Guideline for the Document Preparation for Safety Evaluation of Probiotics in Food

By

Committee on Safety Assessment for Use of Probiotic Microorganisms in Food

National Center for Genetic Engineering and Biotechnology (BIOTEC)

National Science and Technology Development Agency (NSTDA)

(Version: December 2023)

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Please read: To expedite the evaluation process and avoid unnecessary delay, please do the following:

- Submit only accurate and complete information in a concise format. Promotional /advertising/redundant documents with the same information/without added information should be avoided.
- Strictly follow the recommendations suggested in this guideline. If there are deviations, the rationale for those deviations should be clearly stated and reference(s) submitted.
- Carefully check for completeness and accuracy of the submitted dossier. Conflicting results obtained from different methodologies should be avoided or clarified and discussed.
- The submitted dossier should be certified for accuracy and completeness by qualified authorities. The certified version will be used for the evaluation process. (This is to ensure that the dossier has been checked and approved by the applicant and that it is the most current and correct version, to the best of the applicant's knowledge).
- Clearly identify the files submitted (e.g., starting from the heading number, heading subject, and running number if several files are submitted). Additional information may be submitted in a separate folder (the subject title should be identified).
- Complete the checklist.

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Table of Contents

1. General information

1.1 Scientific name and Trade name

1.1.1 Scientific name and strain ID. The species and a unique strain identification (strain name or code) of the organism seeking evaluation should be provided. The most up-to-date and valid nomenclature should be used (The List of Prokaryotic names with Standing in Nomenclature (LPSN) is available at https://lpsn.dsmz.de).

1.1.2 Trade name, if applicable**.** All trade names (or other alternative names) referring to the organism seeking evaluation should be provided.

Table 1 Scientific and Trade name*

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	*Lactiplantibacillus*
Species	*Lactiplantibacillus plantarum* (synonym: *Lactobacillus plantarum*)
Strain ID	ABC123
Trade name	xxx™xxx®

* Examples are shown in gray

1.2 Source of isolation and depository

Please specify where the strain was isolated from, e.g., plant materials, kimchi, human feces, blood, etc. The strain should be deposited in at least one internationally recognized culture collection and maintained throughout the commercialization period.

Table 2 Source of isolation and depository*

Source of isolation	plant materials, kimchi, human feces, blood, etc.	
Depository	**Name, Country**	**Accession No.**
1	American Type Culture Collection (ATCC), USA	1234
2	Thailand Bioresource Research Center (TBRC), Thailand	1234

* Examples are shown in gray

1.3 Product Specification

Please provide your product specification. The specification should be clear and specific to the product seeking approval. The specification for food supplements as set by the Thai FDA (Table 3) should be used as the minimum requirement.

Table 3 Specification for food supplements, Thai FDA

Description	Limits
Microbial contamination	
Staphylococcus aureus	Not found in 0.1 gram
Clostridium spp.	Not found in 0.1 gram
Salmonella spp.	Not found in 25 grams
Escherichia coli	Less than 3 MPN/g
Heavy metals	
Cadmium	Not more than 0.3 ppm
Lead	Not more than 1.0 ppm
Mercury	Not more than 0.5 ppm
Arsenic, total	Not more than 2.0 ppm

Table 4 Product specification, example*

Characteristic, appearance	Dry powder, off-white, etc.
Moisture content	Not higher than xx%
Bacterial count (target strain)	Not lower than xx CFU/g
Non-target strain (yeast, mold)	Not found in 1 g
Microbial contamination	
• *Staphylococcus aureus*	Not found in 0.1 gram
• *Clostridium* spp.	Not found in 0.1 gram
• *Salmonella* spp.	Not found in 25 grams
• *Escherichia coli*	Less than 3 MPN/g
Heavy metals	
• Cadmium	Not more than 0.3 ppm
• Lead	Not more than 1.0 ppm
• Mercury	Not more than 0.5 ppm
• Arsenic, total	Not more than 2.0 ppm

* Examples are shown in gray

1.4 Cover letter and non-GMO statement

The applicant should provide a cover letter that includes the strain ID of the microorganism seeking approval, the name of the applicant, including manufacturer and distributor/importer, and a statement certifying (to the best of the applicant's knowledge) the accuracy and completeness of the submitted dossier.

A statement declaring that the strain under evaluation was not genetically modified through modern biotechnology, or manipulated beyond its natural boundary should also be provided.

