Feed Unit

# Frequently asked questions to applicants during the assessment of feed additives

## Version 1.0




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# 1 Introduction

## 1.1 Scope of this document

The aim of this document is to make applicants aware of the topics for which questions are frequently issued by EFSA to applicants in relation to section II: Identity, characterisation and conditions of use of the additive in the assessment of applications for the authorisation of feed additives. It provides information on the topics for which questions are more often sent and provides a rational of the need for the data/information and advice on how these questions could be addressed in the technical dossiers when submitting an application. Addressing these points in the dossier will likely reduce the number of questions sent to applicants from EFSA and consequently, will shorten the duration of the risk assessment.

### 1.1.1 Frequency of questions

EFSA has a database with all the questions sent to applicants during the assessment of applications for the authorisation of feed additives under Regulation (EC) No 1831/2003.[1]

In this exercise EFSA screened the topics for which questions were requested to the applicants with a higher frequency for the period 2017-2020.

From a total of 1354 questions, almost two thirds (898) relate to the Identity and characterization of the additive. The questions were further classified depending on subtopics: 'purity' (with 182 questions), 'characterisation of the strain' (171 questions), and 'qualitative and quantitative composition' (111 questions) were the topics in which more questions were sent to applicants. The categories/functional groups with most questions were submitted are: nutritional additives (397 questions), in particular amino acids (289 questions) and zootechnical additives, in particular, 'digestibility enhancers' (147 questions), 'gut flora stabilisers' (110 questions) and 'other zootechnical additives' (83).

From the pool of questions identified above, a total of 78 questions considered of general interest were selected and comments on how to address these points are provided in the paragraphs below considering the provisions of Regulation 429/2008[2] and the Guidance documents.

The document is structured along the main chapters of the guidance on identity and characterisation of the additive and the active substance/agent (EFSA FEEDAP Panel, 2017), the characterisation of the strains of microorganisms used as feed additives or as production organisms, the presence of cells and DNA of the production strain in the additive, and answers to general practical issues.

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

# 2 Identity and characterisation of the additive and the active substance(s)/agent(s)

## 2.1 Purity

The composition (qualitative and quantitative) of the additive is the first information that should be provided to the assessors who evaluate its safety. The composition includes the amount of the active substance(s)/agent(s) and the other components. The sum of these components should aim to a 100% or the maximum technically achievable.

The composition of the additive and the amount of active substance(s)/agent(s) should be described in the specifications of the additive. The limitations that may preclude the description of the full composition should be stated. The amount of active substance(s)/agent(s) in the additive should be confirmed with the analysis of at least five batches of the additive. The batches analysed should be recent batches, preferably produced within the last 5 years before submission of the application.

## 2.2 Impurities

### 2.2.1 Number of batches required

Data on impurities (e.g., heavy metals, arsenic, mycotoxins, dioxins, and dioxin-like PCBs) including microbial and biological contamination, where applicable, should be provided on at least three batches of the additive. Indication on the impurities to be analysed is provided in the guidance on identity (EFSA FEEDAP Panel, 2017). The impurities to be measured depend basically on the nature of the additive. Ideally all the analytes should be analysed in the same batches, and the corresponding certificates of analyses should be provided. The analysis should be performed in recent batches, preferably produced within the last 5 years before submission of the application.

The nature of the batch should also be considered. In cases in which the final manufacturing is not yet defined, pilot batches will be accepted. It is recommended that the batches used for the analysis of impurities correspond to the batches used for the batch to batch variation of the active substance(s)/agent(s).

### 2.2.2 Content of dioxins and the sum of dioxins and dioxin-like PCBs.

Directive 2002/32/EC[3] on undesirable substances in animal feed indicates, among other, the maximum concentration of dioxins and the sum of dioxins and dioxin-like PCBs allowed in feed, including feed materials and some feed additives (e.g. some technological additives, compounds of trace elements). It is also important that the Units are correct. The method used for the analysis of these impurities should be fit for purpose, considering the legal thresholds.

### 2.2.3 Other impurities: unavoidable impurities from the manufacturing process.

In certain cases the presence in the additive of impurities from the manufacturing process is unavoidable, and therefore should be addressed. Examples are residual solvents, e.g. toluene, hexane. Since the content of these substances may impact in the safety of the additive, analytical data should be submitted. The FEEDAP Panel will evaluate the content of these excipients/residual solvents in the additive against relevant reference values, e.g. those reported in the VICH Guidelines (VICH GL18(R) (impurities solvents).

