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Guidance on the assessment of the efficacy of feed additives

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Abstract

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

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Background and Terms of reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. Moreover, Regulation (EC) No 429/2008[2] 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itself (e.g. substantial or not), consider initiatives like preparatory info-sessions or public consultations of the draft guidance documents.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 efficacy of feed additives.

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 (Commission Regulation No 429/2008). This document is intended to provide guidance to applicants for the assessment of the efficacy of additives intended to be used in animal feed, in order to demonstrate compliance with the requirements of Article 5.3 of Regulation (EC) No 1831/2003. This guidance is divided in seven sections. The first section provides the principles of the assessment of efficacy. The requirements for efficacy demonstration for the different categories of additives are listed in Section 2. Section 3 provides information on the number of efficacy studies required for those additives for which *in vivo* studies are needed. Section 4 and 5 describe the principles for *in vivo* and *in vitro* studies, while sections 6 and 7 provide information on how to report the studies performed by the applicant or those retrieved from the literature.

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

1. General principles of efficacy assessment

[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.

Regulation (EC) No 429/2008 requires that studies should demonstrate the efficacy for each proposed use and satisfy at least one of the characteristics set out in Article 5(3) of Regulation (EC) No 1831/2003, according to the categories and functional groups of feed additives as provided by Article 6 and Annex I of the said Regulation. Moreover such studies must permit the evaluation of the efficacy of the additive according to common feed manufacturing and farming practices in the European Union (EU). Studies performed outside the EU must permit conclusions to be drawn on the efficacy of the additive when used in the EU. This does not necessarily exclude the reporting of studies made outside the EU. Any potential impact on the distinctive features of animal products should also be investigated during animal efficacy trials (e.g. off-flavour, colour changes).

All efficacy studies submitted should be properly reported and documented in order to allow an adequate assessment to be made. The studies should be based on the additive(s) for which authorisation is sought. To avoid confusion, in-house identifiers should be avoided unless embedded in third-party documents. In this case a statement is required to confirm that the identifier(s) refers to the additive(s) concerned.

However, the Panel considers that there are some additives for which efficacy is recognised (e.g., many nutritional additives and flavouring compounds). These additives do not require further demonstration of efficacy. For others, it is not practical to assess the additive under all possible conditions of use. Many factors may affect the efficacy of an additive, e.g., nutrition, animal breeds, composition of feed, management, environment, husbandry. For such additives, the Panel is able to conclude on the efficacy under the conditions of the studies submitted. From these data, the Panel may be able to conclude on the potential efficacy of the additive under EU farming conditions.

As a general principle, efficacy can be assessed by means of *in vitro* studies for those additives which are intended only to affect the characteristics of feed (i.e. some technological and sensory additives), while for those which are intended to have an effect in the animal efficacy should be assessed by means of *in vivo* studies or, in specific circumstances, by a combination of *in vitro* and *in vivo* studies. The number of studies required to support the efficacy of an additive will depend on the nature of the intended effect(s) and the conditions of use of the additive (e.g., target species/categories). The studies should be based on the additive(s) for which authorisation is sought. Efficacy should be investigated by comparison of the lowest recommended dose with a control group and designed to allow statistical evaluation.

Reference can be made to published studies to support the efficacy provided 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.

Attention should also be paid to known or potential biological or physico-chemical interactions between the additive, other additives and/or veterinary medicines and/or components of the diet, where this is relevant to the efficacy of the additive concerned e.g., compatibility of a microbial additive with coccidiostats and histomonostats or organic acids. For details on how to perform compatibility studies between microbial additives and other additives showing antimicrobial activity, see the technical guidance on the characterisation of microorganisms used as feed additives or as production organisms.

2. Requirements for the different categories of additives

2.1. Technological additives

When the additive is already authorised for use in food and the intended use of the additive in feed is the same, no further demonstration of efficacy is generally necessary provided that the effect seen when used in food could reasonably be expected to be seen when used in feed at the recommended concentration and that food and feed matrices are of comparable nature.

2.1.1. Technological additives which exert their function in feed

For technological additives intended to affect the characteristics of feed, evidence of the efficacy should be demonstrated using laboratory-based studies by means of appropriate criteria as reflected

in recognised acceptable methods, under the intended practical conditions of use in comparison with appropriate control feed.

The studies (at least three) should be designed to cover a representative range of feeds to which the additive will be applied including water for drinking, if appropriate.

The appropriate end-points are indicated in Table 1 for the various functional groups.

Table 1: Demonstration of efficacy for technological additives exerting their effect in feed

Functional group	Demonstration of efficacy
Preservatives	Inhibition of the growth of spoilage microorganisms. Duration of the study should cover the period for which an effect is claimed. Test materials could be naturally or artificially contaminated.
Antioxidants	Protection against oxidative damage of key nutrients/components during feed processing and/or storage. The period for which a protective effect is claimed should be demonstrated.
Emulsifiers	Formation/maintenance of stable emulsions of otherwise immiscible or poorly miscible feed ingredients.
Stabilisers	Maintenance of the physico-chemical state of feedingstuffs, including use of coating agents.
Thickeners	Viscosity of the feed materials or feedingstuffs.
Gelling agents	Formation of a gel resulting in a change in the texture of the feed.
Binders	Pellet durability (hardness, abrasion) or energy consumed during pellet formation.
Anti-caking agents	Flowability (angle of repose, frictional forces, compressibility).
Acidity regulators	pH and/or buffering capacity in feedingstuffs and/or water.
Silage additives	Improved production of silage (better preservation of nutrients). Inhibition of undesirable microorganisms. Reduction of effluents. Improved aerobic stability.
Denaturants	Indelible identification of feed materials.
Hygiene condition enhancers	Reduction of contamination with specific microorganism(s) relevant to feed safety (e.g. potential human or animal enteropathogens or undesirable bacteria).