Table 5 Cover letter checklist

Strain ID, trade name	☐
Applicant (Name, company, address)	☐
Manufacturer	☐
Distributor/Importer	☐
Statement certifying accuracy and completeness	☐
Signature	☐
Non-GMO statement	☐

2. Strain identification and characterization

The strain seeking approval should be identified to the species level using current and valid methods. The strain should be characterized by both phenotypic and genotypic methods.

2.1 Phenotypic characterization

Please provide information on the specific phenotypic traits of the strain. Examples of some characteristics that should be provided are shown in Table 6.

Table 6 Phenotypic characterization

Gram stain	
Cell morphology, organization	
Growth condition	
Others, e.g., sugar utilization, motility, etc.	

2.2 Genotypic characterization

A whole genome sequence (WGS), including chromosome and extrachromosomal genetic elements, e.g., plasmids, is required for bacterial strains. WGS data provide information for the unequivocal taxonomic identification, as well as for the characterization of the strains regarding their potential 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 as listed in Table 7 should be provided. No field (including all bullets) should be left blank. Irrelevant or missing information should be filled with “N/A” or “no data”, accordingly.

The genome characteristics/statistics should be provided (e.g., number of contigs, coverage, GC contents). The presence/absence of plasmids in the genome should be identified, and how this conclusion has been reached should be described.

For the search for potential concern genes using WGS data, such as antimicrobial resistance (AMR), or virulence, pathogenicity and/or toxigenicity, the search should be done applying the minimum available threshold in the database. Query sequence hits with at least 80% identity and 70% length of the subject sequence should be reported.

For references in this section, please refer to EFSA, 2018 and EFSA, 2021 [1,2]

The WGS/genotypic characterization report should be certified for accuracy and completeness by the analyst and/or authorized person.

Table 7 WGS analysis detail

Sequencing Institute, address	
Sample ID[1]	
DNA extraction method[2]	
Long-read sequencing technology • Instrument/sequencing chemistry • Library construction protocol, including DNA fragmentation and size selection[3], if applicable • PHRED score set for quality trimming • Average read depth • Base calling/trimming software used, including version and relevant parameters	
Short-read sequencing technology • Instrument/sequencing chemistry • Library construction protocol • PHRED score set for quality trimming (≥20 should be set) • Average read depth (≥ 30x) • Base calling/trimming software used, including version and relevant parameters	
Genome assembly • protocol used (De novo assembly is preferred) • assembler software, including version and relevant parameters Genome statistics • Total length (genome size) • The genome size is within +/- 20% of the expected genome size of the species[4] • Presence of plasmids? • Number of contigs and their coverage • GC contents • Others.	
Post-assembly processing, if applicable • approach, software, version, and parameters	
Gene prediction and annotation • protocol used • software used, including version and relevant parameters • database, version (where available), and/or date of accession	
Contamination in the sequencing reads • software used, including version and relevant parameters • database, version (where available), and/or date of accession • result (% contamination) [5]	
The FASTA file(s) of the WGS including: • nucleotide sequence (.fna or .fasta) • amino acid sequence (.faa or .fasta) • gene annotation (.gbk)	

[1] Should have a statement confirming that the strain under analysis is the same as the one seeking approval.
[2] A pure culture should be used. Total DNA, including chromosomal and plasmids, should be extracted.
[3] Any selection of fragments by size should ensure that small plasmids are not lost.
[4] The genome size should be within 20% of the expected genome size for the species, if not, an explanation should be provided.
[5] Assigned reads to an unexpected organism should be less than 5%, if not, an explanation should be provided.

2.3 Species identification

For bacteria, the identity of the strain should be established by average nucleotide identity (ANI) or digital DNA-DNA hybridization (dDDH). The data should be compared with the type strain of the expected species. In case the genome of the type strain is not available, genome sequences of another well-documented strain(s) can be used as a reference. For identification at the species level, the ANI values should be > 95% and the dDDH values should be > 70% identity.

In addition to the type strain of the expected species, additional ANI values compared to the type strain of closely related species should also be calculated, especially for the organism known to have high sequence similarity within the group (e.g. species in *Bacillus subtilis* group), especially when the ANI value is close to the borderline (95%) to ensure accurate identification.

Tool and database used for the species identification (e.g., ANI calculation) should be provided (e.g., software version, parameters, database, version (where available) and/or date of accession). An example of the information required for this section is shown in Table 8. The information can be adjusted according to the analysis protocol.

