety concern. In contrast, when a strain of a typically susceptible species is resistant to a given antimicrobial drug, it is considered to be ‘acquired resistance’, which requires further investigation.

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. The cut-off values provided should be seen as a pragmatic tool intended to distinguish strains with acquired resistance from susceptible strains. The cut-off values given in Table 2 were derived from the references listed in Annex B. For bacteria not listed in Table 2, the antimicrobials tested should be those for Corynebacterium and other Gram-positive (for Gram-positive) or for Enterobacteriaceae (for Gram-negative) as listed in Table 2. Resulting MIC values should be compared with published values for that specific or related species and/or those generated in house.

MICs should be determined using serial twofold dilution procedures in agar or broth, including relevant quality control strains. The tests should be performed according to internationally recognised standards such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST9), the Clinical and Laboratory Standard Institute (CLSI10), 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 except in specific and justified circumstances (e.g. when the antimicrobial is not otherwise available).

The culture medium should allow growth of the strain under assessment. Whenever possible, dedicated media to survey antimicrobial resistance/susceptibility profiles (e.g. Muelle–Hinton or IsoSensiTest) should be used. However, for specific bacterial species or strains, other formulations (such as LAB susceptibility test medium (LSM) 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 vs agar dilution), and culture conditions (pH, temperature, time of incubation) on the susceptibility levels to some antimicrobials should be taken into account.

For the purpose of distinguishing resistant from susceptible strains, the FEEDAP Panel has defined microbiological cut-off values based on published data. On this basis, strains can be categorised as:

- susceptible when its growth is inhibited at a concentration of a specific antimicrobial equal to or lower than the established cut-off value (S ≤ × mg/L).
- resistant when it is able to grow at a concentration of a specific antimicrobial higher than the established cut-off value (R > × mg/L).

9 http://www.eucast.org
10 http://www.clsi.org
Table 2: Microbiological cut-off values (mg/L)

<table>
<thead>
<tr>
<th>Microorganism and other Gram-positive</th>
<th>Ampicillin</th>
<th>Vancomycin</th>
<th>Gentamicin</th>
<th>Kanamycin</th>
<th>Streptomycin</th>
<th>Erythromycin</th>
<th>Clindamycin</th>
<th>Tetracycline</th>
<th>Chloramphenicol</th>
<th>Tylosin</th>
<th>Ciprofloxacin</th>
<th>Colistin</th>
<th>Fosfomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus obligate homofermentative (a)</td>
<td>2</td>
<td>2</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Lactobacillus acidophilus group</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>64</td>
<td>16</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Lactobacillus obligate heterofermentative (b)</td>
<td>2</td>
<td>n.r.</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>(c)</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Lactobacillus reuteri</td>
<td>2</td>
<td>n.r.</td>
<td>8</td>
<td>64</td>
<td>64</td>
<td>1</td>
<td>4</td>
<td>32</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Lactobacillus facultative heterofermentative (d)</td>
<td>4</td>
<td>n.r.</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Lactobacillus casei/paracasei</td>
<td>2</td>
<td>n.r.</td>
<td>16</td>
<td>64</td>
<td>n.r.</td>
<td>1</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>2</td>
<td>2</td>
<td>64</td>
<td>n.r.</td>
<td>128</td>
<td>1</td>
<td>1</td>
<td>32</td>
<td>8</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Pediococcus</td>
<td>4</td>
<td>n.r.</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>2</td>
<td>n.r.</td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Lactococcus lactis</td>
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<td>4</td>
<td>32</td>
<td>64</td>
<td>32</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>2</td>
<td>4</td>
<td>32</td>
<td>n.r.</td>
<td>64</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Bacillus</td>
<td>n.r.</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Propionibacterium</td>
<td>2</td>
<td>4</td>
<td>64</td>
<td>64</td>
<td>0.5</td>
<td>0.25</td>
<td>2</td>
<td>2</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>2</td>
<td>4</td>
<td>32</td>
<td>1,024</td>
<td>128</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Corynebacterium and other Gram-positive</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>16</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>8</td>
<td>n.r.</td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>n.r.</td>
<td>8</td>
<td>n.r.</td>
<td>0.06</td>
<td>2</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.r.: not required.
(a): Including L. delbrueckii, L. helveticus.
(b): Including L. fermentum.
(c): For L. buchneri the cut-off for tetracycline is 128.
(d): Including the homofermentative species L. salivarius.
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 databases should be performed (e.g. CARD, ARG-ANNOT, ResFinder). 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 Sections 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.

