SCIENTIFIC OPINION

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Guidance on the assessment of the safety of feed additives for the target species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guido Rychen, Gabriele Aquilina, Giovanna Azimonti, Vasileios Bampidis, Maria de Lourdes Bastos, Georges Bories, Andrew Chesson, Pier Sandro Cocconcelli, Gerhard Flachowsky, Jürgen Gropp, Boris Kolar, Maryline Kouba, Marta López Alonso, Secundino López Puente, Baltasar Mayo, Fernando Ramos, Maria Saarela, Roberto Edoardo Villa, Robert John Wallace, Pieter Wester, Montserrat Anguita, Jaume Galobart, Matteo L. Innocenti and Laura Martino

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Abstract

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Correspondence: feedap@efsa.europa.eu

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

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Background and Terms of reference

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

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

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

1. identify from the current guidance documents, those that need to be updated, taking into consideration the most recent scientific developments and the experience gained in the assessment of feed additives;
2. update the guidance documents in need of revision accordingly; this activity can be conducted in different rounds of activities on the basis of the priorities identified and on the feasibility of the revision according the resources available;
3. 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 and third terms of reference with regards to the update of the guidance documents dealing with the assessment of the safety of feed additives for the target species.

Scope of the guidance

This guidance document is part of a series of documents intended to assist the applicant in the preparation and the presentation of its application for authorisation of a feed additive, as foreseen in Article 7.6 of Regulation (EC) No 1831/2003. This document does not substitute for the obligation of an applicant to comply with the requirements of Regulation (EC) No 1831/2003 and its implementing rules.

Applicants should justify the omission from the dossier of any data or any deviations from the requirements detailed in this guidance.

1. Introduction

Studies involving animals should respect the rules on animal welfare laid down by European Union legislation, particularly those listed in Directive 63/2010/EU, and they should not be repeated if available elsewhere. The use of methods refining or replacing the tests using experimental animals or reducing the number of animals used in these tests shall be encouraged. Such methods must provide the same level of assurance as the methods they aim to replace.

For certain additives, safety for the target animals can be presumed without the need for specific information. For all other additives, safety for the target animals can be assessed as a first step by extensive literature searches for studies on target animals. If safety cannot be established by literature search, the applicant can use toxicity data (either existing or new) from repeated dose studies in laboratory animals or conduct tolerance studies in target animals.

2. Additives for which safety can be presumed without additional studies

For the following additives, safety for the target animals can be presumed without the need for additional studies:

- additives for which no significant amounts of the active substance(s) (or related substances) or the active agent(s) remain in the feed at time of feeding.
- silage additives where it can be demonstrated that the active substance(s) and agent(s) occur as normal constituents of silage and use of the additive does not substantially increase their concentration compared to silage prepared without use of the additive (i.e. where there is no substantial change in exposure).
- microorganisms satisfying the requirements of the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2017).
- nutritional additives assessed and authorised following the provisions of Regulation (EC) No 1831/2003.
- nutritional additives not already authorised:
    - when the active substance is sufficiently purified. A product will be considered as sufficiently purified if the unidentified fraction would not contribute to more than 1 % when the inclusion rate does not exceed 1,000 mg additive/kg complete feed. Higher inclusion rates would need a higher degree of purity (e.g., 10,000 mg additive/kg feed would correspond to 99.9%).
    - when the additive is produced by fermentation using a production organism that (i) satisfies the requirements of the QPS approach to safety assessment, or (ii) is a genetically modified microorganism (GMM) for which the recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment and for which the molecular/genetic characterisation does not give rise to concern.

3. Extensive literature search for studies with target animals

Extensive literature searches should be used as a first step to provide information on the safety of the feed additive under the proposed conditions of use. Relevant information sources should be searched in a structured manner. The applicant should make reasonable efforts to locate all sources of relevant information and provide reasons for the selection of such sources. Bibliographic databases (including at least agricultural/aquacultural and medical/veterinary databases) which record documents such as journals, reports, conference proceedings and books should be searched. In addition the search should consider sources other than bibliographic databases, such as reference lists of full-text journal articles (e.g. reviews), websites of conferences or organisations.

Applicants should follow the recommendations of the "Technical manual for performing electronic literature searches in food and feed safety" when performing the searches and documenting its outcome. Moreover, applicants are encouraged to refer to Appendix D of the "Tools for critically appraising different study designs, systematic review and literature searches" for assessing the quality of the search.

The search methodology must be documented and reported in detail to ensure transparency and enable the evaluation and replication of the strategy. The following must be reported:

For database searches:

- The name of the database and the service provider used;
- The date of the search, and the date range searched;
- Any limits placed on the search such as language or publication status;
- The full search strategy (all terms and set combinations) and the number of records retrieved.