For yeasts and filamentous fungi, WGS is recommended. Confirmation of identity should be done by phylogenomic analysis (e.g., using a concatenation of several conserved sequences to produce a phylogeny against available related genomes) or by alignment to a complete reference genome from the same species. In the case of no WGS data, the similarity of a suitable discriminatory gene(s) for the yeast/fungi group may be used (such as Internal transcribed spacer region (ITS), D1/D2 region, or full large subunit ribosomal RNA gene).

Table 8 Species identification detail*

Software/tool used for ANI calculation • Name • Version • relevant parameters	
Database • Name • Version (where available), and/or date of accession	
Result: assigned species • ANI value(s) to the type strain of the assigned species • Type strain ID (accession no.)	
Result: closely related species • ANI value(s) to the type strain(s) of other closely related species • Type strain ID (accession no.)	

*Please note that the comparison using full 16S rRNA gene sequence alone is insufficient for the unambiguous identification of several bacterial species. The ANI and/or dDDH values are required for unambiguous species identification.

3. Probiotic properties

3.1 Resistance to gastric acidity

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

At the minimum, the report should include:

- Name and address of the institute/lab/company conducting the test.
- Name and composition of the medium used.
- pH value(s) of the test condition. The pH should be adjusted using hydrochloric acid (HCl) to the values of ≤ 3.0
- Duration(s). The exposure time should be sufficient to imply tolerability of the strain in the human gastric environment. The test duration should be ≥ 2 h. Periodic sampling, e.g., every 0.5-1.0 h is recommended.
- Enumeration method. Plate count on growing media is commonly used. If another method is used, the method should be able to distinguish between live/dead cells.
- Calculation. The property should be expressed as

$$\% \text{ Survival} = \frac{\text{Number of living cells after exposure (CFU/mL)}}{\text{Number of living cells before exposure (CFU/mL)}} \times 100$$

If log-transformed data was used in the calculation (i.e., logCFU/mL), this fact should be clearly stated.

- Positive control. Since the % survival of a strain will be different in different test conditions and since no standardized protocol has been set for this test, the use of appropriate control is crucial. For this purpose, a commercial strain that is accepted as an effective probiotic (e.g., *L. rhamnosus* GG, *L. plantarum* 299V, *Bifidobacterium animalis* subsp. *lactis* BB-12), or a strain that was proven to survive the human gastrointestinal environment (scientific literature should be provided) should be included for comparison.

3.2 Resistance to bile salts

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

At the minimum, the report should include:

- Name and address of the institute/lab/company conducting the test.
- Name and composition of the medium used.
- Type/composition of the bile salts used. The use of mixed bile salts with types and concentrations similar to those in the human intestinal environment is recommended. For this purpose, the bile extracted from bovine (ox gall) is recommended since its composition is similar to human bile. The bile extracted from other animal species (e.g. porcine) containing different species of bile salts should not be used for this purpose.
- The bile salts concentration. Various concentrations of bile salts may be used. A minimum of 0.3% bile salts should be used for this test.
- Duration(s). The exposure time should be sufficient to imply tolerability of the strain to the human intestinal environment. A minimum of 4 h is recommended.
- Enumeration. Plate count on growing media is commonly used. In the case where growth occurred, indirect measurement such as turbidity/OD measurement may be used.
- Calculation. The property should be expressed as

$$\% \text{ Survival} = \frac{\text{Number of living cells after exposure (CFU/mL)}}{\text{Number of living cells before exposure (CFU/mL)}} \times 100$$

If log-transformed data was used in the calculation (i.e., logCFU/mL), this fact should be clearly stated.

- Positive control. In the case where growth was demonstrated in the bile salts-containing medium, the strain may be reported as bile-salts resistance, no positive control is required. In the case of no growth, the % survival should be calculated. In this case, a commercial strain that is accepted as an effective probiotic (e.g., *L. rhamnosus* GG, *L. plantarum* 299V, *Bifidobacterium animalis* subsp. *lactis* BB-12), or a strain that was proven to be able to survive the human gastrointestinal environment (scientific literature should be provided) should be included for comparison.

3.3 Adherence to mucus and/or human epithelial cells and cell line

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

It is accepted that the adherence of bacteria to the host intestinal tract involves several mechanisms with different target sites, such as mucin-binding protein in the mucus layer and specific epitope binding to the host epithelial cells. A bacterium is deemed positive for this property if it is shown to be able to persist for a length of time after the administration of the strain is stopped (*in vivo*).

