Establishment of the Standards for Evaluation of Feed Additives

Director General, Food Safety and Consumer Affairs Bureau, MAFF, Japan, Notification No. 4-Chiku-A-201, March 16, 1992

Note: This translation is made by Ministry of Health, Labour and Welfare. In the case of any discrepancy between the Japanese original and the English translation, the former will take priority.

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(Notification ["4 Chiku A"] No. 201
March 16, 1992)

Director, Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries
Director-General, Fisheries Agency

The Minister of Agriculture, Forestry and Fisheries (MAFF) shall seek opinions from the Agricultural Materials Council (AMC) in accordance with the provisions of Clause 3, Article 2 or Clause 2, Article 2-2 of the Law Concerning the Safety Assurance and Quality Improvement of Feed (Law No. 35 of 1953) in order to:

- Designate feed additives according to the provision of Clause 3, Article 2 of the same law; or
- Establish standards or specifications according to the provision of Clause 1, Article 2-2 of the same law.

The evaluation standards for AMC to consider the above matters have previously been communicated in the notifications titled "Establishment of the Standards for Evaluation of Feed Additives" (Notification ["52 Chiku A"] No. 1200 / ["52 Suigyo"] No. 1111, dated April 5, 1977, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency) and "Establishment of the Standards for Safety Evaluation of Probiotics Used as Feed Additives" (Notification ["3 Chiku A"] No. 1169, dated May 30, 1991, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency). The points to consider in the conduct of animal studies have previously been communicated in the notification titled "Establishment of the Guide for Conducting Studies According to the Standards for Evaluation of Feed Additives" (Notification ["54 Chiku A"] No. 5001 / ["54 Suishin"] No. 3380, dated February 4, 1980, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency). At this time, new "Standards for Evaluation of Feed Additives" (attached) are established, replacing the aforementioned three notifications, which are now repealed. Please be thoroughly informed of the new standards, note the points described later in this document, and advise all parties involved under your jurisdiction accordingly.

Please also be advised that the following items are now revised in response to the establishment of the new "Standards for Evaluation of Feed Additives," and the changes are highlighted in the tables comparing old and new versions (Attachments 1 through 7):

- "Operation of the Law Concerning the Safety Assurance and Quality Improvement of Feed" (Notification ["52 Chiku B"] No. 696, dated June 27, 1977, issued by the Director of Livestock Industry Bureau under MAFF);
- "Data required for Submission for the Designation of a Feed Additive, etc." (Notification ["54 Chiku A"] No. 5002 / ["54 Suishin"] No. 3381, dated February 4, 1980, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency);
- "Enforcement of the Ministerial Ordinance to Revise a Portion of the Ministerial Ordinance Concerning the Ingredient Specifications for Feed and Feed Additives" (Notification ["56 Chiku B"] No. 1594, dated July 27, 1981, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency);
- "Establishment of the Standards for the Safety Evaluation of Feed" (Notification ["63 Chiku B"] No. 617, dated April 12, 1988, issued by the Director of Livestock Industry Bureau under MAFF);
- "Good Laboratory Practice for Feed Additives" (Notification ["63 Chiku A"] No. 3039, dated July 29, 1988, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency);
- "Establishment of the Standards for the Safety Evaluation of the Feed Intended for Farm-Raised Aquatic Animals" (Notification ["2 Chiku B"] No. 2103, dated February 13, 1991, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency); and
- "Submission of Data for the Designation of Probiotics Used as Feed Additives" (Notification ["4 Chiku A"] No. 25, dated January 30, 1992, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency).

Description of the Revised Standards for Evaluation of Feed Additives

1. Purpose of the revision

   The purpose of the current revision is to integrate the previous "Standards for Evaluation of Feed Additives" and "Guide for Conducting Studies According to the Standards for Evaluation of Feed Additives," decrease the number of test animals from the viewpoint of animal welfare, and specify the required studies based on each type of feed additives.

   The new "Standards for Evaluation of Feed Additives" are supported by the current scientific standard. However, the determination of the appropriateness of each feed additive will be based on not only these evaluation standards, but also the latest findings concerning the safety of that feed additive, and its specific properties.

   The efficacy and safety of feed additives shall be determined by taking into consideration the various feeding conditions of livestock, because feed additives are usually added to feed at formula feed manufacturers and sold to numerous, unspecified agents who subsequently use the feed.

2. Designation of feed additives

   As was conventionally done, only the requisite minimum number of feed additives shall be designated from among those feed additives that are particularly needed and proven to be both safe and effective. Therefore, those parties who intend to start manufacturing or importing an article that has not previously been designated as a feed additive shall adequately consult with, and seek direction from the authorities in advance.

3. Timing of the applicability of the new evaluation standards

   All studies shall be conducted according to the newly established "Standards for Evaluation of Feed Additives"; however, those studies that will be initiated on or before September 30, 1992 may be conducted according to the previous guidelines.

Standards for Evaluation of Feed Additives

The standards set forth herein specify basic concepts of and procedures for the evaluation of efficacy and safety of feed additives, which are necessary for the Feed Committee of the Agricultural Materials Council (hereinafter called "the Committee") to hold deliberations to designate feed additives and establish standards and specifications thereof on the basis of the Law concerning the Safety Assurance and Quality Improvement of Feed (Law No. 35 of 1953).

I Basic Requirements for Feed Additives

1. Requirements on Efficacy
   (1) Feed additives shall be effective for the purposes specified in Article 1 of the Regulations for Enforcement of the Law Concerning the Safety Assurance and Quality Improvement of Feed (Ministry of Agriculture and Forestry Ordinance No. 36 of 1976, hereinafter called "the Regulations for Enforcement").
   (2) The efficacy of antibacterial substances as feed additives shall not be intended for purposes other than the following:
      a Prevention of deterioration of feed quality due to growth of fungi and other causes.
      b Promotion of livestock growth (animals as specified in Article 1 of the Enforcement Ordinance for the Law concerning the Safety Assurance and Quality Improvement of Feed (Government Ordinance No. 198 of 1976, hereinafter called "the Enforcement Ordinance") and so on) (in principle, restricted to young livestock) and improvement of feed efficiency.
      c Prevention of reduction of productivity of young livestock due to specific pathogenic parasites.
   (3) The effectiveness of the new feed additives intended for purposes similar to those previously designated shall be at least equivalent to that of the previously designated ones.

2. Requirements on Residues

Antibiotics intended as feed additives shall not be detectable in products of livestock fed a diet containing such substances by adequately sensitive methods of quantitative analysis.

3. Requirements on Safety
   (1) Feed additives shall not produce harmful animal products (meat, milk and other

edible products of livestock that may be harmful to human health) as a result of using feed containing these additives or hinder the production of animal products (products of livestock) by harming the livestock.

(2) Safety of new feed additives that have similar structures and mode of actions, etc. to those previously designated, shall be at least equivalent to that of the previously designated ones.

(3) Feed additives shall have an adequate safety margin for use in livestock.

(4) Feed additives shall not, in principle, be poisonous or dangerous drugs based on the Pharmaceutical Affairs Law (Law No. 145 of 1960) or poisonous or deleterious substances designated under the Poisonous and Deleterious Substances Control Law (Law No. 303 of 1950).

(5) Veterinary medical care shall not be negatively influenced by the use of feed containing additives.

4. Other Requirements

(1) Feed additives shall, in principle, be available for quantitative analysis by physical, chemical or biological means from feed containing such additives.

(2) Addition of feed additives shall not lower the quality of the feed or the effectiveness of the feed additives.

II Items Required for Evaluation

In order to prove whether a proposed food additive meets the requirements specified in I above, the following items need to be clarified:

When, according to known data, an additive does not contain deleterious or poisonous substances, has no residue problems, has a negative mutagenicity test, and is not suspected of carcinogenicity, carcinogenicity testing will not be required. When the determination of long term repeated-dose toxicity is considered to be unnecessary based on known data and repeated-dose toxicity studies (short-term), repeated-dose toxicity studies (long-term) will not be required. When known data shows that there is no negative influence on reproduction, Multi-generation reproduction studies will not be required.

Furthermore, a substance already designated as a food additive or used widely as a food will not require further safety testing.

However, when an applicant omits a test because of the above statements, the applicant shall clearly state the reason for, and validity of the omission.

1. Feed additives except for probiotics (direct-fed microbes)

(1) Details of origin or discovery and status of approval, usage, etc. in foreign countries.

(2) Specifications

a Name

(a) Generic name

(b) Chemical name

b Chemical structure

c Manufacturing process

d Biological and physicochemical properties

(a) Properties

(b) Identification test

(c) Purity test

(d) Content and method of quantitative analysis

e Method of quantitative analysis in feed

f Change with time (stability of feed additives and feed additives contained in feed)

(3) Efficacy

a Basic studies proving effectiveness

b Field trial studies proving effectiveness

(4) Residues

Residue studies using target livestock, etc. for which feed additives are intended.

(5) Safety

a Toxicity studies

(a) General toxicity

[1] Single-dose toxicity studies

[2] Repeated-dose toxicity studies (short-term)

[3] Repeated-dose toxicity studies (long-term)

(b) Special toxicity

[1] Multi-generation reproduction studies

[2] Teratogenicity

[3] Carcinogenicity

[4] Mutagenicity

[5] Other toxicity tests (local toxicity, inhalation toxicity)

(c) Pharmacology
(d) Metabolism studies (absorption, distribution, metabolism, excretion, and accumulation)

b Feeding studies using target animals
c Studies regarding development of resistant bacteria
d Other studies
(a) Studies on environmental impact (plant toxicity, fish toxicity, and environmental pollution)
(b) Others

2. Probiotics

(1) Details of origin or discovery and status of approval, usage, etc. in foreign countries.

(2) Specifications
a Name
(a) Generic name
(b) Scientific name
b Manufacturing process
c Bacterial properties
(a) Properties
(b) Identification test (handy identification method)
(c) Purity test (contamination of other bacteria, etc.)
(d) Content (number of live bacteria) and assay procedure (determination method of number of live bacteria)
d Method of quantitative analysis in feed
e Change with time (stability of feed additives and feed additives contained in feed)
f Specifications of the bacteria employed
(a) Method of successive cultivation
(b) Storage
g Method of quality control
h Physical properties of formulation

(3) Efficacy
a Basic studies proving effectiveness
b Studies proving the effects caused by combination with antibacterial feed

additives

c Field trial studies proving effectiveness

(4) Safety

a Taxonomy of bacteria

b Toxicity studies

(a) Single-dose toxicity studies

(b) Repeated-dose toxicity studies (short-term)

(c) Metabolism studies (distribution)

c Feeding studies using target animals

d Studies on environmental impact

III Data for Evaluation

Data to clarify the items listed in II above, which are required for evaluation, shall meet the following conditions:

(1) Studies to collect data shall be conducted using appropriate procedures at facilities that allow for sufficient tests, and the data shall be discussed precisely and objectively. In particular, the studies shall be conducted according to the Good Laboratory Practice for Feed Additives (Notification No. 63-Chiku-A-3039 issued on July 29, 1988, by the Directors of the Livestock Industry Bureau and Fisheries Agency under the Ministry of Agriculture, Forestry and Fisheries).

(2) Some of the items for evaluation mentioned in II above may be omitted or added, as deemed appropriate by the Committee.

(3) Procedures for major studies to collect data should be conducted as outlined in the attachment.

The standardized procedures describe how studies are to be conducted to evaluate efficacy and safety, etc. of feed additive substances. Other procedures to collect data that allow for sufficient evaluation may be applicable.

Outline of Procedures for Major Studies

I Efficacy Studies

1. Objective

The objective of these studies is to prove that a test article is effective for the purpose it

is intended for.

2. Studies for a feed additive intended to prevent deterioration of feed quality

(1) Basic studies

To determine the effectiveness of a test article and the optimum amount to be added.

(2) Studies for the determination of sustained efficacy

Studies shall be conducted to determine sustained efficacy by adding the test feed additive into a commonly used diet under natural conditions, as well as under severe conditions (light, temperature, humidity, etc.). Studies shall be conducted according to the procedures of stability testing of feed additives.

3. Studies of supplemental nutritive substances and other active ingredients intended as additives

Studies shall be conducted to determine usefulness of the test feed additive by using laboratory animals or target animals. In addition, as needed, a comparison study to feed additives already designated shall be conducted.