[3] Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

### 2.2.4 Microbial impurities

The microbial impurities of the additive include, in general, spoilage microorganisms, bacteria, yeasts and filamentous fungi. For products containing or produced by bacilli, levels of *Bacillus cereus* should be reported.

The specifications or maximum allowance of the various microbial impurities set by the applicant should be also reported.

Certificates of analysis should be provided, therefore, statements (e.g. "the final products are proven not to contain microorganisms") are not valid.

For some microbial contaminants the amount of the additive in which the test was carried out should be indicated (i.e. 25 g for *Salmonella*).

### 2.2.5 Other impurities: intrinsic impurities from the production strains.

#### *2.2.5.1 Endotoxins.*

For the additives consisting on or produced by Gram-negative bacteria, levels of lipopolysaccharides (LPS) should be analysed in the final product (three batches) (see Section 2.1.4 Guidance on the identity, EFSA FEEDAP Panel, 2017)).

The results of the tests should be provided with the original certificate of the laboratory that performed the analysis. The certificates should include the description of the method used. It is recommended to use the methods of the European Pharmacopoeia (2019; 2.6.14).

#### *2.2.5.2 Potentially toxic metabolites*

In case of additives produced by microorganisms, the capacity of the production strains to produce toxic secondary metabolites, in particular, when grown on solid substrates, should be addressed. Literature data are available to understand which should be the analytes to track. For example, for *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei*, information on secondary metabolites of toxicological relevance as listed in Blumenthal (2004) and in the AINIA (2017) should be provided. Any statement should be supported with data (e.g., studying the presence of the metabolite/s in the additive).

Analytical evidence should be provided either on the strain itself or in the final product.

## 2.3 Certificates of analysis

The certificates of analysis of the identity and characterisation of the additive and the active substance/agent should be complete, thus including at least the date of the analysis, the name and address of the laboratory, the batch/lot/test item analysed, the analytical technique/s (and method used), the result/s and the signature of the responsible person of the analysis. Statements of adequacy from the laboratory are generally not adequate.

Whenever the interpretation of the given result needs the limit of detection (LOD) or limit of quantification (LOQ) of the analytical method, those values should be always reported.

Certificates of analysis should include the actual analysed values. Certificates indicating only compliance with specifications or legal limits are not acceptable.

## 2.4 Manufacturing Process

A detailed description of the production process (e.g. chemical synthesis, fermentation, cultivation, extraction from organic material or distillation and downstream purification steps) used in the production of the active substance(s)/agent(s) of the additive should be submitted. For additives produced by fermentation, the quantitative composition of

the fermentation/cultivation media should be provided. The use of any antimicrobial substances during the production process should be declared and analysed in the final product.

The manufacturing process should be described in detail including all the steps of the process and be complemented by a detailed flowchart. The link of the manufacturing process with the HACCP system of the manufacturing company should be also addressed. This includes quality control of incoming materials and any steps in which an important contaminant/impurity may occur; a description of the control points and any preventive-corrective measures should be also indicated.

## 2.5 Physical properties of the additive

### 2.5.1 Specific optical rotation of the additive.

The specific rotation of a molecule is defined as the change in orientation of monochromatic plane-polarized light, per unit distance–concentration product, as the light passes through a sample of a compound in solution. This property is necessary in the case of additives which active substance of which is a chiral chemical compound. Thus, information on the specific rotation allows to identify the active compound of the additive with certainty. It is important to highlight that different optical rotations of a molecule identify different chemical characteristics related to the metabolism and toxicity of the compound (paragraph 2.2.2.1 of the Guidance on identity, EFSA FEEDAP Panel, 2017).

The acceptable range of rotation depends on the substance and it is established in the corresponding monograph of the European Pharmacopoeia[4] or other internationally recognised methods (methods described in the United States Pharmacopeia). Its determination is only possible in pure substances. The applicant should provide the corresponding monograph containing the acceptable range for the given active substance. If the substance is not described in any monograph, the applicant should: i) provide information on the reliable acceptable range for that substance; and ii) describe the method used.