In place of the *in vivo* test, since there is no consensus methodology and interpretation criteria for the *in vitro* determination, we deemed that a scientifically sound methodology published in the reputable scientific literature may be used as a reference (a reference from a potential predatory journal/publisher listed in the Beall's list should be avoided). The methodology should strictly adhere to the cited reference. If there is any modification or deviation, the deviation should be clearly stated, and the reason provided. The deviation(s) should not be in the critical step(s) affecting the result interpretation.

At the minimum, the report should include:

- Name and address of the institute/lab/company conducting the test.
- Type and preparation of the target site (e.g. Caco-2 cells, growth conditions, days of cultivation, etc.; mucus, the source and preparation of the mucus, the plate coating procedure, etc.)
- Status and preparation of the bacterial cells (growth conditions, stage of the cells (e.g., acid-bile-stressed cells, labeling with a radioisotope, labeling with fluorescence, etc.)
- Adhesion condition
  - Concentration, number of cells, and/or the ratio of the bacterial cells to the target cells or surface area.
  - The medium used.
  - Duration. Should be ≥ 60 min
  - Temperature.
- Washing conditions.
  - The solution and washing procedure used to get rid of the non-attached bacteria.
  - The number of washings. At least 2x washing should be done. Usually, ≥3x washing is preferred for the complete removal of non-attached bacteria.
- The procedure used for the determination of attached bacteria. Several methods have been used for the determination of attached bacteria, such as viable plate count, direct microscopic count, or measuring the radioactivity/fluorescence signals.

- Calculation. The property may be expressed, depending on the detection method, as:
  - For viable plate counts:

$$\% \text{ attachment} = \frac{\text{Number of bacterial cells attached (CFU/mL)}}{\text{Number of bacterial cells added (CFU/mL)}} \times 100$$

Or Number of attached bacteria/well (or other suitable units)

  - For radioactivity/fluorescence signals:

$$\% \text{ attachment} = \frac{\text{Signals of bacterial cells attached (AU/mL)}}{\text{Signals of bacterial cells added (AU/mL)}} \times 100$$

  - For microscopic counts

$$\text{attachment} = \frac{\text{Number of bacterial cells (cells)}}{\text{Number of epithelial cells (cells) or surface area}}$$

- Positive control. In the case of *in vitro* test, the result obtained from a positive control using the same test procedure should be provided for comparison. A commercial strain that is accepted as an effective probiotic (e.g., *L. rhamnosus* GG, *L. plantarum* 299V, *Bifidobacterium animalis* subsp. *lactis* BB-12), or a strain that was proven to be able to persist *in vivo* (scientific literature should be provided) should be included for comparison.

3.4 Bile salt hydrolase activity

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

A scientifically sound methodology published in reputable scientific literature may be used (a reference from a potential predatory journal/publisher listed in Beall's list should be avoided). Special precautions should be paid on the objective and limitation of the selected reference. The methodology should strictly adhere to the reference. If there is any modification or deviation, the deviation should be clearly stated, and the reason provided. The deviation(s) should not be in the critical step(s) affecting the result interpretation. A simple streak plate method as described by Dashkevicz, 1989[3] is commonly used.

At the minimum, the report should include:

- Name and address of the institute/lab/company conducting the test.
- Type and composition of the bile-salt(s). Typically, the MRS containing 0.5% taurodeoxycholic acid (TDCA) is used. However, since glycine-conjugated bile salts constitute the majority of human bile, at least one glycine-conjugated bile salt should also be tested, especially in the case where the TDCA plate gives a negative result. [consideration: human bile component consists mainly (~ 96%) of GCA, GCDCA, GDCA, TCA, TCDCA, and TDCA in a molar ratio of ~ 6:6:4:3:3:2]. Mixed bile salts extracted from bovine (ox gall) may be used for this purpose.
- Result and interpretation. The plate pictures of the bacterial growth on the negative control plate (MRS) and the test plate (MRS+bile salts) should be presented side-by-side, with sufficient quality/clarity supporting the result interpretation. A positive reaction can be presented as i) the formation of precipitate halos around colonies, or ii) the formation of opaque granular white colonies without precipitate halos.
- Positive control. In the case of a negative result, the result obtained from a positive control using the same culture medium preparation (same lot) should be presented to confirm the validity of the test. A commercial probiotic strain such as *L. plantarum* 299V, or any bacterial strain shown to be positive for this property may be used. It is noted that *L. rhamnosus* GG is inappropriate as a positive control in this test since it has very low activity against TDCA and no activity against other bile salts.