4. Studies for a feed additive intended to promote efficient use of nutritive substance(s) included in the feed

(1) Studies to confirm promotion of growth or improvement of feed efficiency (all feed additives except for probiotics)

These studies shall provide the data for antibiotics, synthesized antibacterial substances or enzymes, etc.

a Basic studies

These studies aim to prove or estimate effectiveness of the test feed additive. Feed additives already designated as having similar effectiveness are to be tested as controls for comparison.

(a) *In vitro* studies

(b) *In vivo* studies

The studies shall be conducted using laboratory animals or target animals

b Field trial studies

These studies aim to confirm, statistically, the effectiveness of the test feed additive under field feeding conditions using target animals.

In addition, there are other ways to increase study precision, such as randomized blocks design, split-plot design, etc. For an enzyme, the study shall be conducted

according to (2)-c, and animals, replications and facilities of (2)-c-(a)-[1] shall be: cattle, at least 5 head per group (one head x 5 replications x one facility); swine, at least 20 head per group (4 head x 5 replications x one facility); chickens, at least 100 birds per group (20 birds x 5 replications x one facility); farm-raised aquatic animals, at least 60 aquatic animals per group (30 aquatic animals x 2 replications x one facility).

(a) Animals and replications

Livestock for which the test feed additive is intended should be employed. In principle, the number of animals shall be: cattle, at least 1 head per one dosage; swine, at least 4 head per one dosage; chicken, at least 20 birds per one dosage. As to replication, degrees of freedom of measurement error in each replication shall be within 10, or, if possible, 20. In principle, the number of animals shall be at least 30 per one dosage and the number of replications shall be at least 2 per facility in farm-raised aquatic animals. In addition, arrangement of each group in the feeding facility shall be randomized. Further, concerning farm-raised aquatic animals, environmental conditions shall be considered during the study, and feeding water temperature shall be within the following ranges: yellowtail, red sea bream, carp, and eel, 18-28°C; rainbow trout and silver salmon, 8-18°C; sweetfish, 15-25°C.

(b) Duration of administration

It should be the same period as the test feed additive is actually used. However, when administration extends over a long period, the duration should be until the average body weight of the control group is at least three-fold the initial weight of the farm-raised aquatic animals, except when the test animals are hatchlings.

(c) Administration method

The test feed additive shall be administered continuously after it is added to the diet. In addition, the diet used shall be complete nutritionally, and the feed ingredients and their formulation ratio shall be clearly stated.

(d) Dose

Concerning the efficacy of the test feed additive, a preliminary study to estimate, statistically, the relation between dose and response shall be conducted in advance. The optimum additive dose shall be determined after considering the data of the preliminary study, the basic study and the residue study, etc. In principle, three doses shall be set up, including the highest and lowest doses of the optimum dose. A control is also needed. In

addition, as needed, one group, to which a feed additive already designated with a recommended dose and duration is administered, shall be set up.

(e) Number of facilities

At least three facilities will be required in Japan.

(f) Items for observation

[1] Body weight, feed consumption (feeding amount), feed additive consumption (administration amount of test feed additive), and feed efficiency (body weight increase divided by feed consumption, and so on). If the test period is about 1 week, the measurements shall be done at the start and the finish. If the test period is about 1 month, the measurements shall be done weekly, and when it is 2 months, the measurements shall be done every two weeks.

[2] Clinical signs

Clinical signs of test animals (in farm-raised aquatic animals, whether intake situations, behavior, body color, body shape are normal or not, and so on) shall be observed during the test period. In addition, if the feces are unusually soft, the amount of water intake shall be measured.

[3] Pathological examination

Unhealthy or dead animals should, whenever possible, undergo pathological examination.

(g) Analysis of variance of test result

In principle, analysis of variance shall be done in each facility, and at the same time, total combined data shall be analyzed. In addition, in the case of group feeding, one group is considered as one unit.

However, when feed consumption (feeding amount) of an individual animal is recorded, and each animal used an independent feeding facility, the test results can be considered as the results collected for individual feeding.

(2) Studies to confirm promotion of growth or improvement of feed efficiency (probiotics)

These studies shall provide the data for probiotics.

a Basic studies

These studies aim to prove the effectiveness of the test feed additive on at least one of the following: maintenance or normalization of intestinal flora, reduction of harmful intestinal substances, growth promotion.

(a) *In vitro* studies

(b) *In vivo* studies

The studies shall be conducted using laboratory animals or target animals. Studies to confirm distribution and fixing of the test feed additive (bacteria) are desirable.

b Studies proving the effects caused by combination with antibacterial feed additives.

(a) *In vitro* studies

This study aims to investigate sensitivity of the test feed additive (bacteria) to antibacterial feed additives already designated.

(b) *In vivo* studies

When an *in vitro* study shows high sensitivity and there is the possibility live bacteria will be unable to survive in digestive organs, the following study to determine their influence will be necessary.

In addition, if antibacterial feed additives are the same, the study shall be done with a representative one.

[1] Animals

Livestock for which the test feed additive is intended should be employed. The number of animals shall be: cattle and swine, at least 5 head per group; chicken, at least 10 birds per group.

[2] Duration of administration

Duration of administration of test feed additive (bacteria) combined with antibacterial feed additives shall be at least 1 week. In addition, administration of antibacterial feed additives shall be initiated after the recovery amount (number of bacteria) of the test feed additive (bacteria) from feces is uniform.

[3] Administration method

The test feed additive and antibacterial feed additives, in principle, shall be administered continuously after addition to the diet.

[4] Dose

Concerning the test feed additive (bacteria), the average dose of the highest and lowest doses of the optimum additive dose shall be administered. Concerning antibacterial feed additives, the highest dose of the recommended doses shall be administered.

[5] Items for observation

i Before administration of the antibacterial feed additive

Sampling of feces shall be done every few days after the

initiation of administration of the test feed additive (bacteria), then, it shall be confirmed whether or not the amount (number of bacteria) of the test feed additive (bacteria) per 1 g of feces has become uniform. The uniformity shall be confirmed by observing a similar degree of uniformity of the amount (number of bacteria) of the test feed additive (bacteria) at least 3 times consecutively.

ii After administration of the antibacterial feed additive

Sampling of feces shall be done every day during administration of the test articles combined with antibacterial feed additives to observe the increases and decreases of the test feed additive (bacteria).

c Field trial studies

(a) Efficacy of probiotics as feed additives in the field, in principle, shall be evaluated by the following studies.

In addition, unhealthy or dead animals should, whenever possible, undergo pathological examination.

[1] Animals, replications and facilities

Livestock for which the test feed additive is intended should be employed. In principle, the number of animals shall be: cattle, at least 15 head per group (1 head x 5 replications x 3 facilities, or 1 head x 5 replications x 1 facility x 3 different times); swine, at least 60 head per group (4 head x 5 replications x 3 facilities, or 4 head x 5 replications x 1 facility x 3 different times); chickens, at least 300 birds per group (20 birds x 5 replications x 3 facilities, or 20 birds x 5 replications x 1 facility x 3 different times); farm-raised aquatic animals, at least 180 aquatic animals per group (30 aquatic animals x 2 replications x 3 facilities, or 30 aquatic animals x 2 replications x 1 facility x 3 different times).

In addition, at least one facility shall be located in Japan.

However, for farm-raised aquatic animals, studies are not necessary for each species of animal for which the test feed additive is intended, and studies should be done with at least 1 species of animals for which the test feed additive is intended after subdividing the group into 2: one group raised in seawater and the other in fresh water.

Further, environmental conditions shall be considered during the study, and the feeding water temperature shall be within the following

ranges: yellowtail, red sea bream, carp, and eel, 18-28°C; rainbow trout and silver salmon, 8-18°C; sweetfish, 15-25°C.

[2] Duration of administration

According to (1)-b-(b)

[3] Dose

The treatment group considered as receiving the optimum additive dose (hereinafter called [estimated optimum additive dose group]) is the study group. A control group is also needed.

In addition, it is acceptable to set up a number of treatment groups (including the estimated optimum additive dose group).

[4] Items for observation

During the test period, in principle, the following items shall be observed.

i Body weight

ii Feed consumption (feeding amount)

iii Amount of intake of the test feed additive (bacteria)
(administration amount of the test feed additive (bacteria))

iv Feed efficiency

v Clinical signs

b Where statistical analysis according to (a) does not show a significant difference, an additional study shall be conducted, then total data, including the additional study data, shall be analyzed.

In addition, before the additional study is conducted, it would be desirable to consider the study scale after analyzing the results collected in (a).

(Example of statistical analysis)

$n>2t^2 \cdot s^2/d^2$

n: number of replications required

t: degrees of freedom 2n-2, t value against significance level α found in the t - table

s^2: error variance in the previous study

d: difference of means in the previous study

(3) Studies to confirm improvement of digestibility

These studies shall provide the data for enzymes, etc.

a Basic studies

(a) In vitro study

(b) In vivo study

b Field trial studies

These studies aim to confirm the improvement of digestibility of each feed ingredient by the test feed additive using target animals.

Livestock for which the test feed additive is intended should be employed.

Further, at least one facility shall be located in Japan.

In addition, unhealthy or dead animals should, whenever possible, undergo pathological examination.

(a) Chickens

[1] Animals

Chickens fitted with an artificial anus shall be used. The number shall be at least 4 birds per group.

[2] Duration of administration

At least 6 days

[3] Dose

The study group shall be an estimated optimum additive dose group. A control group is also needed.

In addition, it is acceptable to set up a number of treatment groups (including the estimated optimum additive dose group).

[4] Diet

The basal diet shall be formula feed that includes sufficient and well-balanced nutrients as shown in the Japanese Feeding Standards; 0.5-1.0% of chromium oxide as an indicator substance shall be mixed 0in uniformly. Particle size of the diet shall be a mash that cannot be sorted by animals while eating.

However, the addition of oils and fats shall be avoided.

[5] Sampling of feces

The test animals shall be kept individually in metabolism cages, etc., and administered about 80 g of diet per animal per day, after taking care that there is no clogging of feces. Then, sampling shall be done by collecting individual feces that have accumulated during at least 2 days, 5 days after the initiation of feeding. Samples shall be dried by forced air oven (about 60°C) and then air-dried. Samples shall be prepared for analysis by grinding.

[6] Analysis

Proximate analysis shall be done using test methods based on prescriptions of the Regulations for Enforcement. Chromium oxide shall be analyzed by colorimetry.

[7] Calculation of digestibility

Calculation will be done using an index method formula.

(b) Swine

[1] Index method with chromium oxide as indicator substance

i Animals

25-50 kg fattening pigs shall be employed. The number shall be at least 4 head per group.

ii Duration of administration

At least 9 days

iii Dose

The study group shall be the estimated optimum additive dose group, and a control group is also needed.

In addition, it is acceptable to set up a number of treatment groups (including the estimated optimum additive dose group).

iv Diet

The basal diet shall be formula feed that includes sufficient and well-balanced nutrients as shown in the Japanese Feeding Standards; 0.1-0.2% of chromium oxide as an indicator substance shall be mixed in uniformly. Further, a stiff kneaded state of feed, by adding water, is applicable.

v Sampling of feces

The test animals shall be kept individually in metabolism cages, etc., and administered about 3% of body weight of feed per head per day, to maintain the body weights of test animals. Then, sampling shall be done by collecting individual feces that have accumulated during at least 5 days, 5 days after the initiation of feeding. Samples shall be dried by forced air oven (about 60°C) and then air-dried. Samples shall be prepared for analysis by grinding.

Sampling shall be done at fixed times, at least twice (in the morning and the afternoon) every day. In addition, in principle, all feces sampled shall be dried. However, dried feces with some moist parts that have been mixed uniformly are also applicable instead of feces that are all dried.

vi Analysis

Proximate analysis shall be done using test methods based on prescriptions of the Regulations for Enforcement.

Chromium oxide shall be analyzed by colorimetry.

vii Calculation of digestibility

Calculation shall be done using an index method formula.

[2] Total collection method (in the case of fibrous feed)

i Animals

Adult swine that are older than 8 months shall be employed. The number shall be at least 4 head per group.

ii Duration of administration

At least 10 days

iii Dose

The study group shall be the estimated optimum additive dose group, and a control group is also needed.