For other technological additives, the end-points used to assess the function/effect of the additive should be defined and justified.

2.1.1.1. Silage additives

For additives intended for the preparation of silage from all forages, a minimum of three separate tests should be made including one example of each of the following categories;

- Easy to ensile forage: >3% soluble carbohydrates in the fresh material (e.g., whole plant maize, ryegrass, brome grass, sugar beet pulp);
- Moderately difficult to ensile forage: 1.5 – 3.0% soluble carbohydrates in the fresh material (e.g., meadow grass, fescue, wilted alfalfa);
- Difficult to ensile forage: <1.5% soluble carbohydrates in the fresh material (e.g., orchard grass, leguminous plants).

For additives intended for the preparation of silage from specific sub-categories of forage described in terms of dry matter, the dry matter range should be explicitly stated. Three tests should then be made with material representative of the claimed range, where possible using examples of different botanical origin.

Claims restricted to, or including, feedingstuffs other than forages, require tests specific to the particular feedingstuffs. This would include fish ensiled for use with production of fur animals.

All studies should demonstrate efficacy in comparison to a negative control made with the same material for ensiling.

As a general guide, all replicate tests should be made with approximately one kg or more of homogeneous fresh material in a closed laboratory silo with the potential to vent gas and drain effluent. Other test systems (e.g., wrapped bales) may be used provided that they are consistent with the claims made and meet the general requirements above (including negative controls). The harvesting and preparation of the test material must be similar to normal practice. Compaction in the silos should be constant across replicates. The duration of the study normally should be 90 days or longer at a constant temperature (recommended range 15-25 °C). Use of a shorter duration must be justified.

Claims made for silage additives differ and may relate to the preservation process in general, to specific aspects of the preservation process or to the aerobic stability of silage once the clamp/silo has been opened. The observations needed to demonstrate a significant benefit for the lowest dose claimed will differ both in nature and sampling time and frequency. As a rule measurements of the following parameters should be provided in comparison to the negative control:

- dry matter and calculated dry matter losses (corrected for volatiles);
- pH
- concentration of volatile fatty acids and lactic acid
- concentration of alcohols
- ammonia nitrogen

In addition, other microbiological and chemical parameters should be included as appropriate to substantiate the specific claim made (e.g., numbers of clostridia, numbers of *Listeria* in silage for sheep).

A claim for effluent reduction will be judged against the total volume of effluent produced over the entire experimental period taking into account the likely effect on the environment (e.g., ecotoxicity of the effluent, biological oxygen demand). Reduction of effluent production should be demonstrated directly. The duration of the study should normally be 50 days.

Aerobic stability studies should be of at least seven days duration after exposure to air and the additive should provide evidence of stability for at least two days longer than that shown by the untreated control. It is recommended that the experiment is made at an ambient temperature of 20 °C, and a rise in temperature of 3 °C or more above background taken as indicative of instability. Temperature measures may be replaced by measurement of CO_2 production. Measurement of dry matter loss and direct counts of aerobic spoilage organisms may be used as supportive evidence of improved stability.

2.1.2. Technological additives which exert their function in the animal

'Substances for control of radionuclide contamination' and 'substances for the reduction of contamination of feed by mycotoxins' are not expected to exert their intended effect until after their ingestion by the animal. Therefore, demonstration of efficacy should be based on *in vivo* studies.

The appropriate end-points are indicated in Table 2 for the two functional groups.

Table 2: Demonstration of efficacy for technological additives exerting their effect in the animal

Functional group	Demonstration of efficacy
Substances for control of radionuclides	Evidence of reduced contamination of food of animal origin.
Substances for the reduction of contamination of feed by mycotoxins	Reduction of the absorption of mycotoxins. Increased excretion of mycotoxins. Degradation/transformation of mycotoxins. Reduced concentration of mycotoxins in food of animal origin.

For other technological additives exerting their effect in the animal, the end-points used for assessing the functionality of the additive should be defined and justified.

2.1.2.1. Substances for reduction of the contamination of feed by mycotoxins

The mycotoxin(s) against which the additive will exert its function and the target species should be specified.

In vitro studies may provide evidence of the intended effect of the additive but do not sufficiently mimic the conditions in the digestive tract and the differences between target animals and their metabolism, to fully demonstrate efficacy under practical conditions.

A minimum of three *in vivo* studies (generally short term) showing significant effects should be provided to demonstrate efficacy at the lowest recommended dose for each target species. *In vitro* tests related to gastrointestinal conditions could substitute for one of the *in vivo* studies required for one animal species, provided that taken together they support the intended effect of the additive.

For additives intended to be used in all terrestrial species, efficacy should be demonstrated as indicated in the paragraph above in at least three major species representing different digestive systems (a poultry species, a monogastric mammal and a ruminant), in each case including the animal category for which the lowest maximum content of the respective mycotoxin in feed is set in Directive 2002/32/EC or recommended in Commission Recommendation 2006/576/EC (see Table 3). For additives intended to be used in fish, specific studies in fish (preferably salmonids) are required.