# 3 Characterisation of microorganisms used as feed additives or as production organisms

## 3.1 Identity and taxonomical classification

### 3.1.1 Certificate of deposition

Microbial strains 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) and should be maintained by the culture collection for the authorised period of the additive (paragraph 2.1 of the Guidance on identity, EFSA FEEDAP Panel, 2017). Therefore, applicants should provide the certificate of deposition with clear information on the duration of the deposit (i.e., the date until it is valid). In case the certificate has expired, a new valid certificate of deposition should be submitted.

### 3.1.2 Taxonomic identification

The name and taxonomic classification of the organism under assessment should be provided according to the latest published information in the International Codes of Nomenclature (ICN). The taxonomic identification of the organism under assessment should be confirmed using updated data and using up-to-date molecular identification techniques.

[4] European Pharmacopoeia, 10th Edition, 2019. European Directorate for the Quality of Medicines and Health

Molecular methods represent in fact the most complete source of information for the unambiguous taxonomic identification of strains at species level and are routinely used in many laboratories. The whole genome sequence (WGS) should be used for identification purposes for bacteria and yeasts. The same approach is recommended for filamentous fungi. The methodologies followed and the results obtained, should be reported. The Guidance on the characterisation of microorganisms (FEEDAP Panel, 2018) provides different methods that can be used for microorganisms identification, including computational taxonomic assignments (e.g. average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH)), comparison of sequences commonly used for taxonomic identification to relevant databases (e.g. 18S rRNA gene and/or ITS (internal transcribed spacer) regions or other housekeeping genes) or phylogenomic methods. The databases used for comparison should be up to date and the sequences used should include those of type strains for the expected species or well-and widely-recognised related strains.

## 3.2 Antimicrobial susceptibility for bacterial species

The assessment of the antimicrobial susceptibility in bacteria, used as viable cells in feed additives or as production strains, is based on the results from phenotypic and genotypic tests. The antimicrobial susceptibility profile of the strain under assessment should be investigated using internationally recognised and standardised methods. Qualitative or semi-quantitative methods (e.g. diffusion test) should be avoided. The report of the analysis should be provided including the culture conditions (e.g. medium, incubation time), the antimicrobials tested and the resulting minimum inhibitory concentration (MIC) values. The aim of the analysis is to distinguish resistant from susceptible strains by comparing the MIC values obtained to the cut-off values established in Table 2 of the FEEDAP Guidance on the characterisation of microorganisms (EFSA FEEDAP Panel, 2018). A statement that the strain is resistant or susceptible to a specific antimicrobial is not considered sufficient and experimental data supporting it should be provided in the technical dossier.

If the strain under assessment belongs to a species for which cut-off values have not been set in the FEEDAP Guidance on the characterisation of microorganisms, applicants can choose to compare the MIC values of the strain with data from literature or to generate their own data to be used for comparison. This can be done by analysing a large number of independent and geographically well distributed isolates for that species.

Regarding the genotypic test, the WGS of the strain should be interrogated for the presence of antimicrobial resistance (AMR) genes. The search should be done using updated and publicly available databases and without targeting a specific gene or resistance.

## 3.3 History of modifications

### 3.3.1 Non-genetically modified strains

The origin of the organism under assessment and its history of modifications, should be reported. Statements saying that the strain is "not genetically modified" should be supported with indications on the origin of the strain, and the description of any technique used and steps followed to select/obtain the strain.

### 3.3.2 Genetically modified strains

The genetic modification should be characterised following the provisions of paragraph 2.5. of the guidance on the characterisation of microorganisms (EFSA FEEDAP Panel, 2018). This can be done either by describing all the steps performed to obtain the genetic modification or by using the WGS of the strain under assessment. The latter is compulsory for bacteria and yeasts and recommended for filamentous fungi. The information provided should allow to characterise all genomic regions harbouring genetic modifications and to evaluate whether the introduced modifications raise a safety concern for the final product. Details on

the genetic elements introduced, deleted and/or modified (including regulatory elements, e.g. promoters and terminators) should be provided.

#### *3.3.2.1 Structure with WGS (mandatory for bacteria and yeast)*

The structure of the genetic modification can be described by comparing the WGS of the genetically modified (GM) strain under assessment with that of the non-modified parental or recipient strain. The methodology and the reference sequence used for comparison should be described in detail to allow the risk assessor to evaluate the appropriateness of the strategy followed. The resulting structure should be presented as a map or graphic presentation of all genomic regions harbouring genetic modifications. The use of the WGS waives the need to describe step-by-step the genetic modification process performed since it provides an accurate characterisation of the resulting modifications present in the GM strain, including the potential or intentional introduction of genes of concern.