In addition, it is acceptable to set up a number of treatment groups

(including the estimated optimum additive dose group).

iv Diet

The basal diet shall be formula feed that includes sufficient and well-balanced nutrients as shown in the Japanese Feeding Standards. Further, a stiff kneaded state of feed, by adding water, is applicable.

v Sampling of feces

The test animals shall be kept individually in metabolism cages, etc. and administered less than 3 kg (on a dry basis) per head per day, in such a way that no feed is left over. Then, sampling shall be done by collecting the whole feces of individuals that have accumulated during at least 5 days, 5 days after the initiation of feeding. Samples shall be dried by forced air oven (about 60°C) and then air-dried Samples shall be prepared for analysis by grinding. Sampling shall be done at fixed times, at least twice (in the morning and the afternoon) every day.

vi Analysis

Proximate analysis shall be done using test methods based on prescriptions of the Regulations for Enforcement.

vii Calculation of digestibility

Calculation is to be done using the total collection method based on feed consumption, amount of feces and those analyzed values.

(c) Cattle

[1] Animals

The number of test animals shall be at least 4 head per group.

Sheep or goats instead of cattle are also applicable.

If the feeding style is to be changed radically, at least 14 days of preconditioning time will be necessary before the initiation of study.

[2] Duration of administration

At least 14 days

[3] Dose

The study group shall be the estimated optimum additive dose group, and a control group is also needed.

In addition, it is acceptable to set up a number of treatment groups (including the estimated optimum additive dose group).

[4] Diet

The basal diet shall include less than 60% of concentrated feed (on a dry basis), at least 12% of crude protein content, and at least 15% of crude fiber content. Other sufficient and well-balanced nutrients shall be included. Hay shall be used as roughage.

[5] Sampling of feces

The test animals shall be kept individually in metabolism cages, etc. and administered the estimated amount of feed, including more for energy maintenance, and crude protein content shall be at least that required for maintenance. Adjust the feed amount so that none is left over. Then sampling shall be done by collecting whole feces of individuals that have accumulated during at least 7 days, 8 days after the initiation of feeding. The sampling shall be done at a fixed time every 24 hours (as the longest interval). Feces samples shall be collected at a proportionally fixed rate against the total amount of feces after uniform mixing and weighing. Samples collected shall be sealed and stored in a freezer.

After collecting all samples, the stored samples shall be mixed well, dried by forced air oven (about 60°C) and then air-dried. Samples shall be prepared for analysis by grinding. However, regarding water and crude protein content, in principle, fresh mixed samples shall be used for analysis.

In addition, when sheep or goats are employed as test animals, whole feces of individuals shall be collected and dried by forced air oven

(about 60°C). Then, dried samples shall be restored to air-dry state, weighed, sealed and stored. After collecting all samples, stored samples shall be mixed well. Some of the samples shall be prepared for analysis by grinding.

[6] Analysis

Proximate analysis shall be done using test methods based on prescriptions of the Regulations for Enforcement.

[7] Calculation of digestibility

Calculation will be done using the total collection method based on feed consumption, amount of feces and those analyzed values.

(d) Farm-raised aquatic animals

[1] Animals and replications

The number of test animals shall be restricted so as not to prevent normal intake of feed.

In addition, the number of replications shall be at least 2. In these cases, there are two options: one is to conduct tests using at least 2 water tanks at the same time, and the other is to conducted tests using one tank at at least 2 different times. Furthermore, environmental conditions shall be considered during the study, and feeding water temperature shall be within the following ranges: yellowtail, red sea bream, carp, and eel, 18–24°C; rainbow trout and silver salmon, 8–14°C; sweetfish, 15–21°C.

[2] Duration of administration

At least 9 days

[3] Dose

The study group shall be the estimated optimum additive dose group, and a control group is also needed.

In addition, it is acceptable to set up a number of treatment groups (including the estimated optimum additive dose group).

[4] Diet

A nutritionally complete basal diet shall be used. Feed ingredients and formulation ratio shall be clearly stated; 0.1-0.2% of chromium oxide as an indicator substance shall be mixed in uniformly.

[5] Sampling of feces

The test animals shall be kept in tanks for collecting feces; sampling shall be done by collecting feces that are naturally excreted for at least 3 days, 7 days after the initiation of administration.

[6] Analysis

Analysis shall be done in each tank, and proximate analysis shall be done using test methods based on prescriptions of the Regulations for Enforcement. Chromium oxide shall be analyzed by colorimetry.

[7] Calculation

Calculation is to be done using the index method formula.

(4) Studies to confirm improvement of palatability.

This study aims to confirm improvement of feed palatability with the test feed additive using livestock for which the test feed additive is intended. These studies shall provide the data for flavor and taste substances, etc.

a Studies by free choice method

(a) Animals and replications

Livestock for which the test feed additive is intended should be

Employed. In principle, the number of animals shall be: cattle and swine, at least 1 head per dose group; chickens, 10 birds per dose group. Replications, set up as degrees of freedom caused by measurement error of replication, should be at least 10, and if possible, at least 20.

In addition, arrangement of each test group in the feeding facility shall be randomized.

(b) Duration of administration

At least 1-2 weeks

(c) Administration method

There shall be two kinds of diet which can be freely eaten by test animals, one is the treatment diet, which contains the test feed additive, as the highest dose of the estimated optimum dose, and the other is the control diet which has no test feed additive.

In this case, being in the same test barn, the conditions of the placement of the two diets shall be fully considered. The placement of feeders, if possible, should be changed every day.

In addition, the diet used shall be nutritionally complete, and the feed ingredients and their formulation ratio shall be clearly stated.

(d) Number of facilities

At least one facility shall be located in Japan.

(e) Items for observation

[1] Feed consumption (feeding amount) of treatment diet and control diet shall be measured every day.

[2] Clinical signs

Clinical signs of test animals shall be observed during the test period.
In addition, unhealthy or dead animals should, whenever possible, undergo pathological examination.

b Studies by separation method

(a) Animals, replications and facilities

Livestock for which the test feed additive is intended should be employed.
In principle, the number of animals shall be: cattle, at least 5 head per group (1 head x 5 replications x 1 facility); swine, at least 20 head per group (4 head x 5 replications x 1 facility); chickens, at least 100 birds per group (20 birds x 5 replications x 1 facility); farm-raised aquatic animals, at least 60 aquatic animals per group (30 aquatic animals x 2 replications x 1 facility).
At least one facility shall be located in Japan.
In addition, for farm-raised aquatic animals, environmental conditions shall be considered during the study, and the feeding water temperature shall be within the following ranges: yellowtail, red sea bream, carp, and eel, 18-28°C; rainbow trout and silver salmon, 8-18°C; sweetfish, 15-25°C.

(b) Duration of administration

According to (1)-b-(b)

(c) Dose

The study group shall be the estimated optimum additive dose group, and a control group is also needed.
In addition, it is acceptable to set up a number of treatment groups (including the estimated optimum additive dose group).

(d) Items for observation

During the test period, in principle, the following items shall be observed.

[1] Body weight
[2] Feed consumption (feeding amount)
[3] Amount of intake of the test feed additive (administration amount of the test feed additive)
[4] Clinical signs

Clinical signs of test animals shall be observe during the test period.
In addition, unhealthy or dead animals should, whenever possible, undergo pathological examination.

(5) Studies to confirm the effectiveness to prevent a decrease in productivity caused

by specific pathogenic parasites

This study aims to confirm the effectiveness of antibiotics and antibacterial substances, etc. to prevent decrease of productivity caused by specific pathogenic parasites. Livestock for which the test feed additive is intended should be employed. This study aims to confirm the effectiveness of the test feed additive to prevent decrease of productivity caused by specific pathogenic parasites in the field.

In principle, studies shall be conducted according to (1). The study plan shall fully consider whether the study method adopted can clearly evaluate the effectiveness of the test feed additive.

II Residue Studies

1. Usual addition

(1) Objective

The objective of these studies is to make clear information about residue of the test feed additive in target animals and its products.

(2) Place for taking samples

Samples are to be collected from test animals raised in at least two different places, which shall be located in Japan.

(3) Animals

a Livestock for which the concerned test feed additive is intended should be used. Animal(s) should be obvious with the previous feeding record of feed, feed additives, feeding conditions etc. before the study.

b Animal(s) to be used should be in sufficient numbers to obtain samples necessary to measure residue from the test feed additive as well as to make clear the fate of the test feed additive. The number of test animals shall be used as follows: cattle, at least 2 head per dose group per sampling time; swine, at least 3 head per dose group per sampling time; chickens (females are more desirable), at least 3 birds per dose group per sampling time (if one bird is not sufficient for the necessary sample amount, one sample shall be prepared by collecting from 2 or more birds); farm-raised aquatic animals, the number which will offer sufficient sample amount for analysis and will not disturb the appropriate feed intake.

c For farm-raised aquatic animals, environmental conditions shall be considered

during the study, and the feeding water temperature shall be within the following ranges: yellowtail, red sea bream, carp, and eel, 18-28°C; rainbow trout and silver salmon, 8-18°C; sweetfish, 15-25°C.

(4) Duration of administration

It should be the same period as the test feed additive is actually used. However, when it extends over a long period, an appropriate period can be set up based on the data obtained from a preliminary study. In this case, a rational explanation shall be given as to why sample residue levels will become fixed values after consecutive administrations during a certain period and will not change later on.

(5) Administration method

The test feed additive shall be administered continuously after adding it to the diet.

The diet shall be considered to being taken uniformly by test animals during administrations.

(6) Dose

In principle, the highest recommended dose of test feed additive shall be thought of as the lowest dose for residue trials. To investigate the relation between dose and residue, several and tens times the amount of test feed additive shall be administered. A control group is also needed.

The highest dose shall be the dose level that will not apparently reduce the feed consumption of test animals.

(7) Diet

If official standards are set for the formula feeds, then this kind of feed shall be used as the basal diet.

Also, addition of feed additives to the basal diet other than the test feed additive (except for vitamins, minerals, amino-acids, etc.) shall be strictly avoided. However, if these feed additives are critical for the feeding of test animals, only feed additives that never interfere with test feed additive analysis are to be used.

(8) Sampling

a A time schedule necessary to collect samples to make clear the fate of the test feed additive should be established.

b Samples should be collected, in principle, from edible sites. In this case, it is

necessary to collect samples in such a manner as to make clear distribution of the test feed additive in such sites as muscle, fat, liver (hepatopancreas), kidney, small intestine, egg, and milk.

c Samples should be stored at 0-5°C after collection and analyzed promptly. Long-term preservation shall be avoided, however, when unavoidable, samples should be frozen. In such a case, it shall be confirmed that the samples were not be resolved during the process of freezing and thawing.

(9) Analysis

a For these residue studies, it is necessary to establish an adequately sensitive, accurate and reproducible analytical method. For these purposes, adequate sensitivity, accuracy and reproducibility, means that detection limits shall be less than 0.05 ppm, recovery rate shall be at least 70% in the recovery study after adding 1-2 ppm of substance, and coefficient of variation (standard deviation/mean) shall be less than 0.1.

When the sample is an antibiotic, the analytical method shall be based on a biological method or a chemical method.

b Analysis should be made for residue of the active ingredients of a test article. However, when the residue of metabolites needs to be made clear, the metabolites shall also be analyzed.

c Analyzed values shall not be deducted from control values, but shall be recorded as they are measured. In addition, no correction of recovery rate should be made.

d When a measurement is less than the detection limit (X ppm), the results shall not be recorded as “not detectable,” but recorded as less than X ppm.

e When results include values less than X ppm, calculation of the mean should not be done.

2. Micro-amount addition

(1) Objective

The objective of these studies is to clarify residue presence in target animals as well as products by administrating feed that includes a very small amount of test feed additive.

If it is obvious that a test article is difficult to be absorbed, the study can be omitted.

(2) Collection sites for samples

Samples shall be taken from test animals raised in at least one place, which shall be located in Japan.

(3) Animals

a Livestock, including laying hens, for which the test feed additive is intended, shall be used.

b The number of test animals shall be six or more per each dose group in the case of laying hens; for other animals the number shall be as specified in 1., (3), b.

(4) Duration of administration

At least 4 weeks

(5) Administration method

According to 1-(5)

(6) Dose

In principle, the dose shall be set at the lowest dose, which is about one percent of the recommended dose, and then doses shall be set at several times and ten times higher than the lowest dose. A control group is also needed.

(7) Diet

According to 1-(7)

(8) Sampling

According to 1-(8)

(9) Analysis

According to 1-(9)

III Taxonomy of Bacteria, etc.

Concerning probiotics, safety of bacteria shall be confirmed by clarifying the taxonomy of bacteria, origin of bacteria, experience of use of bacteria, and products of bacteria, etc.