Table 3: Target species/categories that should be included in an application for all animal species

Mycotoxin(s) against which the additive is intended to act	Species/category
Aflatoxin B_1	Dairy cow
Deoxynivalenol, Ochratoxin A, Fumonisins B1+B2	Pig
Zearalenone	Piglet or gilt

The target mycotoxin content in feed used in studies should not exceed the values given in Directive 2002/32/EC for aflatoxin B_1 and in Commission Recommendation 2006/576/EC for deoxynivalenol, zearalenone, ochratoxin A and fumonisins B1+B2 for complete feedingstuffs for the respective animal species/category and in Commission recommendation 2013/165/EU for T-2 and HT-2. For mycotoxins without a maximum content established at EU level, the dietary levels chosen should not exert adverse effects in the target animals.

As a source of mycotoxins, naturally contaminated feed materials are preferred. Alternatively, feed supplemented with mycotoxins could be used, if properly justified. An analysis of mycotoxins[3] present in feed should be provided for each trial.

The experimental design of studies should include at least two groups: one group fed the basal contaminated diet as such (control) and the other fed the same basal contaminated diet supplemented with the additive for which authorisation is sought. For mycotoxins without a maximum content set/recommended, and in order to ensure the absence of adverse effects at the concentrations of mycotoxins used, an additional control group should be included. In this group, the feed should be free of these mycotoxins[4] and have, in general, the same composition as the feed given to the other two groups.

In general, mycotoxin/metabolites excretion in faeces/urine, concentration in blood/plasma/serum, tissues or products (milk or eggs) or other relevant biomarkers should be taken as end-points for demonstration of efficacy. The end-points should be selected according to the mycotoxin and target species, and taking into account the availability of sensitive analytical methods validated for the specific matrices. Recommendations on the end-points are given in Table 4: .

Zootechnical parameters should be reported but cannot be used for demonstration of efficacy.

[3] Including at least aflatoxin B_1 and B_2, deoxynivalenol, nivalenol, zearalenone, ochratoxin A, fumonisins B1+B2, T-2 and HT-2, and any other for which a claim is made should be determined.

[4] Below or at least close to the limit of detection.

Table 4: Most relevant end-points/biomarkers for substances reducing the contamination of feed by mycotoxins

Target mycotoxin(s)	Most relevant end-points
Aflatoxin B_1	Aflatoxin M_1 in milk/egg yolk
Deoxynivalenol	DON/metabolites in blood serum
Zearalenone	Zearalenone + α- and β-zearalenol in plasma Excretion of zearalenone/metabolites
Ochratoxin A	Ochratoxin in kidney (or blood serum)
Fumonisins B1+B2	Sphinganine/sphingosine ratio in blood, plasma or tissues

2.1.2.2. Substances for control of radionuclide contamination

For substances for control of radionuclide contamination a similar approach to the one for substances for reduction of the contamination of feed with mycotoxins should be followed. However, a single study demonstrating positive effects would generally suffice to support the efficacy.

2.2. Sensory additives

When the additive is already authorised for use in food and the intended use of the additive in feed is the same, no further demonstration of efficacy is generally necessary provided that the effect seen when used in food could reasonably be expected to be seen when used in feed at the recommended concentration and that food and feed matrices are of comparable nature.

2.2.1. For substances which, when fed to animals, add colour to food of animal origin

A minimum of three independent *in vivo* studies showing significant effects should be provided to demonstrate efficacy for the relevant target species/categories. Evidence of efficacy can be provided by i) reference to published studies, where the relationship between a particular substance and the colour of animal tissues/products is well documented, or ii) *in vivo* long or short term studies. Evidence should generally be provided for each target species/category for which the application is made. The change in colour of tissues/products obtained from animals receiving the additive should be measured using appropriate methodologies (e.g., colour fan, reflectance spectroscopy, image analysis).

2.2.2. For substances that add or restore colour in feedingstuffs

Evidence of the efficacy of the additive should be demonstrated using laboratory-based studies by means of appropriate criteria as reflected in recognised acceptable methods, under the intended practical conditions of use in comparison with an appropriate control feed. The change in colour of feed materials and/or compound feeds should be measured using appropriate methodologies (e.g., reflectance spectroscopy, image analysis). The studies (at least three) should be designed to cover a representative range of feed materials (test material) to which the additive will be applied. The additive should not adversely affect feed quality.

2.2.3. For substances which favourably affect the colour of ornamental fish and birds

Evidence of efficacy can be provided by i) reference to published studies, where the relationship between a particular substance and the colour of the animals has been established, or ii) extrapolation of the colouring effect established in poultry or salmonids, as appropriate, or iii) *in vivo* studies in the target species. For i) or iii), a minimum of three independent long term *in vivo* studies showing significant effects should be provided. The change in colour of animals receiving the additive should be demonstrated.

2.2.4. Flavouring compounds

Evidence of efficacy can be provided by i) reference to literature, or ii) laboratory based studies or, iii) if the application includes an effect on palatability, by short term *in vivo* studies. For iii), a minimum of

three independent studies showing significant effects should be provided for each target species/category for which the application is made.

2.3. Nutritional additives

No evidence of efficacy is necessary for amino acids naturally occurring in proteins of plants and animals and their salts, urea and recognised vitamins, pro-vitamins and compounds of trace elements.

Evidence of efficacy should be provided for amino acid analogues, new forms of compounds of trace elements, chemically well-defined substances having similar effect to vitamin, and urea derivatives. Evidence can be provided by reference to literature or by *in vivo* studies. Where evidence from literature is insufficient to reach a conclusion, a bioequivalence study is considered adequate to demonstrate efficacy for amino acid analogues, new forms of compounds of trace elements and urea derivatives. For chemically well-defined substances having similar effect to vitamin, duration and the endpoints of the *in vivo* study should be determined depending on the nature of the substance and the effect intended.