For sources other than bibliographic databases:

1. Websites and journal table of contents
   - The name of the resource (i.e. website name, the journal name in case of searching in specific tables of contents);
   - The URL (internet address);
   - The date on which the search was conducted and the date range of the search, or the dates, volumes and issues in the case of table of contents;
   - The method of searching e.g. browsing, using the search engine or scanning tables;
   - Any limits applied to the search (e.g. publication types);
   - The search terms used and the number of relevant summary records or full-text documents retrieved.
2. References lists
   - The bibliographic details of the documents whose reference lists were scanned;
   - The number of relevant bibliographic references retrieved.

The extensive literature search should cover at least the last 20 years. The list of relevant references included should be compiled in a reference management software and provided in .RIS format. Copies of the relevant papers should be provided. The applicant must ensure that terms and conditions asserted by any copyright holder of publications or information submitted to EFSA are fully satisfied. The applicant should consult with copyright licensing authorities (i.e. at national level) for guidance on purchasing copyright licenses to reproduce any publications provided to EFSA. The applicant remains solely responsible and liable for obtaining all necessary authorisations and rights to use, reproduce and share the publications provided to EFSA.

The analysis of these data must establish that the active substance/agent in literature studies is identical to that under application or, if not, would still allow conclusions on the additive under application to be made; for additives produced by fermentation identity includes the production strain. The concentration of the active substance/agent in feed should preferably exceed or at least cover that proposed in the application. The target species covered in the literature search should be relevant to the application. Application level, replicates, duration and zootechnical and clinical end-points measured should allow a conclusion on the absence of adverse effects. This may be achieved by the consideration of data from a number of independent studies. The literature search should also cover all available toxicological end-points including genotoxicity.

If safety for one species/category is derived from literature studies and extrapolation to other species/categories is required, the same principles as described under Section 5.7 should be followed.

4. Toxicity data from repeated dose studies in laboratory animals

For all additives with the exception of microorganisms, safety for target animals can be derived from toxicological studies with oral administration in laboratory animals. These data should allow establishing a lowest no observed adverse effect level (NOAEL) or a Benchmark dose level (e.g., $BMDL_{10}$). Ideally, subchronic or chronic toxicity studies should follow either the latest OECD protocols or those in force at the time the study was made.

To derive a safe daily dose in the target species (mg/kg body weight (BW)), the NOAEL or $BMDL_{10}$, expressed in mg/kg BW, is divided by an uncertainty factor of 100. The maximum safe concentration in feed (M; mg/kg complete feed, as is basis) is obtained by dividing this safe daily dose by the default feed intake (FI; expressed as a g dry matter (DM) per kg BW, Table 1). The resulting value (mg additive/g DM feed) is multiplied by 1,000 to express the feed concentration per kg complete feed and multiplied by 0.88 (or 0.945 for milk replacer for veal calves) to transform it to as is basis (assuming 88% DM for complete feed and 94.5% for milk replacers).

Maximum safe concentration in feed = ((NOAEL/100)/FI) × 1,000 × 0.88

Table 1: Default values for daily feed intake scaled to body weight (g dry matter (DM)/kg body weight) for the main animal species/categories

Animal category	Default values daily feed intake (g DM/kg body weight)	Values derived from	
		Body weight (kg)	Feed intake (kg DM/day)
Chicken for fattening	79	2	0.158
Laying hen	53	2	0.106
Turkey for fattening	59	3	0.176
Piglet	44	20	0.88
Pig for fattening	37	60	2.20
Sow lactating	30	175	5.28
Veal calf (milk replacer)	19	100	1.89
Cattle for fattening	20	400	8.0
Dairy cow	31	650	20.0
Salmon	18	0.12	0.0021
Dog	17	15	0.250
Cat	20	3	0.060
Ornamental fish	5	0.012	0.000054

The default values of feed intake in Table 1 are derived from estimated values of body weight and derived feed intake of the animals at the end of a tolerance study.

If specific toxicological data are not available, the thresholds of toxicological concern (TTC)[1] could be applied to flavouring additives only for which a Cramer structural class can be assigned. Assignation to a Cramer class is made using the Organisation for Economic Cooperation and Development (OECD) toolbox[2] or other commercial software. The "maximum acceptable feed concentrations" are derived from the thresholds of the TTC approach and based on the default values of feed intake shown in Table 1. Substances in Cramer class I would result in a maximum acceptable concentration in complete feed (mg/kg feed) between 0.3 and 1.5, for Cramer II between 0.1 and 0.5 and for Cramer III between 0.02 and 0.08.

5. Tolerance studies in target animals

If safety for the target species cannot be established at the maximum proposed dose by the methods described above, then *in vivo* studies in the relevant target species/categories are required. The number of tolerance studies required in different animal species/categories is described in Section 5.7.

The aim of the tolerance study is to provide a limited evaluation of short-term toxicity and a margin of safety[3] of the additive to the target animals. It is recommended to combine the tolerance study with one of the efficacy trials, whenever possible.