IV Single-Dose Toxicity Studies

1. Objective

These studies aim to make clear toxicity of the test feed additive by calculateion of lethal doses following administration of single doses of the test additive to animals.

2. Animals

(1) It is desirable to use at least one non-rodent species, in addition to not less than one rodent species (rat, mouse, etc.). For rodents, not less than 5 each of males and females should be used for each dose group; and for non-rodents, 2-3 males or females for each dose group.

(2) For rats, mice, etc., animals of 5-6 weeks of age should be used and the average body weights of each dose group should be approximately the same at the initiation. (This requirement applies to all animal studies specified hereinafter.)

3. Method of administration

At least two routes of administration, oral and non oral (subcutaneous injection, intraperitoneal injection or intravenous injection), should be used for rodents and the oral route for non-rodents. In the case of probiotics, the oral route should be used. The test feed additive should be dissolved, wherever possible, in water, edible oil or other appropriate solvent. If this is impossible, some suspending medium should be employed.

4. Dose

Study groups should be set up with 3 or more dose groups to results in comprehensive toxicity of the test feed additive. A control group (given solvent alone) is also needed. In principle, the dose levels should be sufficient to obtain the approximate lethal dose for rodents, and to clarify toxicological effects for non-rodents.

In addition, for oral administration, 2,000 mg/kg shall be set as the upper limit, and for probiotics, the maximum volume infusible to animals shall be the upper limit.

5. Items for observation

Test animals should be fed and observed for at least two weeks to determine the approximate lethal dose, severity of toxicological effects, and onset and progression of toxicological signs and recovery from them, etc. Necropsies should be conducted on all animals that die during the study and several of the surviving animals should undergo gross examination. When abnormalities are observed by gross examination results, histological examination should be also conducted.

V Repeated-Dose Toxicity Studies (Short-term)

1. Objective

   These studies aim to clarify toxicity of the test feed additive by administering it to animals continuously for at least three months.

2. Animals

   At least 2 species, including one rodent species or more, should be used. For rodents, not less than 10 each of males and females for each dose group, and for non-rodents not less than 2 each of males or females for each dose group should be used.

   However, for probiotics, rats or mice should be selected for the study.

   In addition, young animals, or soon after weaning, should be used to allow observation during the growth phase.

3. Duration of administration

   The usual duration of administration is a period of 3 months for rodents and 6 months for non-rodents.

4. Administration method

   In principle, the test feed additive should be administered to the test animals by giving continuously diet or drinking water admixed or dissolved it.

5. Dose

   There should be 3 phased doses or more, which shall include one dose that has no effect and another dose that has some toxic effect on the test animals. In addition, a control group is also needed.

   The concentration of the test feed additive added to the diet shall be less than 5 w/w%.

6. Observations and measurements

   Minimally, the animals should be observed for the following items in order to evaluate toxicity of the test feed additive sufficiently.

   In addition, for (1)-(4) mentioned below, observations or measurements shall be conducted every week for the first 13 weeks of the study and then every two weeks for the remaining period of the study.

   (1) Body weight

(2) Feed consumption, the test feed additive intake and water consumption, when the test feed additive is dissolved in drinking water

(3) Feed efficiency

(4) Clinical signs and mortality

(5) Hematological examinations

Hematocrit, hemoglobin, erythrocyte, leukocyte and differential leukocyte counts shall be determined.

(6) Blood chemistry examinations

LDH, GOT, GPT, glucose, urea nitrogen, bilirubin, alkaline phosphatase, cholesterol, albumin, globulin, and total protein shall be determined.

(7) Ophthalmological examinations

For rodents, this examination shall be conducted for at least the highest dose group and the control group before administration of the test feed additive and again at the termination of the study. When abnormalities are observed in the highest dose group, all the test animals shall be examined.

(8) Urinalyses

All of the surviving animals shall be examined as to urine appearance, protein, glucose, ketone bodies, and occult blood, etc. at the termination of the study as needed.

(9) Pathological examinations

The following data should, also be obtained on as many organs as possible, in principle. Furthermore, animals found dead during the study should undergo necropsy to determine the cause of death.

a Gross observation

b Organ weight (absolute and relative to body weight)

For rodents, liver, kidneys, heart, testes (ovaries) and brain shall be weighed. In addition, for non-rodents, thyroid gland, adrenal glands and pituitary gland, etc. shall also be weighed.

c Preservation of organs and tissues

The following organs and tissues shall be preserved in order to conduct future histopathological examinations as necessary.

All gross lesions, skin, brain, pituitary gland, thyroid gland (including parathyroid gland), thymus, lungs (including trachea), heart, sternum, salivary glands, liver, spleen, kidneys, adrenal glands, pancreas, gonads, uterus and genital adnexa, mammary glands of the females, muscles, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, lymph nodes, peripheral nerves, spinal cord, eyes, gallbladder, and aorta.

d Histopathological examinations

For non-rodents, all of the test animals used for the study shall undergo histopathological examinations. For rodents, the following test animals shall undergo histopathological examinations.

[1] All test animals in the control group and the highest dose group

[2] All test animals found dead or sacrificed in moribund status during the study

[3] All gross lesions from the test animals examined grossly

[4] Target organs of all the test animals used for the study

[5] Lungs, liver and kidneys of all the test animals used for the study

Any tissues or organs that reveal changes attributable to the administration of the highest dose shall be examined histopathologically also in the other groups.

VI Repeated-dose Toxicity Studies (Long-term)

1. Objective

These studies aim to make clear toxicity of the test feed additive by long-term continuous administration to animals.

2. Animals

At least 2 species, including one rodent species or more, should be used. For rodents, not less than 10 each of males and females for each dose group, and for non-rodents not less than 4 each of males or females for each dose group should be used.

In addition, in principle, young animals, soon after weaning, should be used.

3. Duration of administration

The usual duration of administration is a period of 24 months for rats, 18 months for mice, or 12 months for non-rodent species.

4. Administration method

In principle, the test feed additive should be administered to the test animals by giving continuously diet or drinking water admixed or dissolved it.

5. Dose

With reference to the results of the repeated-dose toxicity study (short-term), some phased doses should be selected so as to include one dose that has no effect and another dose that has some toxicological effects on the test animals. A control group is also needed.

The test feed additive concentration to be added to the diet shall be less than 5 w/w%.

6. Observations and measurements

Minimally, the animals should be observed for the following items in order to evaluate toxicity of the test feed additive sufficiently.

In addition, for (1)-(4) mentioned below, observations or measurements shall be conducted every week for the first 13 weeks of the study and then every two weeks for the remaining period of the study.

(1) Body weight

(2) Feed consumption, the test feed additive intake and water consumption, when the test feed additive needs to be dissolved in drinking water

(3) Feed efficiency

(4) Clinical signs and mortality

(5) Hematological examinations

Hematocrit, hemoglobin, erythrocyte, leukocyte, and differential leukocyte counts shall be determined.

(6) Blood chemistry examinations

LDH, GOT, GPT, glucose, urea nitrogen, bilirubin, alkaline phosphatase, cholesterol, albumin, globulin, and total protein shall be determined.

(7) Ophthalmological examinations

For rodents, this examination shall be conducted for at least the highest dose group and the control group before administration of the test feed additive and at the termination of the study. When abnormalities are observed in the highest group, all test animals shall be examined.

(8) Urinalyses

All of the surviving animals shall be examined for urine appearance, protein, glucose, ketone bodies, and occult blood, etc. at the termination of the study, as needed.

(9) Pathological examination

The following data should, in principle, also be obtained on as many organs as possible. Furthermore, the animals found dead during the study should undergo necropsy to determine the cause of death.

a Gross observation

b Organ weight (absolute and relative to body weight)

For rodents, liver, kidneys, heart, testes (ovaries), and brain shall be weighed. In addition, for non-rodents, thyroid gland, adrenal glands and pituitary gland, etc. shall also be weighed.

c Preservation of organs and tissues

The following organs and tissues should also be preserved to conduct future histopathological examinations as needed.

All gross lesions, skin, brain, pituitary gland, thyroid gland (including parathyroid gland), thymus, lungs (including trachea), heart, sternum, salivary glands, liver, spleen, kidneys, adrenal glands, pancreas, gonads, uterus and genital adnexa, mammary glands of the females, muscles, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, lymph nodes, peripheral nerve, spinal cord, eyes, gallbladder, and aorta.

d Histopathological examination

For non-rodents, all of the test animals used for the study shall undergo histopathological examination. For rodents, the following test animals shall undergo histopathological examination.

[1] A11 test animals in the control group and the highest dose group

[2] All test animals found dead or sacrificed in moribund status during the study

[3] All gross lesions from the test animals examined grossly

[4] Target organs of all the test animals employed for the study

[5] Lungs, liver and kidneys of all the test animals employed for the study

Any tissues or organs that revealed changes attributable to the administration of the highest dose shall be examined histopathologically also in the other groups.

VII Multi-generation reproduction Studies

l. Objective

The studies aim to make clear the effects of the test feed additive on reproduction of parents as well as the effects on offspring by administering the test feed additive to male and female animals for several-generations.

2. Animals

At least 1 species, including rats, should be used. The number of pregnant female animals for each dose group should be about 20 for rats.

3. Number of generations

In principle, no fewer than two generations should be studied. In addition, mating to produce the third generation shall be done for further evaluation on offsprings as needed.

4. Administration method

In principle, the test feed additive should be administered by adding it to the diet or dissolving it in the drinking water.

5. Dose

At least three phased doses should be set up in order to determine the dose-response. A control group is also needed. The highest dose should be set at a level at which the test feed additive produces toxicological effects but not lethal in parent animals. The lowest dose should be set at a level at which the test feed additive produces no effects on parent and offspring animals. The concentration of the test feed additive in the diet should not exceed 5 w/w%.

6. Mating and administration of the test feed additive

(1) Parent animals (F_0) should begin receiving the test feed additive soon after weaning (in rats, less than five weeks of age), then, after three months, one male

and one female, in principle, are allowed to cohabit for an appropriate period of time (in rats, about two weeks). During this period, each pair is checked daily in order to determine the evidence of copulation.

(2) The female animals with the evidence of copulation should be isolated and raised on the basis of one animal per cage, and allowed to deliver naturally the first litter (F_1) of the first generation. In principle, in the case of rats, each litter size is adjusted to a size of four male and four female pups by random selection and allowed to be nursed by parent animals.

Parent animals should be continuously administered the test feed additive during both gestation and lactation.

(3) F_1, like F_0, should be administered the test feed additive after weaning (in case of rats, about three weeks of age). In principle, at least 20 males and 20 females should be selected and mated three months after birth in the same manner as F_0 to obtain the first litter (F_2) of the second generation.

(4) Non-copulation males and females should be investigated. In order to investigate the causes, histopathological examination of genital organs, re-mating with another female or male, estrus and spermatogenesis cycles should be examined.

7. Observations and measurements

Minimally, the following parameters should be recorded to conduct necessary analyses.

(1) Body weight

(2) Feed consumption, the test feed additive intake, and water consumption, when the test feed additive is dissolved in drinking water

(3) Feed efficiency

(4) Clinical signs and mortality

(5) Mating, fertility and parturition indices

(6) Number of implantations

(7) Birth index

(8) Lactation index

8. Notes

(1) F_0

a After the weaning of F_1, parent animals shall be necropsied to observe thoroughly the internal organs and to investigate the number of implantations, etc.

b. The females which was not pregnant and the males should also be necropsied at appropriate times to investigate thoroughly internal organs, especially genital organs.

(2) F_1

a All delivered pups, alive or dead should be counted, sexed and examined for their external abnormalities. The body weights of newborns shall be recorded.

b Observation of clinical signs and measurement of body weights shall be conducted at least every week. F_1 weanlings shall be raised until sexual maturation to investigate for growth, development, and specific clinical signs.

c F_1, used for mating to produce F_2, shall be investigated as was done for F_0.

(3) F_2

The animals shall be investigated as was done for F_1, and then autopsied after weaning.

(4) Histopathological or biochemical examinations shall be conducted as needed.

VIII Teratogenicity Studies

1. Objective

These studies aim to make clear the effects of the test feed additive administered to pregnant animals for the period of fetal organogenesis on fetal development, in particular, its teratogenicity to fetuses.

2. Animals

From such rodent species as rats and mice, and also from such non-rodent species as rabbits, at least 1 of each species should be selected for the studies. Number of

pregnant female animals for each dose group should be about 20 for rats and mice, and 12 for rabbits.