In all cases, 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 qualifies for the QPS approach or belongs to a taxonomic unit 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. This should be based on updated literature searches (according to the provisions from the Guidance on the assessment of the safety of feed additives for the target species).

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.

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11 [https://card.mcmaster.ca/](https://card.mcmaster.ca/)
13 [https://cge.cbs.dtu.dk/services/ResFinder/](https://cge.cbs.dtu.dk/services/ResFinder/)
2.4.1. Bacteria

For bacterial strains belonging to a species not included in the QPS list, 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, PAI DB, MvirDB, CGE) 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 the assessment of the safety of feed additives for the target species, toxicological studies according to the Guidance on the assessment of the safety of feed additives for the consumer) may be required.

Exceptions to the above requirements are:

- strains which qualify for the QPS approach to safety assessment;
- Other strains for which safety can be established by specific tests (e.g. Enterococcus faecium and Bacillus species, Sections 2.4.1.1 and 2.4.1.2).

2.4.1.1. Enterococcus faecium

*Enterococcus 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, hylEfm, 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, hylEfm, 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 *Bacillus 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 A). 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 14C-leucine uptake is described, but other methods 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*). If there is evidence for similarity, the non-functionality of the genes should be demonstrated. Strains with toxigenic potential are not considered safe.

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14 http://www.mgc.ac.cn/VFs/main.htm
15 http://www.paidb.re.kr/about_paidb.php
16 http://mvirdb.linnl.gov
17 http://www.genomicepidemiology.org/
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 3 of the Guidance on the assessment of the safety of feed additives for the target species). Further information on known toxic secondary metabolites potentially produced by several microbial species can be found in scientific publications such as AINIA Technology Centre (2017). When WGS is available, targeted searches can be performed to identify the presence/absence of known metabolic pathways involved in toxigenicity.

Where known 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\(^{18}\), 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. In case of sequences obtained from environmental samples, the closest orthologous gene(s) should be indicated. 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, EC 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;
- 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\(^{19}\), NCBI\(^{20}\), UniProt\(^{21}\)). This should identify the functional domains of the recombinant protein; the best hits should be reported and described.

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\(^{18}\) Article 2(2) of Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms defines ‘genetically modified organism’ as an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

\(^{19}\) http://www.ebi.ac.uk/ena


\(^{21}\) http://www.uniprot.org/
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 should be done using WGS data for bacteria and yeasts, and is recommended for filamentous fungi.

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 preferably by comparing the WGS of the GMM with that of the non-modified parental or recipient strain.

The sequences/databases and the methodology used for analyses and comparison should be described in detail.

2.5.3.2. Structure of the genetic modification without WGS data
For filamentous fungi for which 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.

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 2.5.3.1 (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 polymerase chain reaction (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 Guidance on the identity, characterisation and conditions of use of feed additives.

3.1. Absence of the production strain

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 of the production strain should be investigated using a well-described method for the detection:

- by means of a culture-based method targeted to the detection of the viable cell. Cultivation-independent methods are not acceptable;
- 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 two 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;
- the 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 of product 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. 10–1,000 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 determined in compliance with regulatory requirements;
• products obtained using non-genetically modified production strains carrying acquired 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, to calculate the limit of detection;
  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 investigate whether the target DNA is detected in analyses having detection threshold of 10 ng of DNA per gram or mL of product or lower.

4. In vivo microbial 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), 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. For more information on the conditions of use, see the Guidance on the identity, characterisation and conditions of use of feed additives.

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 2.2.1. 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

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22 Short-term experiments are defined as studies with duration shorter than the minimum duration of long-term studies according to the FEEDAP Guidance on efficacy (under preparation).
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.