Studies should be performed and documented according to appropriate quality standards and should respect the rules on animal welfare laid down by European Union legislation, particularly those listed in Directive 63/2010/EU. Trials should be compliant with the criteria established by a recognised, externally-audited, quality assurance scheme (e.g., good laboratory practice (GLP) in accordance with Directive 2004/10/EC). Evidence should be provided that the work was done by qualified personnel

[1] JECFA (FAO/WHO, 1996, Food additive series 35, IPCS, WHO Geneva); Barlow, S. 2005. Threshold of toxicological Concern (TTC). A tool for assessing substances of unknown toxicity present at low levels in the diet. ILSI Europe Concise Monograph Series.
Review of the Threshold of Toxicological Concern (TTC) approach and development of new TTC decision tree. EFSA supporting publication 2016: EN-1006. 50 pp. http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2016.EN-1006/pdf

[2] http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm

[3] Margin of safety: ratio of the highest tolerated additive concentration in feed and the highest use level proposed.

using appropriate facilities and equipment and responsible to a named study director. Studies conducted outside the European Union must follow the same quality standards.

5.1. Test item

Tolerance studies should be based on the additive(s) for which application is made, except in cases where a concentrated form of the additive(s) is recommended for testing (e.g., enzymes and microorganisms). Any other deviations because of practical or other considerations should be justified. A certificate of analysis of the test item used in the study should be provided. The additive could be administered via feed or water for drinking depending on the conditions of use. The concentration of the active substance(s) or agent(s) in the feedingstuffs/water should be confirmed by analysis.

5.2. Experimental groups

The design of a tolerance test includes a minimum of three groups:

- a control group
  The diet/water of the control group should normally not contain the additive tested. However, in case of additives for which a nutritional requirement exists, the control group should receive the additive at the lowest requirement level.
- a use-level group
  The diet/water of the use level group should normally contain the additive at the highest recommended dose. For those additives for which a maximum dose is not recommended, the highest typical use level should be used.
- an overdose group with a multi-fold of the use-level.

When the multi-fold dose tested is:

- ≥ 100, test animals shall be routinely monitored for visual evidence of clinical signs, performance characteristics and product quality where relevant. In this case a higher concentration of the active substance or agent can be obtained by omitting or reducing the amount of carrier. For fermentation products, the ratio of active agent(s)/substance(s) to the other fermentation products must remain the same as in the additive.
- > 10 to <100: in addition to the above, haematology and blood chemistry as described in section 5.5 and other parameters likely to be related to the biological properties of the additive.
- ≤ 10: in addition to the above, gross pathology and histopathology if relevant, as described in section 5.5. The study should be designed in such a way that a margin of safety for the additive can be estimated. It is recognised that in some cases ethical and practical considerations will prevent performing necropsy in all animals (e.g., pets, dairy cows, sows, horses).

The setting conditions (e.g. temperature, light exposure) should be the same for the various groups including housing, husbandry and diet/water administration.

5.3. Animals

Animals used should be healthy and preferably from a homogeneous group. Housing and husbandry conditions should be adequate for the purpose of the study and conform to animal welfare regulations. Preventive treatments with antibiotics/antimicrobials before the start of the trial should be avoided. The acceptability of trials in which animals are treated with antibiotics/antimicrobials during the course of the study will depend on a variety of factors, including the number of animals treated, duration of the treatment, distribution between experimental groups and severity of the disease. The acceptability of these studies will be assessed on a case by case basis. Any therapeutic/preventive treatments should not interact with the proposed mode of action of the additive and should be recorded individually. Studies with an abnormally high mortality in the control group will not be accepted. This would be judged against European industry standards.

For food-producing animals, the conditions of the study should be such that optimal performance as described for the breed (e.g., performance standards of broiler breeder companies) could be reached. The higher the zootechnical performance of the animals in a given physiological stage, the more sensitive the end-point(s) would be to adverse situations. Therefore, it is recommended to use in studies with:

- chickens for fattening: only male birds
- laying hens: birds in the first third of the laying period
- dairy cows: high yielding animals in the first third of the lactation period
- growing pigs: weaned piglets of both sexes
- cattle: weaned male bovines at the beginning of the fattening period
- salmonids: juvenile phase

The recommended age/weight for the different species/categories at the start of the study is detailed in Section 5.6.

5.4. Statistical considerations

5.4.1. Design of the experiment

The experimental unit is the smallest entity to which a given treatment is applied. If animals are penned in groups and all the animals in the pen share the same feed source (and feed intake is not measured individually), then the experimental unit for all parameters is the pen, not the individual animal. For all endpoints which are measured on individual animals in a pen, a summary parameter of the endpoint in the experimental unit should be used (e.g. mean for continuous measurements such as body weight, median and counts for quantal measurements such as severity of an outcome or mortality). Summary parameters should always be adjusted for losses (mortality/culling). The distribution of losses within the treatment groups should be assessed to avoid the risk of introducing a bias.