For other (novel) nutritional additives at least one long term efficacy study should be provided.

Generally, it will be sufficient to demonstrate efficacy in one study in a single animal species or category including laboratory animals. For additives specifically designed to be effective in a particular animal species/category (e.g., protected amino acids for ruminants) the same target species should be selected.

2.4. Zootechnical additives

A minimum of three independent *in vivo* studies showing significant effects should be provided to demonstrate efficacy for the relevant target species/categories. These should be carried out at least at two different locations, at least one of which should be in the EU. Efficacy studies should always include the lowest incorporation level (mg/kg complete feed)/lowest daily level (mg/head per day) proposed by the applicant.

2.4.1. Additives affecting animal production or performance

For those additives affecting animal production or performance of animals, long-term efficacy studies should be provided, unless the use of the additive/active substance is restricted to specific short-term periods (see Section 4.2.2.1). Depending on the properties of the additive, outcome measures may be based on performance characteristics or reproduction parameters.

For enzymes which affect the digestibility of phytate phosphorus, polysaccharides or protein, short-term (balance) studies can substitute for long-term studies provided that properly defined and specific methods are applied. For phytases, polysaccharidases and proteases improved utilisation of dietary phosphorus, metabolisable energy and protein, respectively, should be demonstrated.

2.4.2. Additives favourably affecting the environmental consequences of animal production

For additives which favourably affect the environment by direct or indirect means (e.g., reduction of nitrogen or phosphorus excretion, methane production or odour), efficacy for the target species can be demonstrated by short-term studies. These studies should take into consideration the possibility of an adaptive response to the additive.

2.4.3. Additives affecting the characteristics of food from animal origin

The choice of long-term or short-term studies to demonstrate the efficacy for these additives will depend on the nature of the substance and their intended purpose. The selection of the end-points should be properly justified.

2.4.4. Additives affecting animal welfare

For additives affecting welfare, the choice of long-term or short-term studies to demonstrate the efficacy will depend on the nature of the substance and their intended purpose. The selection of the end-points should be properly justified. For example, long-term studies would be needed to detect changes in morbidity/mortality while short-term studies may be sufficient to measure reduced stress levels as monitored by metabolic indicators.

2.4.5. Other additives

The intended effect of the additive should be clearly specified. The choice of long-term or short-term studies to demonstrate the efficacy for other additives under this category will depend on the nature of the substance and their intended purpose. The selection of the end-points should be properly justified.

2.5. Coccidiostats and histomonostats

These additives protect animals from the consequences of an invasion of *Eimeria* spp. or *Histomonas meleagridis*. The text below provides guidance for the assessment of efficacy of coccidiostats in poultry and rabbits. For applications covering other animal species or histomonostats, the requirements below should be adapted and justified.

The capacity of anticoccidial substances to control coccidiosis should be demonstrated by targeting specific end-points (e.g., morbidity, mortality, lesion/faecal score, oocyst excretion). Data on body weight and on feed to gain ratio should be provided as supportive information.

Efficacy data should derive from three types of target animal experiments:

- screening for response using artificial single and/or mixed infections
- artificial infection to simulate use conditions (e.g., floor pen studies with poultry, battery cage studies with rabbits)
- anticoccidial sensitivity tests (AST) for poultry, field studies for rabbits

The geographical location of the studies is considered of less importance compared to the virulence of the inoculum.

The minimum proposed inclusion level should be tested in all floor pen studies with poultry/battery cage studies with rabbits and anticoccidial sensitivity tests/field studies.

Due to the inherent weaknesses of field trials in poultry (usually no negative control, short duration of use of the coccidiostat under examination in shuttle programs, inadequate characterisation of end-points) these trials may be considered as supporting evidence only.

2.5.1. Screening tests

Experiments with artificial single and/or mixed infections are intended to demonstrate the relative effectiveness against the parasites.

A dose-range test with a limited number of animals should identify the optimum level in treating single or mixed-strain *Eimeria* infections. Animals should be fed the same basal diet until grouping at which time the experimental diets should be introduced. Allocation of replicates to treatment groups should be done one or two days before inoculation at day 13 to 16 of age for poultry and after weaning in rabbits. A single clinical examination of end-points should normally be done 6 to 7 days after inoculation, taking into consideration the life cycle of the parasite. Zootechnical parameters (morbidity/mortality and body weight gain) should be reported for this experimental period (from grouping until completion).

2.5.2. Floor pen studies with poultry/battery cage studies with rabbits

For floor pen studies with poultry/battery cage studies with rabbits, three studies with different inocula from different geographical locations within the EU are required. The studies should be conducted not more than five years before the date of submission of the application. A negative

control (without a coccidiostat) is essential. The design of such a study usually consists of three groups:

- uninfected untreated control (UUC)
- infected untreated control (IUC)
- infected treated (IT)

A fourth optional group may be included:

- uninfected treated (UT)

The study duration is usually equal to that required for long-term efficacy studies (see Section 4.2.2.1). Allocation of replicates to treatment groups should be done one or two days before inoculation at day 13 to 16 of age for poultry and after weaning in rabbits. The measurement of the different end-points should be done at least 6-7 and 14 days after inoculation and at the end of the study. It is recommended to expose all animals in the IUC and IT groups to the inoculum and not to rely on seeder animals.