3. Duration of administration

Administration should be done during the period of fetal organogenesis. Usually this is day 7 to day 17 of pregnancy (or day 6 to day 15 of pregnancy) for rats; day 6 to day 15 of pregnancy for mice; and day 6 to day 18 of pregnancy for rabbits. In principle, administration shall be done once a day consecutively for this period.

The day when a vaginal plug or sperm in a vaginal smear is found, or the next day when mating is confirmed or insemination is done artificially, shall be designated as day 0 of pregnancy.

4. Administration method

In principle, oral administration by gavage should be used.

5. Dose

At least three phased doses should be set up to determine the dose-response relationship. A control group is also needed. The highest dose shall be high enough to cause such toxicological effects as suppressed body weight gain in the parent animals unless the administration of such a high dose is restricted due to the physicochemical properties of the test feed additive.

The lowest dose should be such a level at which the test feed additive produces no toxicological effect on the parent animals and fetuses. The intermediate dose should be determined geometrically between the highest and lowest doses.

It would be desirable to select the doses based on the results of a preliminary study. If the highest dose that was the maximum volume physically infusible to animals did not produce any toxicological effects on the parent animals and fetuses in the preliminary study, a dose of 1,000 mg/kg shall be employed as the highest dose.

6. Observations and measurements

Minimally, the following items should be observed or measured during the study, and necessary analyses on the results should be conducted.

(1) Maternal animals

a Body weight

b Feed consumption, and water consumption where necessary

c Clinical signs and mortality

d Number of corpora lutea
e Number of implantations
f Genital organs

(2) Fetuses
a Body weight
b Sex
c Embryonic or fetal deaths, such as embryonic resorption, etc.
d External anomalies
e Skeletal anomalies
f Visceral anomalies

(3) Histopathological examination if needed.

IX Carcinogenicity Studies

1. Objective

These studies aim to make clear the effects of life-time administration of the test feed additive to animals, particularly its potential carcinogenicity.

2. Animals

At least one rodent species such as rats, mice, etc. should be used. It is desirable to use animals of a strain (e.g. an inbred strain or strain derived from an inbred strain) in which the type and frequency of spontaneous tumors are well documented. It is desirable to use not less than 50 males and 50 females for each dose group and about 50 animals per sex for a control group.

In principle, young animals, soon after weaning, should be used.

3. Duration of administration

For rats, duration shall be in a range from 24 to 30 months, and for mouse, from 18 to 24 months.

Where carcinogenicity by the exposure through placenta or maternal milk is suspected, it is necessary to conduct another carcinogenicity study at least in one species of animals administered throughout two succeeding generations.

4. Administration method

In principle, the test feed additive should be administered continuously by adding it to

the diet or the drinking water.

5. Dose

At least three phased dose groups and a control group should be prepared for the study. The highest dose should be set at a level at which the test feed additive is expected to affect the incidence of tumors and thereby the longevity of test animals.

The concentration of the test feed additive in the diet should not exceed 5 w/w%.

6. Observations and measurements

Minimally, the following observations and measurements should be conducted during the study.

(1) General parameters

a Body weight

Measurements should be conducted every week from the study initiation up to 13 weeks, every two weeks up to 6 months, and then every month until the study completion.

b Feed consumption, the test feed additive intake, and water consumption, when the test feed additive is dissolved in drinking water, should be measured every week.

c Clinical signs

Should be checked daily.

d Survival rate

Should be recorded monthly.

e Others

Hematological examinations, blood chemistry examinations, urinalyses, etc. should be conducted whenever needed.

(2) Pathological examination

All the animals should be examined for the following. Even moribund animals should be sacrificed for the same examinations.

a Gross pathology

(a) Lesions of suspected tumors

(b) Gross findings on various organs

(c) Weights of major organs

b Histopathological examination

(a) Histologically identified tumors

(b) Precancerous lesions in various organs and tissues

(c) Histological examination on serially sectioned tissue specimens is desirable for small organs such as pituitary gland, thyroid gland and adrenal glands.

X Mutagenicity Studies

1. Objective

These studies aim to make clear mutagenicity of the test feed additive.

2. Study method

In principle,an *in vitro* reversion test and a chromosome aberration test should be conducted, and if any of these tests indicates possible mutagenicity of the test article, a micronucleus test should be conducted. Other tests should be conducted if needed.

(1) Reversion test

a Species and strain

TA1535, TA1537, TA1538, TA98, and TA100 of *Salmonella typhimurium* and WP2*uvr*A of *Escherichia coli*, etc. should be used.

Other species and strains should be added if needed.

b Dose levels

5-6 levels of test doses should be set up, in addition to the controls. In principle, the highest dose should be up to 5 mg/plate, and if the test feed additive has antibacterial properties, the dose level should be set at a level that shows antibacterial activities.

c Control substances

Negative and positive controls should be included in the test system. In principle, the negative control shall be a solvent control. The positive control shall be a known mutagenic substance.

Both positive control substances either of which requires or does not require a metabolic activation system, S9 mix, should be used.

d Metabolic activation

The tests should be conducted under a condition with and also without S9 mix.

e. Study method

Pre-incubation method or plate method should be used.

f Results

Actual measurement values of revertant colonies and their average values should be expressed and illustrated.

(2) *In vitro* chromosome aberration test

a Test cells

Primary culture or subculture cells of mammals should be used.

b Dose levels

At least three phased dose levels shall be set up.

The highest dose shall be a concentration to inhibit cellular growth or mitosis at 50% or higher. When no cytotoxicity is observed in a preliminary study, the highest dose shall be a concentration corresponding to 10 mM or 5 mg/ml.

When the test feed additive is not practically insoluble in water, it shall be dissolved or uniformly suspended in dimethylsulfoxide, sodium carboxymethyl cellulose, etc.

c Control substance

In principle, the negative control shall be a solvent control. The positive control shall be a known substance inducing chromosome aberration.

d Metabolic activation

A proper metabolic activation system (S9 mix, etc.) shall be concomitantly employed for the study.

e Examination method

Cell culture specimens shall be prepared 2 times with adequate intervals and adequate timing after treatment with the test feed additive. At least two plates per dose shall be used, and then 100 metaphase cells per plate shall be examined for cells with morphological chromosomal anomalies and polyploid cells.

f Results

The incidences of cells with chromosomal abnormalities and polyploid cells shall be reported.

(3) Micronucleus test

a Animals

Mice, at least 5 animals per dose group, shall be used as the test animals.

b Duration of administration

Single and 4-5 consecutive administrations shall be conducted. In the case of consecutive administrations, an adequate single dose shall be determined.

c Administration method

An intraperitoneal or oral route shall be employed for the administration. For the oral route, as a rule, administration by gavage is required.

d Dose

At least three dose levels shall be set up. The highest dose shall be selected so as to produce some toxicological effefts such as retarded weight gain. If any toxicological effects are not observed, 2,000 mg/kg shall be employed as the highest dose.

Negative and positive controls are also needed. The negative control, as a rule, shall be a solvent control. The positive control shall be a substance known to induce micronuclei.

e Observations and measurements

All animals in each group shall be sacrificed 18-30 hours after administration of the test feed additive and a bone marrow smear shall be prepared.

In principle, 1,000 polychromatic erythrocytes per animal shall be scored for the presence of micronuclei. In addition, the ratio of polychromatic to normochromatic erythrocytes shall be determined. It is acceptable to count the incidence of reticulocytes instead of polychromatic erythrocytes.

3. Other tests

(1) Tests indicating gene mutation

a Gene mutation test in cultured mammalian cells

b Gene mutation test in *Drosophila sp.*

c Spot test in mice

d Specific locus test in mice

(2) Test indicating chromosome aberration

a Chromosome aberration test in rodent reproductive cells

b Dominant lethal test in rodent species

c Reciprocal translocation test in mice

(3) Test indicating DNA damages

a Prophage induction test in bacteria

b DNA repair test in bacteria

c Unscheduled DNA synthesis (UDS) test in mammalian cells

d Sister chromatid exchange (SCE) test in mammalian cells

(4) Other tests

a Somatic cell recombinant test and gene exchange test in yeast

b Test for morphological anomaly of sperm in mice

XI Metabolism Studies

1. All feed additives except for probiotics

(1) Objective

These studies aim to determine absorption, distribution, accumulation, metabolism, and excretion of the test feed additive when administering the test feed additive to animals and to clarify its *in vivo* pharmacokinetics.

(2) Animals

Livestock for which the test feed additive is intended shall be employed, and matured rats, and rabbits, etc. should additionally be employed where needed. The animals to be tested should be of sufficient number to obtain findings that allow evaluation.

(3) Administration method

In principle, the test feed additive should be administered once orally, but it is desirable to employ consecutive administrations as well. In accumulation studies, the test additive should be administered consecutively over sufficient time periods. Furthermore, *in situ* and *in vitro* studies should be conducted as needed.

(4) Dose

The dose should be the lowest level that is sufficient to fulfill the objective of the study, and that produces sufficient residue amounts of the test feed additive or its metabolites in each tissue or excrement after administration from the viewpoint of the analytical methods used.

(5) Analysis

a Absorption and excretion

Blood levels, intestinal residues, fecal and urinary excretion levels of the test feed additive and major metabolites (hereinafter referred to as the test articles) should be measured at appropriate time intervals to determine the rate of absorption via the digestive tract, the excretory route and excretory rate of test articles.

In addition, when the test article labeled radioactively is administered, recovered radioactive materials shall be identified for their chemical types.

b Distribution

A distribution study should be conducted when absorption of test articles has been confirmed by the absorption and excretion study, to measure distribution of the test articles in as many different organs and tissues as possible at appropriate timings, and if possible, to calculate the 50% dissipation time in the tested animal species.

In addition, the following organs and tissues shall be examined for residues: liver (hepatopancreas), kidneys, heart, lungs (gills), spleen, muscles, digestive tracts, brain, skin, gonads, adrenal glands, thyroid gland, thymus, pituitary gland, etc. Major organs and tissues shall be measured at appropriate time intervals. For small animals, however, measurements in endocrine glands at different time intervals can be omitted.

Further examinations such as autoradiography after administration of a labeled compound, as needed, shall be also conducted.

c Accumulation

The accumulation of the test articles in organs and tissues, in which possible accumulation is expected based on results of the distribution study, should be measured at appropriate intervals. In this case, the test articles should be administered consecutively until accumulation reaches a plateau, and then it is desirable to examine any change in accumulation after discontinuing the test articles administration.

d Metabolism

When the test feed additive is metabolized *in vivo*, its major metabolites should be identified and their rates of formation in principal organs and tissues involved in metabolism should be determined as needed.

Metabolism shall be examined *in vitro* using organs and tissues mainly involved in metabolism for the determination of the formation rates of each metabolite. When there are species differences in the formation rates of each metabolite, it is desirable to conduct another study using another species.

2. Probiotics

(1) Objective

These studies aim to clarify the distribution and excretion of the test feed additive (bacteria) in digestive tracts and also its possible penetration into other tissues than the digestive tracts, to obtain comprehensive information on its *in vivo* kinetics.

(2) Animals

In principle, livestock for which the test feed additive is intended should be employed.

(3) Administration method

In principle, the test feed additive (bacteria) should be administered continuously up to the time when the fecal excretory amount of the test feed additive (bacteria) reaches a fixed level.

(4) Dose

The dose should be set at the lowest level sufficient to fulfill the objective of these studies.

(5) Analysis

a Rootage and excretion of the test feed additive (bacteria) in and from digesting tracts, and its fate after discontinuing administration.

b The distribution of the test feed additive (bacteria) should be measured in as many different organs and tissues as possible.

XII Feeding Studies Using Target Animals

1. Objective

These studies aim to clarify effects of the test feed additive administered continuously to animals for which it is intended, in light of the status of usage of feed additives in such animals at the feeders' level.

2. Animals

Livestock for which the test feed additive is intended should be employed. For cattle and swine, about 3-10 head per each dose group should be used. For chickens, about 20-30 birds per each group, and for cultured aquatic animals, at least 30 aquatic animals per each group.

However, for probiotics, animals for which the test feed additive is intended should be subdivided into 4 groups, as specified in Items 1 to 4 of Article 1 of the Enforcement Ordinance. Then, in principle, at least one species in the animal subdivision for which the test feed additive is intended should be employed.