Experimental units allocated to the various experimental groups should not differ in a systematic way. Therefore a recognised method of randomisation should be used to allocate treatments to the experimental unit (e.g. pen, animal). A randomised block design should be preferably used to control for experimental settings like location within facilities. The same design is also recommended in case of large experiments to ensure concurrency in measurements/determination of endpoints across treatments. Other designs might be also appropriate, in which case the applicant should justify the rationale for the design chosen.

In case of a significant variability across animals of factors which could influence the outcome of the study, animals should be stratified before being randomly allocated to pens/cages/treatments. These factors might include initial body weight, age, stage of lactation, milk yield, parity, egg production.

A proper method for randomization should be used in order to allow allocation concealment (no a priori knowledge of group assignment). In practice the randomization process must ensure that investigator cannot influence the allocation of units to the various groups. It is recommended to implement blinding of the care givers and investigators, where possible, for instance using a proper codification of the treatment to be administered.

5.4.2. Sample size

Statistical considerations should be used to determine the size of the sample used to evaluate the potential safety concerns. The setting of the null and alternative hypotheses should be done in light of the problem formulation. Experiments aiming at demonstrating similarity between control and treated groups should test for equivalence or non-inferiority (i.e. alternative hypothesis stating no or minimal difference exists). Difference testing should be used when the purpose is to confirm superiority or inferiority (i.e. alternative hypothesis stating a difference exists). Additional considerations need to include: i) the magnitude of the effect that the study is designed to test and its variability; ii) the expected direction of the effect; iii) an adequate statistical power and iv) the confidence level. The magnitude of the effect considered biologically relevant (for difference testing) or the similarity range (for equivalence testing) should be clearly indicated for each endpoint and the rationale for the choice

explained. For difference testing, when the direction of the effect is predictable, a one sided test should be used. A two-sided test is recommended in all other cases.

The Type 2 (β) error measures the risk of non-detecting an effect (difference)/similarity (equivalence) when it exists. As a guide it should be lower than or equal to 20% in general, and 25% for experiments with ruminants, minor species, pets and non food-producing animals. Hence a power (1-β) greater than or equal to 80% (75% for ruminants, minor species, pets and non food-producing animals) should always be ensured. Generally, a confidence level of 95% is adopted when testing difference, 90% for testing equivalence. Use of levels below these thresholds should be justified.

5.4.3. Statistical analysis

The statistical analysis should be performed using models that allow comparing treated and control groups whilst controlling for factors that could influence the outcome of the experiment whenever possible. The class of generalised linear mixed models (McCullagh and Nelder, 1989), known as GLMM, offers a suite of methods flexible enough to fit most of the experimental settings. Typically this type of models includes the treatment and other stratification variables (e.g. age) as fixed factor and blocking factors, if any, as random (e.g. animal/pen location) or as covariates. The response variable is the endpoint under investigation. Under certain conditions a log or other transformations can be needed in order to linearize the relationship with the explanatory factors. Depending on the type of response variable (i.e. continuous, quantal, dichotomic), different kinds of statistical tests and distributional assumptions could be required. The applicant is requested to assess which one is more appropriate and to provide the rationale of the choice. An indicator of quality of fit should always be provided.

The analysis of variance is one of the simplest models included in the GLMM class. When using this method, a test for group differences should be carried out preferably using the Scheffé, Dunnet, Tukey (Sachs and Hedderich, 2006) or other comparable tests any time multiple comparisons are performed concurrently. Non-parametric tests may be necessary if only a low number of observations is available, but applicants are encouraged to use sufficient replicates to allow for parametric tests to be performed. When different additives are assessed concurrently using the same control, the statistical evaluation should be done considering only the control and the groups treated with the additive under assessment.

5.5. End-points

The end-points to be measured depend on the design of the tolerance study (see Section 5.2). The minimum required parameters for the different groups of end-points are listed below and may be augmented on a case by case basis.

Performance parameters and related parameters

Feed intake, initial and final body weight, body weight gain, feed to gain ratio, water intake for those additives administered via water. Clinical observations including general health status, behaviour, morbidity and mortality.

In addition,

- for laying hens, laying rate, egg weight, shell quality, feed to egg mass ratio, egg mass/hen per day.
- for breeding hens, additionally to those required for laying hens, fertility, hatchability and chick viability.
- for dairy animals, milk production (also fat corrected milk), milk composition (total solids, protein, fat and lactose), somatic cell counts, protein, fat and lactose yield.
- for sows, number of piglets born, piglets born alive, litter weight at birth and at weaning, number of piglets weaned, weaning to oestrus interval.
- for fish, specific growth rate (preferably thermal growth coefficient)

Haematology and clinical chemistry

Generally, samples for haematology and blood chemistry analysis should be taken at the end of the study. Samples should be taken at the start of the trial to establish a baseline in studies involving cattle for fattening, dairy cows, sows, horses, dogs and cats. Samples should be taken from all experimental units, and ideally from all animals. However, when total numbers of animals makes it impractical, subsets of animals/pen should be identified for sampling by a random process carried out at the beginning of the study. Blood samples should not be pooled.