2.5.3. Anticoccidial sensitivity tests for poultry

Three anticoccidial sensitivity studies done with inocula from different geographical locations within the EU and showing significant and positive results are required for poultry. The studies should be conducted within two years before the submission of the application.

Sensitivity tests should be performed according to the principles established by Chapman (1998) and following the guidelines published by Holdsworth et al. (2004).

Animals should be fed the same basal diet until grouping at which time the experimental diets should be introduced. Allocation of replicates to treatment groups should be done one or two days before inoculation at day 13 to 16 of age. Clinical examination of end-points should normally be done at least 6 to 7 days after inoculation. Zootechnical parameters (including morbidity/mortality, initial and final body weight) should be reported for this experimental period (from grouping until completion).

2.5.4. Field trials in rabbits

Three studies made in different geographical locations within the EU should be provided. The group receiving the coccidiostat under application should be compared to either an untreated group (negative control) or to a group given another authorised coccidiostat (positive control). If a negative control is used, the treated group should show significant differences in the relevant end-points. Otherwise, the studies should indicate that the coccidiostat is at least as effective as the coccidiostat used for comparison purposes. Field studies based on shuttle or rotation programs will not be considered.

2.5.5. Inocula

The inoculum is the critical factor in the models used in studies with artificial infection. The inoculum (sporulated oocysts) should represent EU field strains of coccidia that have been exposed to currently approved coccidiostats but should not originate from operations where birds have been vaccinated against coccidia in the previous two flocks. Laboratory strains are not acceptable. For inocula used in the AST the *Eimeria* field strains should ideally undergo one, but in any case not more than three, passage(s) provided virulence is retained.

Mixed inocula should be selected from the following *Eimeria* species based on current prevalence: in chickens: *E. brunetti, E. acervulina, E. maxima, E. mitis, E. tenella and E. necatrix;* in turkeys: *E. meleagrimitis and E. adenoeides*. For minor poultry species, the most typical *Eimeria* species encountered should be selected.

Virulence titration studies should be performed with each inoculum. The study should include birds in an uninfected untreated control group, and multiple groups given increasing numbers of oocysts. The study follows the principle described for screening tests considering the age of animals at inoculation and the experimental period and it is done with a small number of animals per group. Virulence is assumed when weight gain is depressed in the experimental period by 25% in chickens and 15% in

turkeys, lesion score increased by a minimum of two units on a five-point scale in chickens. In addition mortality/morbidity should be reported and faecal score for turkeys.

For rabbits no numerical limits for establishing sufficient virulence of an inoculum can be given. However, the same criteria as described above can be applied and results showing significant differences used to describe a pathogenic dose.

The protocol used in virulence titration studies and the full study report should be submitted.

3. Number of *in vivo* efficacy studies required

The number of independent *in vivo* efficacy studies required depends on the number of target species/categories for which application is made.

3.1. Single animal category

If the application covers only one animal category, the studies required in Section 2 should be performed in that animal category.

3.2. Multiple categories of the same species of food-producing animals

In principle, conclusions from studies in fattening animals are extended to include animals of the same species that are reared for reproduction, e.g., from chickens for fattening to chickens reared for laying, from turkeys for fattening to turkeys reared for breeding.

Conclusions from studies in weaned piglets are taken to include suckling piglets for the period in which solid feed is given.

Efficacy data cannot generally be extrapolated between categories of the same species at different production stages (e.g., from chickens for fattening to laying hens).

3.3. Multiple species of food-producing animals

When the application covers several target species/categories, it is recognised that it may be unrealistic to expect studies in all potential target species for which application is made. Therefore, inter-species extrapolation of data can be applied.

In principle, data can be extrapolated between physiologically similar species (Table 5). The degree to which species are physiologically related is judged predominantly in terms of gastrointestinal function. Similarities in metabolism also are considered. However, the inter-species extrapolation can be applied only in case the animals are kept for the same purpose, i.e., meat production or reproduction (including milk or egg production), the mode of action can reasonably be presumed to be the same between species and the effects claimed are the same.

Table 5: Extrapolation of efficacy data from certain species to other physiologically related species

From	To physiologically related 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, ostrich)
Piglets† or pigs for fattening	other growing *Suidae*
Sows	other reproductive *Suidae*
Calves or cattle for fattening	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

† Piglets: either weaned piglets or suckling and weaned piglets

The minimum effective level in the physiologically related species would be the same as established in the species/category from which data is extrapolated.

When the application covers multiple species/categories, the minimum number of independent studies showing the intended effect is shown in Table 6.

Table 6: Minimum number of independent studies and target species required for the assessment of efficacy in applications covering multiple species/categories.

Application for:	**Number of studies required and species**
All growing poultry species (chickens for fattening, turkeys for fattening and minor growing poultry species)	3 chickens for fattening
All poultry species (chickens/hens, turkeys and minor growing and reproductive)	3 chickens for fattening 3 laying hens
All growing pigs (piglets, pigs for fattening and minor growing porcine)	3 weaned piglets 3 pigs for fattening
All pigs (piglets, pigs for fattening, sows and minor growing and reproductive porcine species)	3 weaned piglets 3 sows
All growing ruminants (calves, cattle for fattening, sheep and goats for fattening, other minor growing ruminants)	3 calves 3 cattle for fattening
All ruminants (calves, cattle for fattening, cows, sheep and goats for fattening and dairy production, other minor ruminants growing and reproductive)	3 calves 3 cows
All fin fish	3 salmonids (salmon or trout) 3 other species (1 study in each)
Crustaceans	3 shrimp
Rabbits (growing and reproductive)	3 covering both growing and reproductive animals

For applications covering all animal species, efficacy should be demonstrated in species with different digestive systems. Therefore, studies should be provided to support efficacy for all pigs, all poultry, all ruminants and all fin fish according to the Table 6: .