3.5 Other properties (if present)

Please specify other properties of the strain seeking approval, accompanied by its supporting evidence.

4. Safety assessment

4.1 Antimicrobial resistance (AMR)

Both phenotypic testing and a search of the WGS for the presence of known AMR genes should be conducted.

4.1.1 Phenotypic testing (antibiotic resistance patterns)

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

For bacteria, the minimum inhibitory concentrations (MICs) to a set of antimicrobial drugs as recommended by EFSA, 2018 (Table 9) should be determined, using microdilution methods according to internationally recognized and standardized methods. For Lactobacilli and Bifidobacterium, the ISO10932|IDF223[4] is commonly used. For other bacteria that grow aerobically (e.g. *Bacillus*), the CLSI M07-A10[5] may be used. The validity of the test should be determined using quality control strain(s) recommended by the standard. The report should identify the QC strain used and its MIC results to confirm the validity of the test.

Qualitative or semi- quantitative methods to determine MIC indirectly, such as diffusion methods, are not acceptable[1]

The MICs provided in Table 9 are used as cut-off values to distinguish between intrinsic and acquired resistance. A strain is identified as susceptible (intrinsic resistance) when the MIC is less than or equal to the cut-off, or resistant (acquired resistance) when the MIC is higher than the cut-off value. Intrinsic AMR is not considered a safety concern, while acquired resistance requires further investigation.

Table 9 List of antimicrobial drugs to be tested and their MIC (mg/L), Adopted from EFSA, 2018

	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol	Tylosin	Ciprofloxacin	Colistin	Fosfomycin
Lactobacillus obligate homofermentative[(a)]	2	2	16	16	16	1	4	4	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus acidophilus group	1	2	16	64	16	1	4	4	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus obligate heterofermentative[(b)]	2	n.r.	16	64	64	1	4	8[(c)]	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus reuteri	2	n.r.	8	64	64	1	4	32	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus facultative heterofermentative[(d)]	4	n.r.	16	64	64	1	4	8	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus plantarum/pentosus	2	n.r.	16	64	n.r.	1	4	32	8	n.r.	n.r.	n.r.	n.r.
Lactobacillus rhamnosus	4	n.r.	16	64	32	1	4	8	4	n.r.	n.r.	n.r.	n.r.
Lactobacillus casei/paracasei	4	n.r.	32	64	64	1	4	4	4	n.r.	n.r.	n.r.	n.r.
Bifidobacterium	2	2	64	n.r.	128	1	1	8	4	n.r.	n.r.	n.r.	n.r.
Pediococcus	4	n.r.	16	64	64	1	1	8	4	n.r.	n.r.	n.r.	n.r.
Leuconostoc	2	n.r.	16	16	64	1	1	8	4	n.r.	n.r.	n.r.	n.r.
Lactococcus lactis	2	4	32	64	32	1	1	4	8	n.r.	n.r.	n.r.	n.r.
Streptococcus thermophilus	2	4	32	n.r.	64	2	2	4	4	n.r.	n.r.	n.r.	n.r.
Bacillus	n.r.	4	4	8	8	4	4	8	8	n.r.	n.r.	n.r.	n.r.
Propionibacterium	2	4	64	64	64	0.5	0.25	2	2	n.r.	n.r.	n.r.	n.r.
Enterococcus faecium	2	4	32	1,024	128	4	4	4	16	4	n.r.	n.r.	n.r.
***Corynebacterium* and other Gram-positive**	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.
(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*.

For yeast, the antibacterial drugs as shown in Table 9 are irrelevant. The strain should be tested for susceptibility to a set of antimycotic drugs clinically used for yeast or fungal infection. The test should include several antimycotics from different classes. Interpretation of the MIC cut-off values (breakpoints) should be done according to the relevant standard(s) (e.g., CLSI or EUCAST) for the species. In the case where no breakpoint has been determined for the drug-species of interest, the breakpoint from other, preferably taxonomically related, species may be used, and this deviation should be clearly stated in the report.