The minimum parameters to be measured are:

Total count for erythrocytes, packed cell volume, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, total and differential counts for leukocytes, platelet counts, prothrombin time and fibrinogen (with the exception of the latter two parameters for fish).

Sodium, potassium, chloride, calcium, phosphate, magnesium, total protein, albumin, globulin, glucose, urea/uric acid (non-protein nitrogen for fish), cholesterol, creatinine, bilirubin, acute phase proteins, amylase, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, gamma-glutamyltransferase, alkaline phosphatase and creatine kinase.

Tissues/organs from necropsy

The following organs and tissues from all dose groups should be examined grossly (including weight of the organs) and preserved for microscopic evaluation: liver, kidneys, spleen, adrenal gland, lung, stomach, pancreas, small intestine, colon, caecum, thymus, thyroid gland, heart, intestinal lymph nodes, ovaries/testes. For fish the following organs should be investigated, kidney, liver, spleen, stomach (where present) and intestinal tract, heart, gonads, gills, bone and eye. Histopathology is normally required only when indicated by findings in the gross pathology.

In all cases, critical end-points known from the toxicological studies in laboratory animals shall be considered. Any adverse effect detected during efficacy trials shall also be reported in this section. All deaths should be explained and, if necessary, investigated by gross pathology and histopathology.

5.6. Duration of the tolerance study

The necessary minimum duration of tolerance trials depends on the animal species/category and is reported below.

Category	Definition of the animal category	Start, from	Duration
Piglets	Young animals having completed the suckling period	Weaning	42 days 35 days if growth rate is ≥ 0.5 kg/day
Pigs for fattening	Animals intended for meat production until day of transport to slaughterhouse	20-35 kg	42 days
Sows	Female animals having been inseminated/mated	From insemination/ mating	From insemination to the end of weaning period (one cycle)

Category	Definition of the animal category	Start, from	Duration
Chickens for fattening	Birds raised for fattening	Hatch	35 days
Laying hens	Productive female birds held for egg production purposes	From 20 weeks of age	56 days
Breeder hens	Female birds held for breeding purposes	From 25 weeks of age	56 days
Turkeys for fattening	Birds raised for fattening	Hatch	56 days
Turkeys for breeding purposes	Female and male birds held for breeding purposes	From 32 weeks of age	56 days

Tolerance studies for chickens for fattening/reared for laying and turkeys for fattening should normally be performed with one-day-old birds. Tolerance studies on laying hens should be performed normally during the first third of the laying period.

Category	Definition of the animal category	Start, from	Duration
Calves	Calves which are reared for reproduction, veal production or beef production	1 week of age*	42 days
Cattle	Bovine animals that have completed the weaning period	Full development of rumination but < 6 months of age	42 days
Cows	Lactating cows	4 weeks after beginning of lactation	56 days

*for veal production, from 1-3 weeks of age

If calves for rearing and cattle for fattening were applied for, a combined study (28 days for each period) would be considered sufficient. Studies on dairy cows should be performed with high yielding animals in the first third of the lactation period.

Category	Definition of the animal category	Start, from	Duration
Lambs/kids	Young animals reared for reproduction or meat production	1-4 weeks of age	42 days
Sheep/goats	Lactating animals	4 weeks after beginning of lactation	56 days

Category	Definition of the animal category	Start, from	Duration
Salmon and trout	Growing salmonids	Trout: 10 g Salmon: 50 g	90 days or until initial body weight is doubled

Other fin fish	Growing fin fish		90 days or until initial body weight is doubled
Crustaceans	Growing crustaceans		90 days

In case of tolerance trials for Salmonidae and other fin fish if the additive is intended to be used for brood stock only, the tolerance tests should be carried out as close to the spawning period as possible. In that case, tolerance tests should last for 90 days and attention should be paid to the egg quality and survival of the eggs.

Category	**Definition of the animal category**	**Start, from**	**Duration**
Rabbits	Rabbits that are reared for reproduction or meat production	Beginning one week after birth	42 days
Breeding does	Does that have become pregnant at least once		From insemination to the end of weaning period (one cycle)

Category	**Definition of the animal category**	**Start, from**	**Duration**
Horses	All categories		42 days

Category	**Definition of the animal category**	**Start, from**	**Duration**
Cats			28 days
Dogs			28 days
Other non food-producing animals			28 days

Where a tolerance study is required for minor species, the duration of the studies (when not indicated in the tables above) should be at least 28 days for growing animals and 42 days for adult animals.

If an additive is applied for a specific and shorter period than that given in the tables above, it should be administered according to the proposed conditions of use. However, the observation period should not be shorter than 28 days and should involve the relevant end-points (e.g., for sows for reproduction the number of piglets born alive when considering the gestation period, or the number and weight of weaned piglets when considering the lactation period).