In addition, for farm-raised aquatic animals, environmental conditions shall be

considered during the study, and feeding water temperature shall be within the following ranges: yellowtail, red sea bream, carp, and eel, 22-28°C; rainbow trout and silver salmon, 12-18°C; sweetfish, 19-25°C.

3. Duration of administration

For animals, the duration of administration should be determined in consideration of the time period for which the test feed additive is actually to be used. For farm-raised aquatic animals, the duration of administration should be at least one-half of the time period for which the test feed additive is used. In principle, duration of administration should be set as when the average control group body weight increases at least 3 times.

4. Administration method

The test feed additive should be continuously administered by adding it to the diet.

5. Dose

At least two graduated dose levels, ranging from the highest dose of the estimated optimum additive dose, to 10 times the highest dose, should be set up. A control group is also needed.

6. Items for observation

Minimally, the following items (1)-(3) should be observed during the study. If an anomaly is observed regarding items (1)-(3), items (4)-(6) should be conducted as needed.

(1) Body weight
(2) Feed consumption (feeding amount) and test feed consumption (administration amount of test articles)
(3) Clinical signs
(4) Hematological examinations
(5) Chemical examinations
(6) Pathological examinations

XIII Tests Regarding Development of Resistant Bacteria

1. Objective

These studies aim to examine and clarify, quantitatively and qualitatively, resistant bacteria-producing potential of an antibacterial substance to be used as feed additive.

2. Testing on antibacterial spectrum

(1) Sensitivity test using preserved bacterial strain in the laboratory

a Bacterial strain

At least 20 strains, including the following strains, shall be employed as test strains. It is desirable to use standard strains (ATCC strain, etc.) that make one-test results easy to compare with other test results.

(a) Gram positive strains

[1] *Staphylococcus aureus*
[2] *Staphylococcus epidermidis*
[3] *Streptococcus agalactiae*
[4] *Streptococcus pyogenes*
[5] *Streptococcus suis*
[6] *Bacillus cereus*
[7] *Clostridium perfringens*
[8] *Actinomyces pyogenes*
[9] *Erysipelothrix rhusiopathiae*

(b) Gram negative strains

[1] *Bordetella bronchiseptica*
[2] *Escherichia coli*
[3] *Haemophilus paragallinarum*
[4] *Actinobacillus pleuropneumoniae*
[5] *Pseudomonas aeruginosa*
[6] *Pasteurella multocida*
[7] *Salmonella typhimurium*
[8] *Salmonella enteritidis*
[9] *Salmonella pullorum*

b Test method

The minimal inhibitory concentrations of the antibacterial substance against test bacterial strains are to be determined according to the newest issue of the [Standard Methods of Japan the Chemotherapy Association].

(2) Sensitivity test using field bacterial strains

a Bacterial strains

About 50 kinds of bacterial strains, which shall be fresh strains isolated from animals and poultry, corresponding to the following bacterial subdivisions.

Concerning the selection of bacterial strains, it is desirable they be selected from strains collected from as wide a range of places as possible.

(a) If active ingredients of test articles mainly effect gram positive bacteria

[1] *Staphylococcus aureus*

[2] *Streptococcus pyogenes*

(b) If active ingredients of test articles mainly effect gram negative bacteria

[1] *Escherichia coli*

[2] *Salmonella typhimurium*

(c) If active ingredients of test articles effect gram positive and negative bacteria

Bacterial strains shown in (a) and (b)

b Test method

According to (1)-b

3. Testing on effects on other antibacterial substances

(1) A study using bacterial strains that have already acquired resistance against representative antibacterial substances in each system.

The effectiveness of antibacterial substances to be used as a feed additive and its biochemical mechanism of resistance development should be studied using a R plasmid-carrying organism, or a chromosomally resistant bacteria, or another appropriate organism in which biochemical mechanism of resistance development is known.

a Bacterial strains

Bacterial strains that correspond to the following subdivisions

(a) If test articles effect mainly gram positive bacteria.

Staphylococcus aureus, which is resistant to many drugs, should be used for aminobenzylpenicillin, cefazolin, dihydrostreptomycin, gentamycin, oxytetracycline, chloramphenicol, erythromycin, sulfa drugs, etc.

(b) If test articles effect mainly gram negative bacteria.

Escherichia coli, which is resistant to many drugs, should be used for aminobenzylpenicillin, cefazolin, dihydrostreptomycin, gentamycin, oxytetracycline, chloramphenicol, sulfa drugs, etc.

(c) If test articles have wide spectrum effects on both gram positive and negative bacteria.

Bacterial strains shown in (a) and (b).

b Test method

According to 2-(1)– b.

(2) Studies using field bacterial strains that have cross-resistance to test articles

This study shall only be conducted when the field bacterial strain has resistance to test articles. The minimal inhibitory concentrations of known antibacterial substances in each system should be determined using resistant field strains.

a Bacterial strains

Bacterial strains that show resistance in the 2-(2) test should be used.

b Drugs

Drugs that include at least one of the following ingredients, except for drugs that effect mainly protozoa, should be selected. In addition, in the case where test articles have effects mainly on gram positive bacteria, erythromycin should be added.

(a) Aminobenzylpenicillin
(b) Cefazolin
(c) Dihydrostreptomycin
(d) Gentamycin
(e) Oxytetracycline
(f) Chloramphenicol
(g) Sulfa drug (One kind)

c Test method

The minimal inhibitory concentration of drugs against each bacterial strain should be determined according to 2-(1)- b.

4. Testing on acquisition of resistance

Testing aim to clarify the incidence of the appearance of resistant strains and the degree of resistance to test articles.

(1) *In vitro* study

The mechanism of resistance acquisition should be investigated using standard strains that are succeeded in test tubes.

a Bacterial strains

Bacterial strains that correspond to the following subdivisions should be used.

(a) If test articles effect mainly gram positive bacteria
[1] *Staphylococcus aureus*
[2] *Streptococcus pyogenes*
(b) If test articles effect mainly gram negative bacteria
[1] *Escherichia coli*

[2] *Salmonella typhimurium*

(c) If test articles have wide spectrum effects on both gram positive and negative bacteria

Bacterial strains shown in (a) and (b).

b Test method

A subculture test using liquid culture media including test articles (increased and fixed amounts of test articles) should be conducted.

In addition, if resistance acquisition is observed, subculture shall be succeeded at least 20 generations, and the state of resistance disappearance shall be made clear.

(2) *In vivo* study

The effects of the test antibacterial substance on intestinal bacteria, protozoa, etc. in excreted feces after test articles administration to test animals at the same concentration and for the same time period as it is actually to be used should be studied. At least Escherichia coli shall be present in rectal feces, and using plates containing the test antibacterial substance and major known antibacterial substances of each system, it is necessary to determine, at appropriate intervals, the ratio of the resistant E. coli count to the total E. coli count per gram of feces, as well as to clarify the mechanism and the degree of resistance.

a Animals

Livestock for which the test feed additive is intended should be employed, and number of animals should be: cattle and swine, at least 5 head per each dose group; chickens, at least 10 birds per each dose group. There should be at least 3 test animal groups, including the highest and lowest recommended dose groups and a control group.

b Duration of administration

At least 7 days longer than the time period for which the test feed additive is administered.

c Drugs and concentrations of drugs

Drugs should be selected from the following table.

Drugs	Concentration of drug
Aminobenzylpenicillin	25.0 μg (titer)/ml

Dihydrostreptomycin	12.5 μg (titer)/ml
Gentamycin	25.0 μg (titer)/ml
Oxytetracycline	25.0 μg (titer)/ml
Chloramphenicol	25.0 μg (titer)/ml
The test feed additive	The resistance limit concentration of E. coli measured in 1–(1) and (2)

d Test method

Rectal feces of test animals of each test group should be collected once before the test and at least once a week thereafter. The feces, diluted with sterile saline, should be coated on plates with and without the test drug. The number of E. coli per 1 g of feces should be measured by counting the number of colonies that have grown on the plates.

XIV Studies on Environmental Impact

For probiotics, it is necessary to confirm or estimate that test feed additive use will not impact the ecology of bacteria, etc. in the environment by indicating articles (bacteria) origin, distribution, etc. in the environment.
In addition, it is necessary to evaluate test articles' impact by investigating its existence in the environment by conducting field or laboratory tests.

XV Stability Tests of Feed Additives

1. Objective

These studies aim to clarify stability of the test feed additive under various conditions of actual use.

2. Test methods

(1) Stability testing of test articles stored at room temperature

At least three lots of adequate amounts of test articles, including the ingredients used in manufacturing formulated products, should be stored, in their standard packaging, indoors at room temperature. Then stability of test articles should be examined at 0, 3, 6, 9, 12, 18, and 24 months of each storage period (storage period can be extended or shortened depending on the term of validity or use

period).

Package size can be reduced as needed.

(2) Heat tolerance testing

At least three lots of adequate amounts of test articles, including the ingredients used in manufacturing formulated products, should be sealed in a glass bottle or airtight container at 40°C, and examined for stability at 0, 1, 2, 3, and 6 months of each storage period (storage period can be extended or shortened depending on handling specifications and physical properties of the feed additive).

(3) Humidity tolerance testing

At least three lots of adequate amounts of test articles, including the ingredients used in manufacturing formulated products, should be put in uncovered laboratory dishes at 25-30°C and subjected to at least 2 levels of humidity, and examined for stability at 0, 1, 2, 3, and 6 months of each storage period (storage period can be extended or shortened depending on handling specifications and physical properties of the feed additive). At least 2 humidity levels should be selected, including near the highest level within the range where a clear reduction of quality, such as change of appearance, occurrence of fungi, deterioration, deliquescence, consolidation, etc. should not occur after conducting the preliminary test.

(4) Photostability testing

At least three lots of adequate amounts of test articles, including the ingredients used in manufacturing formulated products, should be placed in lidded laboratory dishes and sealed with adhesive tape or paraffin, and examined for stability under conditions of room temperature (1-30°C) and 500 lux of fluorescent lighting at 0, 1, 2, 3, and 6 months of each storage period (storage period can be extended or shortened depending on handling specifications and physical properties of the feed additive).

(5) Acceleration tests

At least three lots of adequate amounts of test articles, including the ingredients used in manufacturing formulated products, should be put into their standard packages and stored under usual conditions of 40°C, 75% humidity and indoor room temperature, and examined for stability at 0, 1, 3, and 6 months of each storage period (storage period can be extended or shortened depending on

handling specifications and physical properties of the feed additive). Package size can be reduced as needed.

(6) Stability testing of test articles in feed

Test articles(formulation product) should be added, at the actual concentration to be used, to at least three kinds of common formula feed, and adequate amounts of those feeds should be put into the usual packaging under usual indoor storehouse conditions, and examined for stability at 0, 0.5, 1, 2, and 3 months (and 6 months as needed) of each storage period.

In addition, concerning quantitative determination of test articles in feed, in principle, the analytical methods should conform to the following items.

a Average recovery rate shall be at least 90%, and accuracy of reproducibility (standard error is calculated by adding the error caused by repeat laboratory tests to the error caused by bias among laboratories) shall be less than 0.1 as the coefficient of variation.

The recovery test should be conducted three times to determine the average recovery rate and accuracy of reproducibility by adding formulation product as test articles to feed at the concentration actually used, in principle, in at least three laboratories using a two-point parallel line bioassay on a different test date.

b To limit quantitative determination, analysis should determine test articles content that will be added to feed at less than 10% of the concentration regularly used.

c The amount of active ingredient should be identified from decomposition or other contamination.

3. Items for measurement

All items set as specifications of the ingredient should be measured at at least 3 time points, including the starting and ending times. At other time points, observations of appearance and measurements of content of active ingredient should be conducted. In addition, the amount reduced by drying or water content should be measured in the stability testing in feed, stability testing in room temperature, and humidity test. Measurement items should be added to each test where necessary.

4. Statistical analysis of test results

Regression analysis should be conducted to calculate 90% confidence limit of population mean by applying the relation between values of measurements of test periods and contents of active ingredients to the most suitable model.

Data required for submission for the designation of a feed additive, etc.

Data Required for Submission for the Designation of a Feed Additive, etc.

(February 4, 1980
Notification ["54 Chiku A"] No. 5002 / ["54 Suishin"] No. 3381)
Director, Livestock Industry Bureau, Ministry of Agriculture, Forestry and Fisheries
Director-General, Fisheries Agency
Revised: March 16, 1992
Notification ["4 Chiku A"] No. 201

The Minister of Agriculture, Forestry and Fisheries shall seek opinions from the Agricultural Materials Council (AMC) in order to designate feed additives or establish standards or specifications in accordance with the provisions of the Law Concerning the Safety Assurance and Quality Improvement of Feed (Law No. 35 of 1953).