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

- For substances for reduction of the contamination of feed by mycotoxins, radionuclide binders and nutritional additives, the number of studies and the target species are given in Section 2.
- For coccidiostats and histomonostats, specific studies are required for chickens and turkeys. For those intended to be used in minor species, if efficacy has been demonstrated in a major species, then one additional study (preferably a floor pen study) incorporating the most typical *Eimeria* species encountered should be provided for each additional species to a maximum of three.

3.4. Pets and other non food-producing animals

The requirements for the different categories/functional groups of additives apply.

1. For additives for which efficacy has been demonstrated in a food-producing animal species one *in vivo* study is required for each target pet/non food-producing species to a maximum of three studies in total provided that the intended effect is the same.
2. Where the intended effect in the pet/non food-producing species is not the same as that described for the food-producing animal species or when efficacy has not been demonstrated in food-producing animal species, three *in vivo* studies in one pet/non food-producing species are required. If the application is made for more than one pet/non food-producing species, a single additional study would be required for each additional target species to a maximum of three species in total.

4. *In vivo* efficacy studies

In vivo animal studies are foreseen for all additives which exert the intended effect in the target species. Generally, zootechnical parameters (e.g., growth, feed conversion, milk yield, laying performance, carcass composition, reproduction performance) can only be reliably measured in long term efficacy studies, whereas effects on other parameters (e.g., absorption, digestibility, excretion, retention) may be better demonstrated in short term studies. The choice of short or long term studies or a combination of both will depend on the effect and/or mode of action of the additive.

Such experiments should use numbers and species/categories of animals appropriate to the conditions of use proposed. Studies should be designed to demonstrate the efficacy of the lowest recommended level of the additive by targeting sensitive parameters usually in comparison to a negative and, optionally, a positive control group. No single design is recommended, flexibility being provided to allow for scientific discretion in the design and conduct of the studies.

The experimental design used must be justified according to the additive function, use, animal species and category. The trials should be conducted such that their health and husbandry conditions do not adversely affect the interpretation of the results. The positive and negative effects should be described for each experiment. Trials should follow the criteria established by recognised, externally-audited, quality assurance schemes (e.g., good laboratory practice, good clinical practice). Evidence should be provided that the work was done by qualified personnel using appropriate facilities and equipment and responsible to a named study director. Studies conducted outside the European Union must follow the same quality standards.

4.1. General requirements for the *in vivo* studies

4.1.1. Test item

Efficacy studies should be based on the additive(s) for which application is made. Any 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.

4.1.2. Route of delivery

Use of the additive in efficacy studies should respect the proposed conditions of use (e.g., with regard to use level, route, number of administrations, duration).

For additives intended for use in feed and water the oral administration routes are principally considered as bioequivalent. Therefore studies can be made in either feed or water, or a mixture of both, provided that the exposure of the animal is the same. Otherwise, studies for each route would be required.

For an additive for which data is already available allowing a minimum effective level to be established in feed, the corresponding concentration in water can be derived from feed intake. The same principle would apply when the effective level has been established in water. For poultry, pigs and rabbits, the water intake would be 2-3 times the amount of dry matter feed intake. In ruminants and horses, concentrations of an additive cannot be consistently extrapolated from feed to water using a fixed ratio of feed to water intake. However, these concentrations can be converted to daily amounts which can then be equally administered via feed or water. Consequently, the conversion of feed concentration to water concentration should be done on the basis of the daily ration.

The concentration of the active substance(s)/agent(s) in the feedingstuffs/water should be confirmed by analysis.

4.1.3. Experimental groups

The design of an efficacy study includes a minimum of two groups:

- a control group

  The feed and water of the control group should normally not contain the additive tested.

  Where studies are required to demonstrate that the additive contributes to the animals' nutritional requirements, the control group should contain the nutrient at concentrations marginally below the animals' requirements.

- a use-level group

  The feed/water of the use level group should normally be supplemented with the additive at the lowest proposed use level.

  For some additives (e.g., nutritional, some colouring agents) the appropriate level of incorporation may be defined by the diet to which it is applied.

Additional groups with the additive supplemented at different levels or a positive control may be included, as appropriate.

4.1.4. 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. Routine vaccinations across all groups are acceptable but 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. Studies with an abnormally high mortality will not be accepted. This would be judged against current European commercial production standards.

The recommended age/weight for the different species/categories at the start of the study is detailed in Section 4.2.2.1.

4.1.5. General end-points

For all *in vivo* studies the following parameters should be measured and reported: clinical observations including general health status, behaviour, morbidity/mortality, feed intake and water intake for those additives administered via water, initial and final body weight, milk/egg production (as appropriate).

Specific end-points will depend on the nature of the additive and its intended effects. More information can be found in Section 2 and Section 4.2.2.2 below.

4.1.6. Statistical considerations

4.1.6.1. 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.