#### *3.3.2.2 Structure with WGS (for filamentous fungi)*

The description of the genetic modification process performed should allow the identification of all genetic material introduced and/or modified into the recipient/parental strain. Therefore, details on the genealogy, methods, selection steps (if any) and vectors used for the development of the genetically modified micro-organism (GMM) should be provided. Applicants should clearly indicate the parental strain and its origin as well as the relationship between any strain obtained during the development of the strain under assessment.

Any gene of potential concern (e.g. AMR, toxin and virulence factors coding gene) inserted and/or transiently used to develop the GM production strain should be indicated and, if not intended to be present in the final production strain, its absence should be experimentally demonstrated. If the absence of the gene is analysed by polymerase chain reaction (PCR), the primers should be designed to specifically target the gene of concern. Otherwise, a Southern blot analysis could be performed and indications on the probe used and its length should be included. The protocol followed (including controls used) and the results should be provided.

## 4 Presence of viable cells of the production strain in the additive

For fermentation products, absence of viable cells of the production organism in the additive is a legal requirement of implementing Regulation (EC) No 429/2008. The FEEDAP guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018) describes in section 3.1 the methodology to be followed, the amount and number of samples to be tested and the controls to be included. For products for which more than one production strain is involved, absence of viable cells of each of them should be demonstrated.

### 4.1 Sampling

The test item used and the amount tested are of utmost importance to ensure representativity of the additive and good sensitivity of the analysis. In case the additive has several formulations, the analysis should be performed either in all final formulations, or in a common intermediate which could be representative of all the final formulations. The suitability of the test item used will be evaluated considering its concentration (whether it is equally or more concentrated than the final product(s)) and the manufacturing process. At least 1 gram/mL of the product needs to be tested. This must be obtained from at least 9 samples (of at least 10 gram or mL) from a minimum of three independent recent batches of product. Any dilution made should be clearly stated and the amount of product plated should correspond to at least 1 gram/mL of starting material. Small samples would reduce detection sensitivity leading to uncertainties on the presence of viable

cells of the production strain in the final product. The corresponding certificates of analysis of the batches tested should be provided.

## 4.2 Methodology

The presence of viable cells of the production strain should be investigated using a culture-based method targeted to the detection of the production strain. The culture conditions including medium used, time and temperature of incubation should be reported. Appropriate controls should be included in the analysis to prove that the methodology enables the growth of any possible viable cell remaining in the product. The protocol followed should avoid overgrowth of contaminants. In case colonies are detected in the samples tested, all the colonies should be properly identified using molecular methods (e.g. PCR, sequencing). Exclusion of colonies based on morphology may be considered not conclusive. The analysis should also exclude the presence of stressed cells of the production strain in the final product. This should be done by including a resuscitation step in the culturing method. Resuscitation should be done in cultivation media with a minimal selective pressure and/or by providing a longer incubation time compared to the normal culturing of the viable organism. If the strain under assessment can form spores, the analysis should allow the risk assessor also to exclude the presence of the organism under assessment in the sporulated form. The results of the analysis (e.g. pictures of the plates used) should be provided.

# 5 Presence of DNA of the production strain in the additive

The FEEDAP guidance on the characterisation of microorganisms used as feed additives or as production organisms describes in section 3.2 the methodology to be followed, the amount and number of samples to be tested and the controls to be included. The presence of DNA should be investigated when the production strain of the product under assessment is GM or is not GM but carries acquired AMR genes. For products for which more than one production strain is involved, absence of DNA of each of them (when applicable) should be demonstrated.

## 5.1 Sampling

In case the additive has several formulations, the analysis should be performed either in all final formulations, or a common intermediate which could be representative of all the final formulations. The suitability of the test item used will be evaluated considering its concentration (whether it is equally or more concentrated than the final product(s)) and the manufacturing process. At least 1 gram/mL of the product needs to be tested. This must be obtained from at least 9 samples from a minimum of three independent recent batches of product. The corresponding certificates of analysis of the batches tested should be provided.