5.7. Requirement for tolerance studies

In principle, tolerance tests should provide evidence of the safety of the additive for each of the target species/animal categories for which an application is made. It is recognised that it may be unrealistic to expect studies in all potential target species for which application is made, especially when the application is for all animal species. Therefore, inter-species extrapolation of data can be applied. In principle, data can be extrapolated between species if they are physiologically similar. The degree to which species are physiologically related is judged predominantly in terms of gastrointestinal function.

Similarities in metabolism also are considered. In general, the extrapolation is limited between animals which are kept for the same purpose, i.e., meat production or reproduction (including milk or egg production).

For the purpose of this guidance, the following food-producing animals are considered as major species from which safety data is normally extrapolated: chicken (*Gallus gallus* ssp. *domesticus*), pig (*Sus scrofa* ssp. *domesticus*), cattle (*Bos taurus*) and Salmonidae (*Salmo salar* or *Onchorynchus mykiss*).

The number of tolerance studies needed to demonstrate the safety for the target species will depend on the target species for which application is made:

- If the application is for all animal species, tolerance studies should be provided in salmonids and with at least three major terrestrial animal species representing different physiologic/metabolic capacities and should include chickens for fattening, piglets and dairy cows.
- If the application is for all terrestrial animal species, tolerance studies should be provided with at least three major animal species representing different physiologic/metabolic capacities and should include chickens for fattening, piglets and dairy cows. If the margin of safety (the ratio of tolerated to maximum proposed use level) is similar between these species, no further studies in other species would be required.
- If the application is restricted to all poultry/avian species, then tolerance studies should be provided with chickens for fattening and laying hens. In order to cover species for breeding, an additional limited study in breeding hens considering only performance end-points (see Section 5.5) should be submitted. Tolerance data from chickens or turkeys for fattening are generally taken to include chickens reared for laying or turkeys reared for breeding, respectively.
- If the application is restricted to all pigs/porcine species, then tolerance studies should be submitted for weaned piglets and sows. Tolerance studies for pigs for fattening are not needed if safety for weaned piglets is established.
- If the application is restricted to ruminant species, then tolerance studies should be submitted in cattle for fattening and dairy cows.
- If the application covers two animal species (e.g., pigs and poultry), then the requirement would be limited to a total of three tolerance studies including both species and covering growing and reproductive animals.
- If the application is restricted to all fish, then tolerance studies should be submitted in a salmonid (salmon or trout) and another species (e.g., carp, sea bream or sea bass). If the application includes crustaceans, then an additional study in shrimp would be required.
- If the application is restricted to all pets and non-food producing animals, tolerance studies would be required for cats, dogs and a third species (e.g. a laboratory animal).
- If the application covers horses, a tolerance study in horses is required unless safety is established for cattle for fattening or dairy cows and pigs for fattening or sows.
- If the application covers only one animal category (as defined in Annex IV of Regulation (EC) No 429/2008), at least one study in this category is required. The same principle should be applied if an application is for ornamental fish and/or ornamental birds. However, safety for ornamental fish can be extrapolated from studies in salmonids, safety for ornamental birds from studies with poultry species for fattening, in both cases provided a sufficient wide margin of safety[4] has been shown in the major species.

Tolerance data from major species can be used to support the safety for other species as follows, provided a wide margin of safety is established for the major species:

[4] A sufficiently wide margin of safety generally is at least ten, meaning that a concentration of at least ten times the highest recommended (approved) dose of the additive was tolerated by the major species without any adverse effects. However, for some substances a lower margin of safety may be considered (e.g., organic acids)

Major species	Other species
Chickens for fattening	other poultry for fattening (e.g., turkeys, ducks, goose, pheasants, quail, guinea fowl, ostrich) and ornamental birds
Laying hens	other birds kept for egg production (e.g., ducks, goose, pheasants, quail, guinea fowl)*
Pigs	other porcine species
Calves or cattle	other growing ruminants (e.g., sheep goat, buffalo) at the corresponding developmental stage
Dairy cows	other dairy ruminants (e.g., goat, sheep, buffalo)
Salmon or trout	ornamental fish

* Extrapolation to breeders (including turkeys) is only possible if additional data on breeding end-points are available.

For certain types of additives, the requirements for tolerance studies above may be modified:

- For nutritional additives where a tolerance study is required, target animal safety data can be derived from one study in a target species or laboratory animal.
- For silage additives for which tolerance studies are required it is usually sufficient to restrict tolerance to a ruminant species, normally the dairy cow. Studies involving other species are required only when the nature of the ensiled material makes it more appropriate for use with non-ruminants or when there are particular concerns when treated silage is used for categories other than adult ruminants (e.g., moist corn for pigs or fish silage for fur animals).
- For coccidiostats, tolerance studies should be performed in the relevant species/category for which application is made.