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

4.1.6.2. Sample size

Statistical considerations should be used to determine the size of the sample used to evaluate the intended effect(s). The setting of the null and alternative hypotheses should be done in light of the problem formulation. Difference testing should be used to confirm statistical superiority or inferiority (i.e. alternative hypothesis stating a difference exists) and tests for non-inferiority should be done for experiments aiming at demonstrating non-inferiority between treated groups and control. Additional considerations need to include: i) the magnitude of the effect that the study is designed to detect at the substance lowest recommended level; ii) the expected variability of the effect; ii) the expected direction of the effect; iii) an adequate statistical power; and iv) the confidence level. 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 applicant should justify the selection of the endpoints chosen to determine the sample size.

As a guide, a power greater than or equal to 80% (75% for ruminants, minor species, pets and non food-producing animals) should be ensured. Generally, when testing difference a confidence level of 90% is adopted for ruminants, minor species, pets and non-food producing animals and 95% for all other animal species and categories.

4.1.6.3. Statistical analysis

The statistical analysis should be performed at the level of experimental unit 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). 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 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. Independently from the outcome of tests of normality, non-parametric tests should be used when only a low number of observations is available. However, applicants are encouraged to use a sufficient number of experimental units to allow for parametric tests to be performed. When different substances 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.

Pooling of data from different studies may be done, and may substitute for a single efficacy study. A minimum of four independent studies of comparable design should be used, provided that the interaction treatment x study is not significant.

4.2. Typology of *in vivo* studies

4.2.1. Short term efficacy studies

Short term studies are defined as studies with a duration shorter than the minimum duration given in Section 4.2.2.1. They find particular application in the measurement of bioavailability/bioequivalence of an additive, intestinal absorption and/or excretion of nutrients or other substances, for the assessment of feed palatability and colouring potency in food of animal origin. Other short term efficacy studies with animals may be proposed as appropriate.

4.2.1.1. Bioavailability/bioequivalence studies

Bioavailability is defined as absorption/transport of the active substance(s)/metabolite(s) to the target cells/tissue(s) where it exerts a typical function/effect. Bioavailability will be evaluated by the corresponding specific end-points (observable or measurable biological, chemical, or functional events), depending on the nature of the additive.

Bioequivalence is used to assess the expected *in vivo* biological equivalence of two additives. If two products are said to be bioequivalent it means that they would be expected to be, for all relevant effects, the same (needs statistical confirmation by a non-inferiority test). Such studies may also be used to demonstrate the extent to which a novel form or source of a nutritional additive or an additive which colour food of animal origin can substitute for an equivalent additive already authorised or established.

4.2.1.2. Digestion/balance studies

The outcome of a digestion study is digestibility (e.g., apparent or true, faecal or ileal) of a nutrient as influenced by the additive. Balance studies are preferred because they deliver additional information on quantitative excretion and retention of a nutrient/energy.

Digestibility/balance studies should be performed considering an adequate period of adaptation to the diet (and experimental conditions). The minimum duration of this pre-period depends on the species: 4 days for poultry and pigs, 14 days for ruminants and 7 days for equidae.

For studies using the total collection method of faeces/excreta the duration of the collection should be 3-4 days in poultry, 4-6 days in pigs and horses, 5-7 days in ruminants. In balance studies where urine collection is needed, the collection of urine should be done at the same time as the faeces are collected. Measures should be taken to ensure that the same quantity of feed is consumed sufficiently before the start of collection (e.g., at least 1 day for poultry, 2 days for pigs, ruminants and equidae) and during the collection period. The use of a marker in the diet would avoid the need for quantitative collection of faeces.

In studies in layers, cows and sows, special considerations should also be given to the output (e.g., eggs, milk, litter). For applications in gestating and lactating sows, digestibility studies should be performed in both gestating and lactating sows.

Digestibility studies in fish are discouraged and balance (retention) studies should be made instead.

4.2.1.3. Palatability studies

Palatability studies should provide a free choice of feed (simultaneous access to control and test feed) to the animal. The experimental design should exclude the possibility that the results are influenced by the position in which the individual feed types are offered. The minimum duration of studies of this type is two periods of five days each, with an intermediate period in which only the control feed should be provided.

The two diets should be essentially equal in composition, with the only difference being the presence of the additive in the test diet at the proposed inclusion rate (analytically confirmed).

Feed intake should be recorded at least once daily and reported accordingly.

A similar design should be applied for additives intended to be used in water. In that case, feed intake should also be monitored.

4.2.2. Long term efficacy studies

4.2.2.1. Duration of the long-term efficacy studies

Generally, the duration of efficacy trials should correspond to the application period claimed. The necessary minimum duration of efficacy trials depends on the animal species/category and is reported in Table 7.

Table 7: Minimum duration of long term efficacy studies

Category	Definition of the animal category	Start, from	Minimum duration
Piglets (weaned)	Young animals having completed the suckling period	Weaning (or not more than 7 days after 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	≤35 kg	Until slaughter, but not less than 70 days
Sows	Female animals having been inseminated/mated	From insemination/ mating	For effects on reproduction: two cycles (from insemination/mating until weaning). For effects on piglets, at least two weeks before parturition until weaning
Chickens for fattening	Birds raised for fattening	1 day of age	35 days
Laying hens	Productive female birds held for egg production purposes	From 25 weeks of age	84 days
Turkeys for fattening	Birds raised for fattening	1 day of age	84 days
Calves	Calves which are reared for reproduction, veal production or beef production	1 week of age (for veal production, from 1-3 weeks of age)	56 days
Cattle	Bovine animals that have completed the weaning period	Full development of rumination but < 6 months of age	84 days
Cows	Lactating cows	4 weeks after beginning of lactation	84 days
Lambs/kids	Young animals reared for reproduction or meat production	1-4 weeks of age	56 days
Sheep/goats	Lactating animals	4 weeks after beginning of lactation	84 days
Salmon and trout	Growing salmonids	Trout: 10 g Salmon: 50 g	84 days
Other fin fish	Growing fin fish		84 days
Crustaceans	Growing crustaceans		84 days
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		For effects on reproduction: Two cycles For effects on young rabbits: From two