Only the requisite minimum number of feed additives shall be designated from among those feed additives that are particularly needed and proven to be both safe and effective. Therefore, as communicated in the notification titled "Establishment of the Standards for Evaluation of Feed Additives" (Notification ["52 Chiku A"] No. 1200 / ["52 Suigyo"] No. 1111, dated April 5, 1977, issued by the Director of Livestock Industry Bureau under MAFF and the Director-General of Fisheries Agency), those parties who intend to start manufacturing or importing an article that has not previously been designated as a feed additive shall adequately consult with, and seek direction from the authorities in advance. At this time, the required data for the consultation with the authorities and deliberation by AMC are specified and described in the attachment of this notification. Please be thoroughly informed of the details of this requirement, and advise all parties involved under your jurisdiction accordingly.

Data required for submission for the designation of a feed additive, etc.

I Data required for submission when meeting with the authorities

When meeting with the authorities for designation, etc. of a feed additive, five sets of documents that clarify the following matters shall be submitted.

1. Details of origin or discovery and status of approval, usage, etc. in foreign countries.

2. Specifications
   (1) Name
      a Generic name
      b Chemical name (in case of probaiotics, the scientific name)
      c Trade name
   (2) Chemical structure

3. Efficacy
   Basic studies proving effectiveness

4. Residue
   Residue data conducted by using livestock for which the test feed additive is intended (Data produced at one facility).

5. Safety
   (1) Single-dose toxicity study
   (2) Mutagenicity data (back mutation test)

II Data required for submission for deliberation of the Agricultural Material Council

If the application is forwarded for deliberation to the Agricultural Material Council, as a result of the meeting mentioned in I above, the following data shall be submitted.

1. Summary of the test results, etc.

   (1) Form for written summary of the test results (hereinafter called [summary]) is according to separate paragraph 1 (written page of items and specified items shall be properly aligned).

(2) Separate tables (separate table forms 1-8), which make each test result easily understood, shall be attached to the Summary. If a test item has no corresponding table form, a table that makes the summary of the test result clearly understand shall be prepared. In addition, for preparation of efficacy data, which addresses quality reduction prevention by adding agents to prevent mold, and prevention of livestock productivity reduction caused by specified pathogenic microorganisms, an adequate table for the test results shall be prepared independently from table form 1.

(3) The paper sheet for the Summary, shall be A4 size and of high quality sheet, based on Japanese Industrial Standards “The Summary” shall be written on top of the page. The binding of the document should be done on the left.

(4) The Summary shall be typed or produced by a printer.

(5) Number of submissions of the Summary shall be 50.

2. Abstract of the test results, etc.

(1) Written form of Abstract of the test results, etc. (hereinafter called [Abstract]) shall be according to Separate paragraph 2. In principle, it shall be prepared within one sheet.

(2) Specifications and printing of the paper sheet for the Abstract shall be according to 1-(3) and (4).

(3) Number of submissions of the Abstract shall be 150.

3. Test results, etc. of each test

(1) Test results, etc. of each test (hereinafter called [Document]) shall be prepared so that the test plan and result are clearly understood.

(2) Specifications of the paper sheet for the Document, in principle, shall be A4 size of high quality sheet based on Japanese Industrial Standards. The binding of the document should be done on the left.

(3) Printing of the Document shall be typed or produced by a printer.

(4) If the Document is written in a language other than Japanese, the entire document shall be translated into Japanese (however, figures and tables, in principle, need no translation). The name and position of the person responsible for the translation shall be stated in the translated Document.

(5) Kinds and number of submissions of the Document shall be as follows.

a Document for specifications (15-20 sets)

(a) Details of origin or discovery and status of approval, usage, etc. in foreign countries.

(b) Specifications

[1] Name, [2] Chemical structure, [3] Manufacturing process, [4] Biological and physicochemical properties, [5] Method of quantitative analysis in feed, [6] Change with time, [7] Other specifications

(c) Residue

Residue test using target animal, etc.

b Document for efficacy and safety (25-30 sets)

(a) Efficacy

[1] Basic studies proving effectiveness, [2] Field trial studies proving effectiveness

(b) Residue

Results of residue test using target animal, etc.

(c) Safety

[1] Results of toxicity test

a. General toxicity test
b. Special toxicity test
c. Pharmacology
d. Metabolism test

[2] Feeding studies using target animals, etc.

[3] Studies regarding development of resistant bacteria

[4] Other studies

a. Studies on environmental impact
b. Others

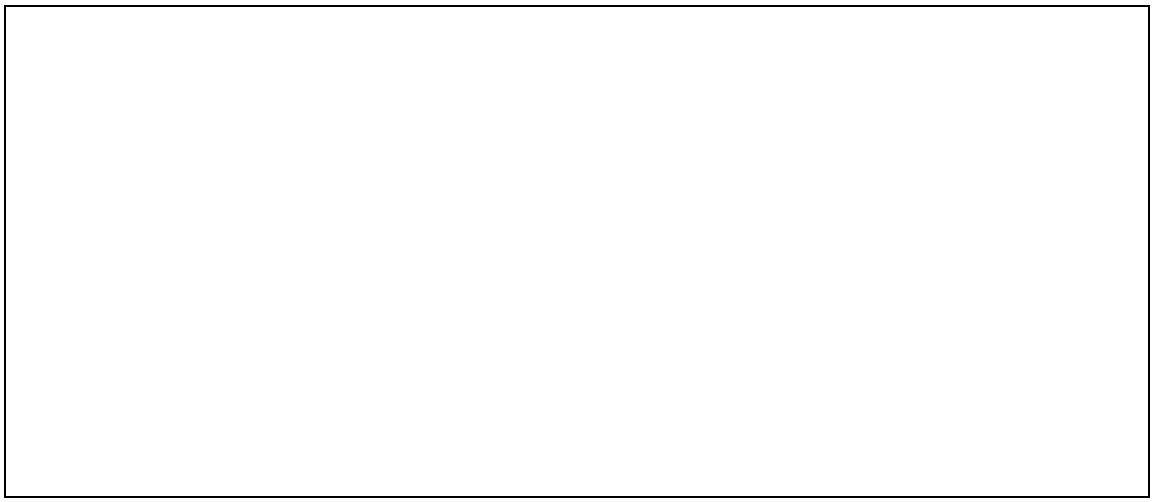
c The entire Document (30-35 sets)

This shall include Documents for specifications, efficacy and safety indicated in a and b.

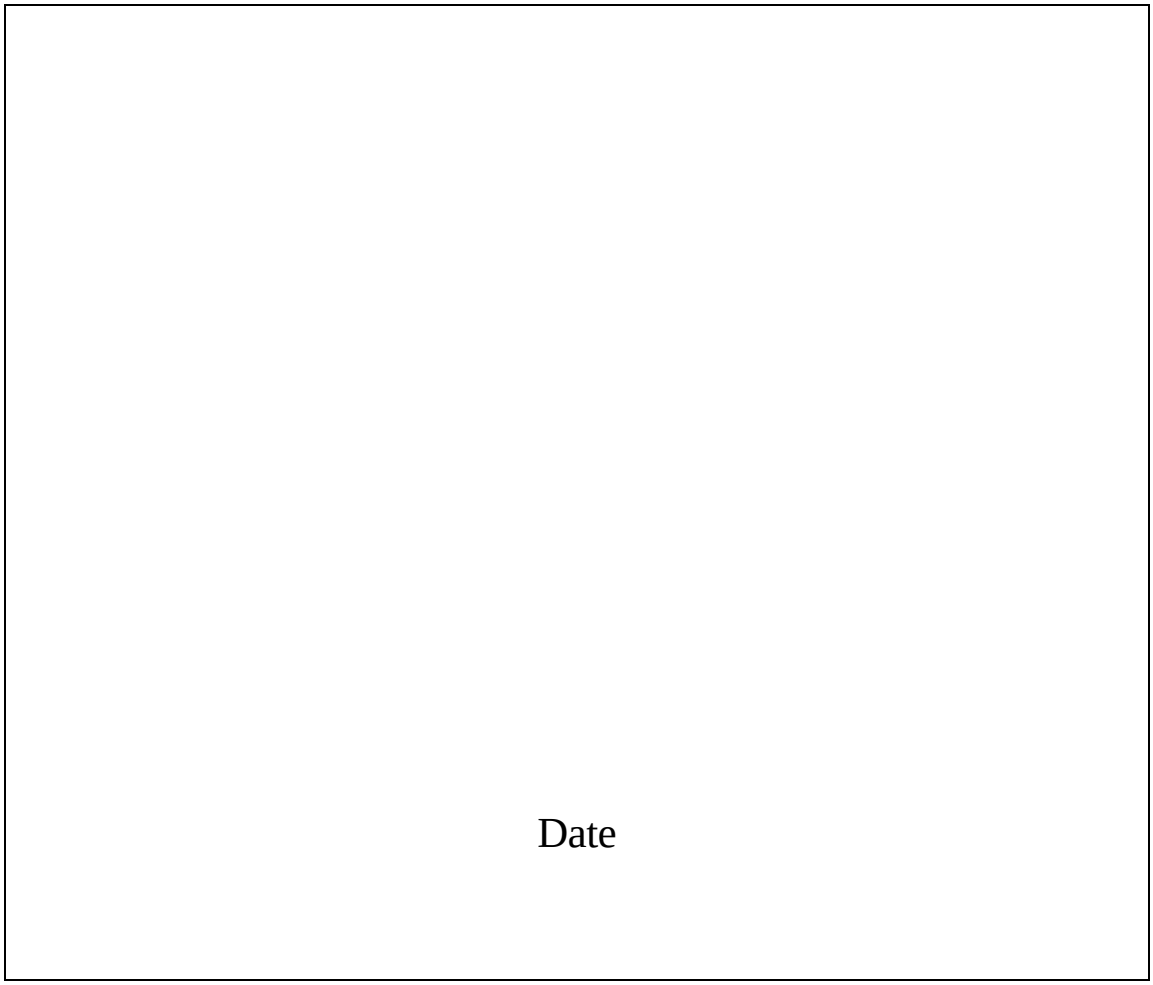
III Submission of samples

Samples shall be put into a glass or plastic container (three containers of samples are to be submitted). Each container shall be labeled with the name of the sample, the expressed value of the active ingredient and the analytical value.
In addition, the volume of the samples shall be 10-100 g.

Separate paragraph 1



Summary of the test results of



General Name	Chemical Name	Trade Name
Use and Dose	Chemical Structure	

List of Summary Items (Page) (No. of separate table form)

1. Details of origin or discovery and status of approval, usage, etc. in foreign countries
2. Specifications
 (1) Name
 (2) Chemical structure
 (3) Manufacturing process
 (4) Biological and physicochemical properties
  a. Properties
  b. Identification test
  c. Purity test
  d. Content and assay procedure
 (5) Method of quantitative analysis in feed
 (6) Change with time
3. Efficacy
 (1) Basic studies proving effectiveness
 (2) Field trial studies proving effectiveness
4. Residue

Residue Studies Using Target Animals

5. Safety
 (1) Toxicity test
  a. General toxicity test
   (a) Single-dose toxicity
   (b) Repeated-dose toxicity (short-term)
   (c) Repeated-dose toxicity (long-term)
  b. Special toxicity test
   (a) Multi-generation reproduction
   (b) Teratogenicity
   (c) Carcinogenicity
   (d) Mutagenicity
   (e) Other toxicity test (local toxicity, inhalation toxicity)
  c. Pharmacology
  d. Metabolism
 (2) Feeding studies using target animals
 (3) Studies regarding development of resistant bacteria
 (4) Other studies

Data No.	Item	Specified item	Examined item and summary of results
	1. Details of origin or discovery and status of approval, usage, etc. in foreign countries	Origin or discovery	
		Status of approval, usage, etc. in foreign countries	
		Status of approval for manufacturing or import license of animal drug	
		Derivatives	
	2. Specifications	(1) Name a. Generic name b. Chemical name c. Trade name	
		(2) Chemical structure	
		(3) Manufacturing process	
		(4) Biological and Chemical properties a. Properties b. Identification test c. Purity test d. Content and method of quantitative analysis	(Spec. test method, data etc.)
		(5) Method of quantitative analysis in feed	

Data No.	Item	Specified item	Examined item and summary of results
		(6) Change with time	
	3. Efficacy	(1) Basic studies proving effectiveness	
		(2) Field trial studies proving effectiveness	
	4 . Residue	Residue studies using livestock, etc. for which feed additives are intended	Analytical method for residue in animal products and test results
	5 . Safety	(1) Toxicity test a. General toxicity test (a) Single-dose toxicity	
		(b) Repeated-dose toxicity (short-term)	
		(c) Repeated-dose toxicity (long-term)	
		b. Special toxicity test (a) Multi-generation Reproduction	
		(b) Teratogenicity	

Data No.	Item	Specified item	Examined item and summary of results
		(c) Carcinogenicity	
		(d) Mutagenicity	
		(e) Other toxicity (local toxicity, inhalation toxicity)	
		c. Pharmacology	
		d. Metabolism	
		(2) Feeding studies using target livestock	
		(3) Studies regarding development of resistant bacteria	
		(4) Other studies a. Studies on environmental impact	
		b. Others	

Separate Table Form 1-1 Efficacy (Livestock)

Data No.	Facility Place and Test Period	Animal		Test Methods					Test Results						(Note) 3 Remarks
		Type	No. of Animals per Group	Test Group	Dose of Test Material (ppm)	Replica-tion	No. of Total Animals	Feeding Period	(Note) 2 Av. Body Wt. Gain (Figures) (%)		Av. Feed Consumption (Figures)	(Note) 2 Av. Feed Conversion ratio (Figures) (%)		Survival Rate (%)	Pathological Findings	
									(Note) 1							
				Control	0				750b 769b 834a 801ab	100			100			

(Note) 1. Numbers in the same column with different superscripts are significantly different.