The result in this section should be present as illustrated in Table 10

Table 10 MICs to antimicrobials of the test strain*

Antimicrobials	Test range (mg/L)	cut off MIC (mg/L)	Test strain MIC (mg/L)	Interpretation
Vancomycin	0.25 – 32	4	x	S/R
Chloramphenicol	0.25 – 32	8	x	S/R
Clindamycin	0.063 – 8	4	x	S/R
Erythromycin	0.031 – 4	4	x	S/R
Gentamicin	0.063 – 8	4	x	S/R
Kanamycin	0.5 – 64	8	x	S/R
Streptomycin	0.5 – 64	8	x	S/R
Tetracycline	0.125 – 16	8	x	S/R

*Antimicrobials and MIC cut-offs for the species as stated in Table 10; S: susceptible; R: resistant

4.1.2 Genotypic testing (WGS search for AMR genes)

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

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-see WHO-CIA list for reference[6]).

It is recommended to search against at least two well-maintained databases. In the case of microorganisms for which no or few AMR genes are present in databases (such as in the case of probiotics), searches with Hidden-Markov model tools are recommended[2]. The search using tools in the Kyoto Encyclopedia of Genes and Genomes (KEGG) was proved to be effective[7], hence recommended as the main search tool. Other AMR databases are usually constructed mainly from pathogenic bacteria (such as CARD[8] and ResFinder[9]). These databases/tools may be used to provide supplementary information. Query sequence hits with at least 80% identity (at the protein level or nucleotide level as reported in the database) and 70% length of the subject sequence should be reported.

The search result should be up-to-date, not older than 2 years from the date of analysis.

All hits (not only the genes related to the resistance phenotype) fitting the search criteria should be presented.

If the search reveals AMR genes for antimicrobials considered to be CIAs or HIAs, the MIC values of those antimicrobials should be determined.

The genetic determinant contributing to the resistance phenotype required further investigation. Locations of the AMR genes should be determined. AMR genes located on mobile elements (especially on plasmids or prophages) are considered a high risk of AMR transfer. The organism harboring mobile AMR should not be used in the food chain.

The information required in this section is summarized in Table 11. The search results obtained from several databases may be shown in separate tables or combined as shown in Table 12.

Table 11 AMR gene search

Database 1: (KEGG) • Version/last updated date • Analysis date • Tool (version) • Search parameters/Filter parameter	
Database 2 • Version/last updated date • Analysis date • Tool (version) • Search parameters/Filter parameter	
Search result: • All significant hits and their locations should be provided. See the example in Table 12	
Identification of Mobile elements (if applicable) • Type of mobile elements • Identification protocol	
Conclusion • Presence of AMR gene(s) on mobile element(s) • Discussion and conclusion on the risk of transferring the AMR traits to other organisms. (functional and transferable traits)	

Table 12 AMR gene search result detail *

Drug class	GeneName	GeneID	Contig	Position	Database
Macrolide resistance	K06979 mph; macrolide phosphotransferase	MGA_289	Chr	286358..287278	KEGG, ResFinder, CARD
lincosamide antibiotic	lmrB; ATP-binding cassette (ABC) antibiotic efflux pump	MGA_304	Chr	299172..300605	CARD
Macrolide resistance	K08217 mef; MFS transporter, DHA3 family, macrolide efflux protein	MGA_1444	Chr	1455384..1456676	KEGG
beta-Lactam resistance	K17836 penP; beta-lactamase class A [EC:3.5.2.6]	MGA_1957	Chr	2051420..2052340	KEGG
Aminoglycoside resistance	K05593 aadK; aminoglycoside 6-adenylyltransferase [EC:2.7.7.-]	MGA_2567	Chr	2595688..2596542	KEGG, ResFinder, CARD

* Examples are shown in gray

4.2 Assessment of certain metabolic activities (Deleterious metabolic activities)

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

The ability to produce metabolites with deleterious effects on the host should be assessed for the strain's safety. In the Guidelines for the Evaluation of Probiotics in Food, 2002[10], examples of these activities included D-lactate production and bile salt deconjugation. While these properties should be submitted for completion, they are no longer considered deleterious to the general population and are common in several probiotics.

The main concern due to bile salt deconjugation is the production of carcinogenic secondary bile salts. Therefore, the ability of the strain to produce secondary bile salts should be determined.

The WGS should be interrogated for known deleterious metabolites. Genes related to the production of biogenic amines are considered relevant and should be searched for. If present, the risk of such amine(s) in the intended use conditions should be quantified or discussed.

Examples of genes/pathways related to the production of deleterious metabolites are shown in Table 13.