Category	Definition of the animal category	Start, from	Minimum duration
			weeks before parturition until end of weaning period.
Cats, dogs and other non food-producing animals			28 days

For minor species not included in the table above, the duration of the studies should correspond to that of the physiologically related major species listed in **Error! Reference source not found.**. For all other species/categories, the minimum duration should be 42 days for growing animals and 56 days for adult animals.

If an additive is applied for a specific and shorter period than that given in the table 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).

4.2.2.2. End-points

The end-points to be measured depend on the effects which are expected from the additive (see Section 2). A non-exhaustive list of end-points for some common effects is given below.

Performance parameters and related parameters

For all studies, feed intake, initial and final body weight, body weight gain, feed to gain ratio, water intake for those additives administered via water, should be provided. Additionally, clinical observations including general health status, behaviour, morbidity and mortality should be monitored.

Additional parameters for:

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

Product quality/composition

When measuring changes in the product quality or composition as an intended effect of the use of the additive, the following end-points can be considered as appropriate.

- Composition: e.g., nutrient content
- Physical/technological properties: e.g. water binding capacity, oxidative stability
- Sensory modification of food products: e.g., colour, taste, smell, texture

  The sensory properties of the food products should be measured, preferably by objective methods. However, it is recognised that some parameters can be better assessed by means of e.g., a trained panel or other subjective methods.
- Hygiene quality of food products: e.g., numbers of potential human or animal enteropathogens

  Studies should clearly identify the target microorganisms. These should be enumerated and their prevalence either in faeces or measured in the carcass established. Ideally, the measurements of the pathogens should be done in the food products.

Other end-points may be proposed and justified.

Environmental effects

Direct effects on the environment may include, for example, reduction on methane, ammonia, carbon dioxide emissions, and reduction of odour or odorous compounds.

Indirect effects on the environment may result from an increased nutrient utilisation, and result in a reduced excretion of e.g., nitrogen, phosphorus and sulphur, if appropriate dietary adjustments are made.

Faecal consistency

It is recommended to use objective measurements such as dry matter content of faeces. Subjective observations of faecal consistency alone are discouraged. If used, continuous subjective observations should be complemented with periodic objective measurements.

Welfare

Suitable and reproducible end-points should be proposed and justified.

4.3. Studies on the quality of products when this is not the effect claimed

Evidence should be provided that the additive does not have a negative effect or another unintended effect on sensory and nutritional (and hygienic and technological if appropriate) characteristics of food deriving from animals fed with the highest proposed level of the additive. Evidence can be based on physiological/metabolic considerations or given by reference to published literature. Otherwise, specific studies should be provided. Appropriate end points may be found under Section 4.2.2.2.

Omission of these studies should be adequately justified.

5. *In vitro* studies

For additives affecting the characteristics of feed, efficacy should be demonstrated using laboratory-based studies. Efficacy studies should be based on the additive(s) for which application is made. A certificate of analysis of the test item used in the study should be provided. The concentration of the active substance(s) or agent(s) in the feedingstuffs/water should be confirmed by analysis. The experimental design and methodology used should be appropriate to the intended effects of the additive. Studies should be designed to demonstrate the efficacy of the recommended level(s) of the additive by targeting sensitive parameters in comparison to a control group. The study should be designed to cover a representative range of materials to which the additive will be applied (feed materials, complete or complementary feed or water depending on the intended use).

The experimental design should consider sufficient number of observations to allow an adequate statistical analysis. Results of each test/subset should be statistically evaluated and a confidence level of 95% adopted. Independently from the outcome of tests of normality, non-parametric tests should be used when only a low number of observations is available. However, applicants are encouraged to use sufficient replicates to allow for parametric tests to be performed. When different substances 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.

All trials should follow the criteria established by recognised, externally-audited, quality assurance schemes (e.g., good laboratory practice or ISO standards). Evidence should be provided that the work was done by qualified personnel using appropriate facilities and equipment and responsible to a named study director.

6. Reporting of efficacy studies

For each efficacy study, a study report should be submitted describing the objectives, materials and methods, results and conclusions. The 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 for *in vivo* studies 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). In addition for studies with enzymes, the diets should be analysed for the enzyme-specific substrate.

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 identify outliers and handle missing data. If any relevant data points are excluded from the model (e.g. outliers) a justification should be given.

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. 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.

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 must 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 the 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.

Reports of *in vitro* studies should respect the principles described above, as appropriate.

7. Literature studies

Reference can be made to published studies to support the efficacy of the additive. The additive (active substance(s)/agent(s)) in literature studies should be identical to that under application or, if not, should still allow conclusions on the additive under application to be made. The concentration of the additive (active substance(s)/agent(s)) in feed should reflect the conditions of use specified in the application. The target species covered in the literature studies should be relevant to the application. Application level, replicates, duration and end-points measured should be in line with the requirements listed in this guidance and should allow a conclusion on the efficacy of the additive.

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.

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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:
Concentration(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 concentration:	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

† 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.

959 **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:
Concentration(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 concentration:	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	

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

963