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.

For other microbial strains:

- bacterial strains carrying acquired 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 acquired antibiotic resistance determinants, shown not to produce relevant antimicrobial substances according to Section 2.3 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.
- 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.

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, shown not to produce relevant antimicrobial substances according to Section 2.3 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.

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 acquired 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, 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,23 consumers,24 users and the environment25 should be separately considered as appropriate.

5.3.  Feed additives produced by GM microorganisms

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 or
- which are free from acquired 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,23 consumers,24 users and the environment25 should be separately considered as appropriate.

References


Fagerlund A, Lindbäck T, Storsøe AK, Granum PE and Hardy SP, 2008. Bacillus cereus Nhe is a pore-forming toxin with structural and functional properties similar to the ClyA (HlyE, SheA) family of haemolysins, able to induce osmotic lysis in epithelia. Microbiology, 154, 1554.


Glossary and Abbreviations

**Acquired AMR gene**  A gene acquired by a bacterium conferring antimicrobial resistance to an otherwise typically susceptible microorganism

**Active agent**  Any microorganism intended to be used as a feed additive or in the manufacture of a feed and that provides the intended effect.

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

**Dry feed**  Feed containing at least 88% dry matter

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

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

**Hazard**  A biological, chemical or physical agent in, or conditions of, food or feed with the potential to cause an adverse health effect

**History of use**  Documented information on the microbial strain regarding its previous deliberate introduction or use in the food chain

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

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

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

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

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

**Risk**  A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard

**Vector**  Is understood as the agent containing the introduced DNA sequence used as a vehicle to transfer such sequence into the transformed cell

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26 As defined in Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AMR</td>
<td>antimicrobial resistance</td>
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<tr>
<td>ANI</td>
<td>average nucleotide identity</td>
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<tr>
<td>BHI</td>
<td>brain heart infusion broth</td>
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<td>CFU</td>
<td>colony forming unit</td>
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<td>CIA</td>
<td>critically important antimicrobial</td>
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<td>CLSI</td>
<td>Clinical and Laboratory Standard Institute</td>
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<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<tr>
<td>GM</td>
<td>genetically modified</td>
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<td>GMM</td>
<td>genetically modified microorganism</td>
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<tr>
<td>GMO</td>
<td>genetically modified organism</td>
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<tr>
<td>HIA</td>
<td>highly important antimicrobial</td>
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<tr>
<td>ICN</td>
<td>International Code of Nomenclature for algae, fungi, and plants</td>
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<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
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<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
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<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
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<tr>
<td>LSM</td>
<td>LAB susceptibility test medium</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<td>MIC</td>
<td>minimum inhibitory concentration</td>
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<td>ORF</td>
<td>open reading frames</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>QPS</td>
<td>Qualified Presumption of Safety</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>WGS</td>
<td>whole genome sequence</td>
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</tbody>
</table>
Annex A – Recommended procedure for the detection of cytotoxicity in *Bacillus* species other than those of the *B. cereus* group 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 10^8 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 Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum. Cells should be seeded into 24-well plates 2-3 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 h 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 14C-leucine and add 300 µL of this mixture to each well, incubate the cells for 1 h at 37°C.
- Remove the radioactive medium and add 1 mL 5% trichloroacetic acid (TCA) to each well, incubate at room temperature for 10 min. 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 min. 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 min.

Percentage inhibition of protein synthesis is calculated using the following formula: \((\text{Neg. ctrl} - \text{sample})/\text{Neg. ctrl} \times 100\); the negative control is Vero cells from wells without addition of sample. Above 20% inhibition is considered to indicate cytotoxicity. As a positive control, surfactin or supernatants from known cytotoxic *B. cereus* strains may be used.

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 10^6 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 s 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 PI 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.
Annex B – List of bibliographic references used to derive microbiological cut-off values


Jeong DW and Lee JH, 2105. Antibiotic resistance, hemolysis and biogenic amine production assessments of *Leuconostoc* and *Weissella* isolates for kimchi starter development. LWT-Food Science and Technology, 64, 1078–1084.