(Note) 2. Figures calculated according to the index of 100 that indicate results of control group must be recorded.

(Note) 3. Feeding conditions and other remarkable results of observation, etc. must be recorded.

Separate Table Form 1-2 Efficacy (Farm-Raised Aquatic Animals)

Data No.	Facility Place and Test Period	Animal		Test Methods			Test Results																Remarks
		Type	No. of Animals per Group	Test Group	Dose of Test Material (ppm)	Feeding Period	Av. Body Wt.				Increase Wt. Rate		Feed Efficiency		Perish rate (%)	Average Degree of Obesity	Av. Wt. Relative Liver (hepa/pancr) Wt.	Hematological Examination				Patho-logical findings	
							Start		End									Hb ( )	Plasma Protein ( )	Hct ( )	R B C ( )		
							Figures	(%)	Figures	(%)	Actual; No.	(%)	Actual; No.	(%)									

(Note) 1. Results must be reported for each replicated group and other entries must be according to Separate Table Form 1-1, Efficacy (Livestock)

2. Unit for test method must be noted within ( ) in hematological examination

Separate Table Form 2 List of toxicity studies

Data No.	Type of Study	Purity of Test Material	Animals (Strain, etc.)	No. of Animals per Group	Administration Method	Dose Levels	Approx. Lethal Dose or No Observed Effect Level	Facility and Test Period

Separate Table Form 3 Single-dose toxicity studies

Data No.			
Facility and Test Period			
Animals (Strain, etc.)			
No. of Animals per Group			
Administration Method			
Purity of Test Material			
Dose Levels (mg/kg)			
Observation Period			
Lethal Dose (mg/kg)			
Clinical Signs			
Range of Time from First to Last Death			
Remarks			

Separate Table Form 4 Repeated-dose toxicity study (Short-term)
(or Repeated-dose toxicity study (Long-term))

Data No.	Facility and Test period	Animals (Strain, etc.)	No. of Animals per Group	Administration Method	Purity of Test Material
Test Groups and Dose Levels (ppm) (mg/kg)					
Clinical Signs and Mortality					
Av. Body Wt. Gain					
Feed	Av. Feed Consumption (g/day)				
	Av. Feed Efficiency				
Total Test Material Intake (mg/animal)					
Lab. Test Results	Hematological Examination				
	Blood Chemistry Examination				
	Urinalyses				
Pathological Findings	Gross Observations				
	Organ weights				
	Histological Examination				
No Observed Effect Level and Toxic Level					
Remarks					

Separate Table Form 5 Multi-generation reproduction study

Data No.	Facility and Test Period	Animal (Strain, etc.)	No. of Animals per Group	Administration Method	Purity of Test Material

Generation		F_0 Feeding Period Day				F_1 Feeding Period Day				F_2 Feeding Period Day			
Test Groups and Dose Levels (ppm) (mg/kg)													
General Feeding Parameters	Clinical Signs												
	Mortality												
	Av. body Wt. Gain												
	Av. Feed Consumption (g/day)												
	Av. Feed Efficiency												
	Findings												
Reproduction Parameters	No. of Animals Positively Mated												
	Mating Index												
	No. of PregnantFemales												
	Fertility Index												
	No. of Live Births												
	Av. Body Wt. of Newborns												
	No. of Stillborns												
	Parturition Index												
	No. of Newborns/Litter												
	Av. Body Wt. Gain of Pups (At 21 days of Age) (g)												
	Surviving Rate of 21-day old Live Pups (%)												
	Sex Ratio												
	Findings												
Remarks													

Separate Table Form 6 Teratogenicity study

Data No.	Facility and Test Period	Animal (Strain, etc.)	No. of Animals per Group	Administration Method	Dosed Period	Purity of Test Material
Test Groups and Dose Levels (mg/kg)						
No. of Maternal Animals						
Clinical Signs						
Av. Body Wt.						
Av. Feed Consumption (g/day)						
Av. Feed Efficiency						
Mortality						
No. of Corpora Lutea per Dam						
Implantation Findings	No. of Implantations per Dam Av. No. of Implantations Rate of Live Fetuses (%) Av. No. of Live Fetuses No. of Resorptions No. of Dead Fetuses No. of Macerated Fetuses Others					
Fetal Sex Ratio						
Fetal Body Wt. Mean ± S.D. (g)						
External Anomalies						
Skeletal Anomalies						
Visceral Anomalies						
Developmental Abnormalities after Birth						
Remarks						

Separate Table Form 7 Carcinogenicity study

No. of Data	Facility and Test Period	Animal (Strain, etc.)	No. of Animals per group	Administration Method	Purity of Test Material
Test Groups and Dose Levels (ppm) (mg/kg)					
Cumulative Mortality					
Av. Body Wt. Gain					
Av. Feed Consumption					
Clinical Signs					
Organ Wts.					
Histopathological Findings					
Incidence of Tumor (and incidence of specified tumor)	( )	( )	( )	( )	( )
Findings of Other Examinations					
Remarks					

Separate Table Form 8-1 Feeding Studies Using Target Animal

Data No.	Facility and Test Period	Animal		Test method					Test Results							Remarks
		Type	No. of Animals per Group	Test Group	Dose (ppm)	Replications	No. of Animals	Feeding Period	Av. Body Wt. Gain (Figures) (%)		Av. Feed Consumption (Figures)	Av. feed Conversion Ratio (Figures) (%)		Survive Rate (%)	Hematological Examination	Blood chemistry Examination	Patho-logical Findings	

(Note) Reporting methods must be according to Separate Table Form 1-1 for Efficacy.

Separate table form 8-2 Feeding Experiment Using Target Animal (Farm-Raised Aquatic Animals)

Data No.	Facility and test period	Animal		Test method			Test results																Remarks
							Av. body wt.				Wt. gain		Feed efficiency		Perish rate (%)	Av. fat degree	Av. wt. liver (hepa/pancr) rate	Hematological examination				Patho-logical findings	
							Start		End														
		Kind	No. of animals per group	Test group	Dose (ppm)	Feeding period	Figures	(%)	Figures	(%)	Actual	(%)	Actual	(%)				Hb ( )	Plasma protein ( )	Hct ( )	RBC ( )		

(Note) Writing methods must be according to Separate table form 1-2 for Efficacy (Farm-Raised Aquatic Animals)

Separate paragraph 2

Chapter 1. Outline of the test results of

1. Summary (details of origin or discovery and status of approval, usage, etc. in foreign countries)
2. Specifications
   (1) Name (general name, chemical name, trade name, and chemical structure)
   (2) Properties
   (3) Stability
   (4) Method of quantitative analysis
3. Efficacy
4. Residue
5. Safety
   (1) Single-dose toxicity
   (2) Repeated-dose toxicity (short-term)
   (3) Repeated-dose toxicity (long-term)
   (4) Multi-generation reproduction
   (5) Teratogenicity
   (6) Carcinogenicity
   (7) Mutagenicity
   (8) Other toxicity (local toxicity, inhalation toxicity)
   (9) Pharmacology
   (10) Metabolism
   (11) Feeding studies using target animals
   (12) Studies regarding development of resistant bacteria
   (13) Studies on environmental impact
   (14) Other items regarding safety
6. Others

List of Data Required to Submit for New Designation of a Feed Additive

Class / Types of Data	(1) Antibiotics of feed class		(2) Anti-bacterial substances responding to poisonous and deleterious substances	(3) Anti-bacterial substances except for (1) and (2)	(4) Materials that prevent reduction of feed quality	(5) Enzyme drug (not including bacteria)	(6) Probiotics
	Refined class	Feed class					
1 Origin or discovery and approval, usage, etc. in foreign countries		○	○	○	○	○	○
2 Specifications							
(1) Name		○	○	○	○	○	○
(2) Chemical structure		○	○	○	○	○	
(3) Manufacturing process		○	○	○	○	○	○
(4) Biological, chemical or bacterial properties		○	○	○	○	○	○
(5) Method of quantitative analysis in feed		○	○	○	○	○	○
(6) Change with time	○	○	○	○	○	○	○
(7) Fungal toxin		△ (Note2)				△ (Note2)	
3 Efficacy							
(1) Basic studies proving effectiveness							
a in vitro study	○		○	○	○	○	○
b in vivo study		○ (Note3)	○	○	○	○	○
(2) Studies proving the effects caused by combination with antibacterial feed additives							○
(3) Field trial studies proving effectiveness		○ (Note3)	○	○	○	○	○
4 Residue		○	○	○	○		
5 Safety							
(1) Taxonomic situation of the bacteria							○
(2) Toxicity							
a General toxicity							
(a) Single-dose toxicity	○	○	○	○	○	○	○
(b) Repeated-dose toxicity (short-term)	○	○	○	○	○		○

Class / Types of Data	(1)Antibiotics of feed class		(2) Anti-bacterial substances responding to poisonous and deleterious substances	(3) Anti-bacterial substances except for (1) and (2)	(4) Materials that prevent reduction of feed quality	(5) Enzyme drugs (not including bacteria)	(6) Probiotics
	Refined class	Feed class					
(c) Repeated-dose toxicity (short-term)	○	○	○	△ (Note4)	△ (Note4)		
b Special toxicity							
(a) Multi-generation Reproduction	○	○	○	△ (Note5)	△ (Note5)		
(b) Teratogenicity	○	○	○	○	○		
(c) Carcinogenicity	○	○	○	△ (Note6)	△ (Note6)		
(d) Mutagenicity	○	○ (Note7)	○	○	○	○	
(e) Other toxicity (local toxicity, inhalation toxicity)	○	○	○	○	○		
c Pharmacology	○		○	○	○		
d Metabolism	○		○	○	○		○
(3) Feeding experiment using target animals	○	○	○	○	○	○	○
(4) Studies regarding development of resistant bacteria	○		○	○			
(5) Other studies (Studies on environmental impact		○	○	○	○		○

Notes 1 ○ mark indicates that the data, as a rule, is required.
In addition, safety data of a substance to be applied that is designated as a food additive or is used widely in foods will not be required.

2 This shall be conducted when production of a fungal toxin is suspected.

3 Study data on a refined grade of the test article is also acceptable.

4 When repeated-dose toxicity (long-term) data is considered not to be needed judging from the residue data and repeated-dose toxicity (short-term) data and also known data, this study can be omitted.

5 Multi-generation reproduction data can be omitted where adverse effects on reproduction are not suspected based on the residue data and known data.

6 Carcinogenicity data can be omitted where the carcinogenicity is not suspected based on the residue data, mutagenicity data, and also known data.

7 If a feed additive grade is available as the test article, this study shall be conducted using it.

8 In the case of a feed additive grade of an antibiotic, a test article for an indicated study/test shall be conducted with the indicated material, i.e. the feed additive grade or refined grade.