5.8. Reporting

For each tolerance study, a study report should be submitted describing the objectives, materials and methods, results and conclusions. The initial protocol should be included; any deviations from the protocol should be clearly indicated and justified in the final report. The reports should include the raw data in digital format and detailed results including descriptive statistics, statistical tests and model outcomes. Reports should start with a trial protocol data sheet (Appendix A) followed by the full study report. International units should be used to express the results.

It is recommended that the study report follows the structure detailed below and contain the following information. Applicants are encouraged to follow the recommendations of the EFSA guidance on statistical reporting.

Title: The title should provide a concise and clear description of the study, including the type of study, the product under assessment and animal species/category.

Summary: The summary should include the objectives, a description of the design and methods, the main results and the conclusions of the study.

Objectives: The objectives of the study should be clearly described.

Materials and methods: methods, apparatus and materials used, details of the species, breed or strain of the animals, their number and the conditions under which they were housed and fed. In particular, the following should be recorded and reported:

Ethical statement

1) Indicate compliance with national or institutional guidelines for the care and use of animals.

Animals, housing and husbandry

2) Animals: species (for aquatic species intended for human consumption: identification should be made by their colloquial name followed in parenthesis by the Latin binomial), breed, age

(and size/length for aquatic species), initial body weight, sex, identification procedure, physiological stage and general health.

3) Husbandry conditions: feeding and rearing conditions (pen/tank size, stocking density, temperature, lighting); for aquatic species water quality including water flow rate, water temperature and salinity, where relevant;

4) Diets: description of manufacture and quantitative composition of the diet(s) in terms of ingredients used, relevant nutrients (calculated and analysed values) and energy (digestible, metabolisable or net).

Study design

5) Study location, dates and responsible individuals.

6) Study duration.

7) The type of design of the study (e.g. factorial, stratified, cross-over).

8) Experimental groups: number of treatment and control groups, numbers of replicates (experimental unit) per group and number of animals per replicate.

9) The experimental unit (e.g., individual animal, pen) should be indicated.

10) The basis for the different measurements (e.g., individual animal, pen) should be indicated for each parameter measured.

11) Rationale for the selection of the number of animals/replicates used (sample size calculation). Power analysis should be provided.

12) Steps taken to minimise bias including randomisation and blinding (see section 5.1.1 of the EFSA guidance on statistical reporting).

13) Test item: intended concentration of the active substance(s) or agent(s) in the feedingstuffs.

Experimental procedures

14) The procedures carried out to the different experimental groups should be detailed. These should include the parameters/end points measured, indicating when and how they were measured, and information on the methods of analysis.

15) The health of the animals should be monitored, morbidity and mortality (including culling) recorded.

16) The methodology to correct feed to gain ratio for mortality (including culling) should be reported.

Statistical methods

17) The result of the power analysis should be reported.

18) The methods to perform statistical analysis should be stated, including those used to handle missing data.

19) Describe any methods used to assess whether the data met the assumptions of the statistical approach.

Results: Results of the study should be presented for all end points considered in the study. Tables should be used to summarise the results from treatments.

20) Health status of the animals, morbidity and mortality including culling. The timing and prevalence of any unexpected/undesirable incident/effect in individuals or groups. Therapeutic/preventive treatments, if any should be recorded. Likely cause of death should be established by a veterinarian and reported.

21) The report should include data from all animals or experimental units involved in the trials. Cases which cannot be assessed due to a lack or loss of data should be reported, and their distribution within the groups of animals indicated.

22) Concentration of the active substance(s) or agent(s) in the feedingstuffs should be periodically analysed and reported. A certificate of analysis of the test item used in the study should be provided.

23) Report the results for each end-point measured/analysis carried out, with a measure of precision (e.g. standard error or confidence interval).

24) The report should include descriptive statistics plus detailed outcome of any statistical analysis performed for all measured end points and each time-point.

25) The measurement units should be specified for any result reported.

Discussion

26) Interpretation of the results, taking into account the study objectives and hypotheses and other relevant studies in the literature.

27) Comments on the study limitations including any potential sources of bias, any limitations of the animal model and the imprecision associated with the results.

Conclusions

28) The conclusions from the study should be drawn considering the objectives of the study, the hypothesis and the outcome of the study.

Raw data, certificates of analysis

29) The raw data should be provided in a form of an electronic database and should be accompanied by a data dictionary containing the description of the variables and the metadata needed to properly analyse them.

30) All codes, log and complete outputs for the final statistical analysis (i.e. the results and analysis reported) should be provided in electronic format.

31) The report should include the certificates of analysis for the different analysis performed, reports of the veterinary observations/gross pathology/histopathology, haematology/clinical chemistry, etc.

6. Toxicological studies

The toxicological studies available for the active substance(s) should be taken into account when assessing the safety for the target species.