Table 13 Examples of genes/pathways related to deleterious metabolites from KEGG search[7]

Map ID	Name	Enzyme	Function	Result in strainxxx*
Under "Brite"				
Ko02042	Bacterial toxins	toxins	Virulence	*tlyC (1), hlyIII (1)*
Under "Pathway"				
Carbohydrate metabolism				
00620	Pyruvate metabolism	lactate racemase [EC 5.1.2.1]	D-lactate <-> L-lactate	Yes (1)
		D-lactate dehydrogenase [EC:1.1.1.28]	Pyruvate <-> D-lactate	Yes (2)
Lipid metabolism				
00120	Primary bile acid biosynthesis	Choloylglycine hydrolase [EC 3.5.1.24]	Bile salt deconjugation	Yes (1)
00121	Secondary bile acid biosynthesis	complete pathway	possible carcinogenic secondary bile acids	No
Amino acid metabolism				
00310	Lysine degradation	Lysine decarboxylase [EC 4.1.1.18]	production of cadaverine	No

Map ID	Name	Enzyme	Function	Result in strainxxx*
00330	Arginine and proline metabolism	Ornithine decarboxylase [EC 4.1.1.17]	ornithine -> putrescine	No
		Arginine decarboxylase [EC 4.1.1.19]	arginine -> agmatine	No
		Agmatinase [EC 3.5.3.11]	agmatine -> putrescine	No
		Spermidine synthase [EC 2.5.1.16]	putrescine -> spermidine	No
		Arginase [EC 3.5.3.1]	arginine -> ornithine	No
		spermine synthase [EC:2.5.1.22]	spermidine-> spermine	No
00340	Histidine metabolism	Histidine decarboxylase [EC 4.1.1.22]	histidine -> histamine	No
00350	Tyrosine metabolism	Tyrosine decarboxylase [EC 4.1.1.25]	tyrosine -> tyramine	No
00380	Tryptophan metabolism	Tryptophan decarboxylase [EC 4.1.1.28]	tryptophan -> tryptamine	No

***** Examples are shown in gray

4.3 Assessment of side-effects during human studies

Sufficient evidence should be submitted to support the strain's safety on the consumers. The results from efficacy studies may be used if such studies also have safety parameters measured. Information as shown in Table 14 and appropriate reference(s) should be provided.

Table 14 Assessment of side-effects during human studies

Human study#1	**Result (document#)**
Safety parameter measured: • Parameter 1 • Parameter 2 • …	
Human study#2	**Result (document#)**
Safety parameter measured: • Parameter 1 • Parameter 2 • …	

In the case where there is no human study with appropriate safety parameters determined, the safety of the species and/or strain of interest may be demonstrated using appropriate systematic review(s) or referenced from the country/organization with a safety evaluation system in place.

The Qualified Presumption of Safety (QPS) list maintained by the European Food Safety Authority (EFSA) is an example of acceptable documentation. The most recent version of the QPS list should be used. The evidence of the strain's compliance with the QPS qualification for the species should be submitted, along with the published Scientific Opinion (from the EFSA journal) for inclusion of the species in the QPS list.

4.4 Epidemiological surveillance of adverse incidents (post-market)

If the strain has been commercialized, please provide the information as shown in Table 15. If not yet commercialized, please provide the surveillance plan.

Table 15 Epidemiological surveillance of adverse incidents in consumers (post-market)

Country#1 Name: ……	**Result (document#)**
• Year started • Year terminated (if applicable, provide the reason for termination) • Sale volume (approximate number of doses) • Surveillance method/how to accept complaint report • Surveillance result	
Country#2 Name: ……	**Result (document#)**
• Year started • Year terminated (if applicable, provide the reason for termination) • Sale volume (approximate number of doses) • Surveillance method/how to accept complaint report • Surveillance result	
For not yet commercialized product	
• Surveillance plan: method/how to accept complaint report • Response/complaint management plan	

4.5 Toxin production (for the species that is a known mammalian toxin producer)

If the strain belongs to a species of known mammalian toxin producer, the toxin production capability should be determined. Strains in some species of the genus *Bacillus*, *Enterococcus*, and *Escherichia* have been shown for probiotic properties, however, other strains of the same species were recognized as human pathogens. In this case, evidence showing the strain's inability to produce the species' known toxin should be provided. The evidence may be in the form of *in silico* (WGS analysis for the species' known virulence genes), *in vitro* (cytotoxicity), or *in vivo*.