## 5.2 Methodology

The analysis should be carried out to exclude the presence of total DNA of the production strain in the final product, including genomic DNA and extra-chromosomal DNA (if any). The method used to extract the DNA should be described to allow the risk assessor to evaluate its suitability for the strain under assessment. The use of an extraction procedure that includes a lysis step is of utmost importance to ensure the recovery of DNA from non-viable cells potentially remaining in the product. The sequence targeted in the analysis and the expected amplicon size should be reported. The rationale for the choice of the sequence targeted should be explained. In particular, the amplicon size of the sequence targeted should not be more than 1kb, but if the strain harbours AMR genes, a fragment not exceeding the size of the smallest AMR genes should be targeted. This is

needed to exclude the presence of DNA fragments long enough to cover the complete AMR gene which, if present, would represent a risk for the target species, the environment and those exposed to the additive.

### 5.3 Controls and sensitivity tests

Appropriate controls and sensitivity tests should be included in the analysis to prove that the methodology is sufficiently reliable to detect possible DNA of the production strain remaining in the product. A positive control (using total DNA of the production strain) and a negative control should be included in the analysis. Moreover, total DNA of the production strain should be added to the DNA extracted from each samples of the product tested to check any factors that can cause PCR failure. If this is the case, the causes should be investigated and samples taken before formulation may be used. The Limit of Detection (LOD) of the method should be determined. This should be done by adding total DNA of the production strain (spiking) at different concentrations to the product samples prior to any step of DNA extraction. The methodology used to extract DNA from samples and to calculate the LOD should be the same and should include the use of a lytic agent. The method should allow to detect at least 10 ng of DNA per g or mL of product potentially remaining in the product. Results of the analysis (e.g. gel images) should be provided.

## 6 General

### 6.1 Language of the supporting documents

Scientific and technical documentation should be submitted in English to facilitate the evaluation of the applications. EFSA may ask the applicant to translate the parts of the dossier that would not be submitted in English. For further information please consult the administrative guidance to applicants on the preparation and presentation of applications for authorisation of additives for use in animal nutrition (European Food Safety Authority, 2007).

Administrative Guidance document:
Administrative guidance on the preparation and presentation of applications for authorisation of additives for use in animal nutrition

Scientific Guidance documents:
Guidance on the identity, characterisation and conditions of use of feed additives
Guidance on the characterisation of microorganisms used as feed additives or as production organisms

**USEFUL LINKS/ADDRESSES**
Ask a Question–: https://connect.efsa.europa.eu/RM/s/
Email FEED Unit: FEEDAP@efsa.europa.eu

# 7 References


AINIA Technology Centre, de Benito A, Ibáñez C, Moncho W, Martínez D, Vettorazzi A and López de Cerain A, 2017. Database on the taxonomical characterisation and potential toxigenic capacities of microorganisms used for the industrial production of food enzymes and feed additives, which do not have a recommendation for Qualified Presumption of Safety. EFSA supporting publication 2017:EN-1274. 185 pp. doi:10.2903/sp.efsa.2017.EN-1274.

Blumenthal CZ, 2004. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: Justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. Regulatory Toxicology and Pharmacology. 39: 214-228.

European Food Safety Authority, 2007. Administrative guidance to applicants on the preparation and presentation of applications for authorisation of additives for use in animal nutrition, EFSA Journal 2007; 5(5):1447, 63 pp. https://doi:10.2903/j.efsa.2007.1447, updated on 22 March 2019.

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Anguita M, Galobart J and Innocenti ML, 2017. Guidance on the identity, characterisation and conditions of use of feed additives. EFSA Journal 2017;15(10):5023, 12 pp. https://doi.org/10.2903/j.efsa.2017.5023

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, López-Alonso M, López Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, Kärenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206 .

European Pharmacopoeia, 10th Edition, 2019. European Directorate for the Quality of Medicines and Health, Monograph 01:2018/20614

# 8 List of acronyms and abbreviations

| | |
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| AMR | antimicrobial resistance |
| ANI | average nucleotide identity |
| dDDh | digital DNA-DNA hybridization |
| DNA | Deoxyribonucleic acid |
| EC | European Commission |
| EFSA | European Food Safety Authority |
| FEEDAP | Scientific Panel on Additives and Products or Substances used in Animal Feed |
| GM | Genetically modified |
| GMM | Genetically Modified Micro-organisms |
| HACCP | Hazard analysis and critical control points |
| ICN | International Codes of Nomenclature |
| ITS | Internal transcribed spacer |
| MIC | Minimum inhibitory concentration |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| PCB | Polychlorinated biphenyl |
| PCR | Polymerase chain reaction |
| rRNA | ribosomal ribonucleic acid |
| VICH | International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products |
| WGS | Whole genome sequence |