Depending on structural alerts or other toxicological considerations, genotoxicity studies may be required when the additive is intended for use in long living animals (e.g., pets) and reproduction animals (e.g., cows, sows, breeder hens). This could be achieved by reference to published studies.

7. Interactions *in vivo*

Any known interactions of the additive with feed materials, other approved additives, or medicinal products should be documented.

For those additives which exert their activity mainly by binding (e.g., clays) there is the possibility that the availability of crucial nutrients, micronutrients and other additives could also be affected. It is recognised that it is not practical to consider all possible nutrients/additives. Therefore, it is recommended to measure apparent digestibility of crude protein, zinc, retinyl or tocopheryl esters, thiamin or pyridoxine and an ionophore coccidiostat, the latter in the case the additive is intended to be used in poultry/rabbits. Such studies should be performed with the highest recommended dose of the additive and could be made in the context of a tolerance/efficacy study. For other additives which may have a negative impact on the absorption of nutrients a similar approach should be taken.

8. Microbial studies

Studies are only required when:

- the tolerance test give an indication of an adverse effect related to digestive tract disturbances; or
- an adverse effect on the gut microbiota can otherwise be anticipated; or
- the additive shows specific antimicrobial activity at the feed concentration; or
- the additive is an ionophoric coccidiostat.

For the details on how to perform the studies see the technical guidance on microbial studies.

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679 # Abbreviations

ANOVA	Analysis of Variance
$BMDL_{10}$	Benchmark Dose Level 10
BW	Body weight
DM	Dry matter
FI	Feed intake
GLMMs	Generalised Linear Models
GLP	Good Laboratory Practice
GMMs	Genetically Modified Microorganisms
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Cooperation and Development
QPS	Qualified Presumption of Safety
TTC	Threshold of Toxicological Concern

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Appendix A – Trial Protocol data sheet

FOR TERRESTRIAL ANIMALS

Identification of the additive:	Batch number:
Trial ID:	Location:
Start date and exact duration of the study:	
Number of treatment groups (+ control(s)):	Replicates per group:
Total number of animals:	Animals per replicate:
Dose(s) of the additive/active substance(s)/agent(s) (mg or Units of activity or CFU/kg complete feed or L water)	
Intended: [†]	Analysed:
Substances used for comparative purposes:	
Intended dose:	Analysed:
Animal species/category:	
Breed:	Identification procedure:
Sex: Age at start:	Body weight at start:
Physiological stage:	General health:
Additional information for field trials:	
Location and size of herd or flock:	
Feeding and rearing conditions:	
Method of feeding:	
Diets (type(s)):	
Presentation of the diet: Mash ☐ Pellet ☐ Extruded ☐ Other	
Composition (main feedingstuffs):	
Nutrient content (relevant nutrients and energy content)	
Intended values:	
Analysed values:	
Date and nature of the examinations performed:	
Method(s) of statistical evaluation used:	
Therapeutic/preventive treatments (reason, timing, kind, duration):	
Timing and prevalence of any undesirable consequences of treatment:	
Date	Signature Study Director or Signature of applicant or representative

[†] In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive can be given per animal/day or mg/kg body weight or as concentration in complementary feed.

686 **FOR AQUATIC ANIMALS**

Identification of the additive:		Batch number:
Trial ID:		Location:
Start date and exact duration of the study:		
Number of treatment groups (+ control(s)):		Replicates per group:
Total number of animals:		Animals per replicate:
Dose(s) of the additive/active substance(s)/agent(s) (mg, Units of activity, CFU/kg complete feed or L water)		
Intended:	Analysed:	
†		
Substances used for comparative purposes:		
Intended dose:	Analysed:	
Route of administration:		
Animal species/category:		
Colloquial name:		Latin binomial:
Breed:	Identification procedure:	
Sex*:	Age at start:	Body weight at start:
Physiological stage:	General health:	
Fork length at start:		Lighting conditions:
Water quality including temperature, salinity, O_2 and CO_2:		
Additional information for field trials:		
Location, size and number of tanks or pens at the farm, production volume:		
Feeding and rearing conditions:		
Method of feeding:		
Diets (type(s)):		
Presentation of the diet: Mash ☐ Pellet ☐ Extruded ☐ Live feed ☐ Other		
Composition (main feedingstuffs):		
Nutrient content (relevant nutrients and energy content of the feed)		
Intended values:		
Analysed values:		
Date and nature of the examinations performed:		
Response measures for efficacy and tolerance:		
Method(s) of statistical evaluation used:		
Therapeutic/preventive treatments (reason, timing, kind, duration):		
Timing and prevalence of any undesirable consequences of treatment:		
Date	Signature Study Director or Signature of applicant or representative	

687 † In case the concentration of the additive in complete feed/water may reflect insufficient accuracy, the dose of the additive
688 can be given per animal/day or mg/kg body weight or as concentration in complementary feed.
689 * Where possible