In the case of WGS analysis, it is recommended to search against at least two well-maintained databases. The search using tools in the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Virulence Factor Database are recommended as the main search tool. The search result should be up-to-date, not older than 2 years from the date of analysis. If the search reveals toxigenic potential, the strain's ability to produce such toxin should be determined.

The required information for this section is shown in Table 16.

Table 16 Toxin production (for known mammalian toxin producer)

***In silico*: WGS analysis**	**Detail**
Software/tool #1 • Name • Version • relevant parameters	VFanalyzer* The release (R5) The raw FASTA sequence(s) of a COMPLETE genome
Database • Name • Version (where available), and/or date of accession	The virulence factor database (VFDB) Database last update: Mon Nov 14 20:57:06 2022
Result: • List of known toxins	Negative for all known Bacillus toxins
Software/tool #2 • Name • Version • relevant parameters	BlastKOALA Version 2.2 Last updated: May 15, 2019 Taxonomic group: Bacteria
Database • Name • Version (where available), and/or date of accession	KEGG GENES database: species_prokaryotes Accession date: Jan 3, 2023
Result: • List of known toxins	Negative for all known Bacillus toxins
***In vitro*: cytotoxicity**	**Detail**
Provide a test report including • Method • Result and conclusion	A cytotoxicity test report on Caco-2 cell is included
***In vivo*: animal trial (mouse)**	**Detail**
Provide a test report including • Method: should follow OECD guideline • Result and conclusion	None

* Examples are shown in gray

4.6 Hemolytic activity (for the strain belongs to a species with known hemolytic potential)

Please submit a test report, accompanied by the conclusion of the finding. The report should provide sufficient information to determine the accuracy/suitability of the conclusion. The report should be certified for accuracy and completeness by the analyst and/or authorized person.

At the minimum, the report should include:

- Name and address of the institute/lab/company conducting the test.
- Name of the medium used (including the species of animal which the blood was obtained from).
- Methods (a simple streak plate method on blood agar is commonly used. If a different method was used, please provide a valid reference) including:
  - Medium composition (% blood, species of blood).
  - Incubation condition, temperature.
  - Incubation time. For a non-beta hemolysis conclusion, at least 48 h incubation at an appropriate temperature is needed. As long as 72 h incubation may be necessary for an alpha-hemolysis conclusion.
- Result and interpretation.
  - The plate pictures of the bacterial growth on the blood agar should be submitted.
  - The picture should be clear, with sufficient resolution to support the conclusion. Transmitted light (light source behind the plate) should be used to document the hemolysis activity.
  - Positive and negative control(s) may be included for comparison.

5. Result of safety evaluation from other organization(s), if present

5.1 International

Identify the organization, year of evaluation, and the assessment result.

5.2 National

Identify the organization, year of evaluation, and the assessment result.

6. References:

1 EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed) *et al.* Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal* **16**, e05206, doi:https://doi.org/10.2903/j.efsa.2018.5206 (2018).

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

3 Dashkevicz, M. P. & Feighner, S. D. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. *Applied and environmental microbiology* **55**, 11-16, doi:10.1128/aem.55.1.11-16.1989 (1989).

4 ISO 10932|IDF 223. (International Organization for Standardization (ISO) and International Dairy Federation (IDF), Geneva, 2010).

5 CLSI. *M07-A10: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Tenth Edition*. Vol. 35 (Clinical and Laboratory Standards Institute, 2015).

6 World Health Organization. Critically important antimicrobials for human medicine, 6th revision. Report No. 978-92-4-151552-8, 45-45 (World Health Organization, Geneva, 2019).

7 Chokesajjawatee, N. *et al.* Safety Assessment of a Nham Starter Culture *Lactobacillus plantarum* BCC9546 via Whole-genome Analysis. *Scientific Reports* **10**, 1-12, doi:10.1038/s41598-020-66857-2 (2020).

8 Alcock, B. P. *et al.* CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic acids research* **48**, D517-D525, doi:10.1093/nar/gkz935 (2020).

9 Bortolaia, V. *et al.* ResFinder 4.0 for predictions of phenotypes from genotypes. *J Antimicrob Chemother* **75**, 3491-3500, doi:10.1093/jac/dkaa345 (2020).

10 Joint FAO/WHO Working Group. Guidelines for the Evaluation of Probiotics in Food. (London, Ontario, Canada, 2002).