European Food Safety Authority

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Technical Guidance

Microbial Studies

Prepared by the Panel on Additives and Products or Substances used in Animal Feed

(Question No EFSA-Q-2008-461)

Adopted on 21 October 2008

Microbial studies may be required when:

- the additive is based on micro-organisms not considered by EFSA to qualify for QPS status.
- the additive is or contains a substance known or demonstrated to have a significant antimicrobial effect at feed concentration.
- the tolerance test give an indication of adverse effect possibly related to intestinal disorders.

These studies are used to assess the potential of an additive to:

- produce antibiotics of relevance to human or veterinary medicine
- induce cross-resistance to antibiotics used in human or veterinary medicine
- encourage the growth and/or shedding of zoonotic agents
- cause an adverse change to the target animal gut microbiota

For those substances whose antimicrobial effects are unknown, tests should be made to assess the inhibitory activity (minimum inhibitory activity, MIC) against a list of reference strains known to be susceptible to clinically relevant antibiotics, ionophores or biocides (e.g., *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633).

The MIC should be determined, according to standardised procedures, by using two-fold dilution procedures in agar or broth of the active antimicrobial substance. After incubation, the MIC is defined as the lowest concentration of the substance that inhibits microbial growth. The existing body of scientific information must be considered when the procedure for MIC determination (dilution method, growth media and incubation conditions) is chosen, keeping in mind the possible interference of media and growth conditions.

If the MIC is equal to or below four times the maximum concentration of the antimicrobial in feed/water, the additive is considered to have a potential antimicrobial effect at feed level and, as a minimum, possible effects on cross resistance should be considered.

1. Induction of cross-resistance to antibiotics used in human or veterinary medicine

Additives known to have or considered to have a potential antimicrobial activity at feed use level of an additive should be regarded as an alert for possible selection of cross resistance. At present there are no validated laboratory tests able to substitute for epidemiological methods to monitor the induction of cross-resistance to antibiotics. For those antibiotics included in resistance monitoring programs, evidence of continuing susceptibility can be taken as evidence of absence of cross resistance. For those antibiotics or antimicrobial compounds not included in monitoring programs, consideration should be given to the long term assessments of developing resistance and cross resistance as part of a post-market monitoring requirement.

2. Effects on the gut microbiota of the target animal

Microbial studies are generally not required to examine potential adverse changes in the target animal gut microbiota unless the tolerance study indicates an adverse effect possibly related to intestinal disorders (e.g., poor faecal consistency). If an adverse effect is observed, then studies should be performed on:

- the intestinal microbiota to assess the effect of the additive on the major microbial populations in the gastro-intestinal tract of the target species.
- the overgrowth and/or shedding of zoonotic agents.

If the additive is intended to reduce the shedding or the contamination of animal products by zoonotic agents, then specific studies are required regardless of the outcome of the tolerance study.

2.1. Effects on gut microbiota

These studies should be done at maximum use level and the same overdose used in the (tolerance) study in which the potential adverse effect was detected and should be done in comparison with an untreated control group. The purpose of these studies is to establish the safety of the additive at use level.

The use of faecal samples is acceptable for non-ruminants. However, if samples from the different parts of the gastrointestinal tract (e.g., caecum) are available, they should be included. For ruminants, it may be necessary to distinguish between an effect in the rumen or in the post-ruminal tract. Use of a well-established *in vitro* simulation could substitute for rumen studies made *in vivo*.

Given the large natural variation in the composition of the intestinal microbiota, the test groups should be sufficiently large to exclude the effects of background fluctuation.

In the microbiological analyses at least the easily cultivable microbial groups should be included, such as total anaerobes and aerobes, clostridia, enterobacteriaceae, enterococci, and lactobacilli, and for ruminants, protozoal/fungal and representatives of the bacterial population contributing to fibre degradation and lactate metabolism. Care should be taken in maintaining the anaerobic status of the samples during the collection and processing.

The use of culture independent molecular methods (such as PCR or DNA sequencing based techniques) to analyse the global changes or changes in specific genera of intestinal/ruminal micro-organisms is encouraged. All studies should consider the potential overgrowth of enteropathogens relevant to the target species (e.g., coliforms, clostridia).

Standard statistical tests should be applied to the analysis of the results. If molecular biological methods are used, then computerized algorithms to group the samples according to

the similarities of the molecular data (such as DNA sequences or DNA fingerprints) should be applied.

2.2. Overgrowth and/or shedding of zoonotic agents

These studies should take the form of the comparison between an untreated control and a group treated with the additive at the maximum recommended dose and at multi-fold of the maximum recommended dose that would allow a margin of safety to be established. In both groups, relevant zoonotic agents should be enumerated primarily in faeces. Samples should be taken at the start and end of the experiment, and ideally at one or more intermediate time points. Care should be taken to ensure that the experimental population are selected from a group with a low level of presence of the zoonotic agent. In the absence of a naturally occurring presence, this may require artificial infection. The suggested zoonotic agents to be enumerated depend on the target species and should at least include the following:

- for poultry: *Salmonella* Typhimurium/Enteritidis and *Campylobacter* spp.
- for pigs: *Salmonella* spp. and *Campylobacter* spp.
- for calves/cattle: *Salmonella* spp.
- for dogs and cats: *Campylobacter* spp.

The experimental design used must be justified according to the additive and the target species and must consider statistical power. The trials should be conducted ensuring that husbandry conditions (e.g., veterinary intervention) do not adversely affect the interpretation of the results. Duration should be at least equal to the tolerance study. This could be made in conjunction with a tolerance or efficacy trial, unless the experimental animals are artificially infected.

It is considered that the additive does not have an effect on the shedding of zoonotic agents if the bacterial counts (and the prevalence) between the two groups are equivalent. Estimating equivalence should take account of the variability of the experimental data but, as a guide, equivalence could be assumed when bacterial counts differ by no more than 1 log order.

The results can be extrapolated to other physiologically similar species.

3. Production of antibiotics of relevance to human or veterinary medicine

Micro-organisms used as additives or as a production strain should not be capable of producing antibiotic substances that are relevant as antibiotics in humans and animals.

If the strain belongs to a microbial species known to produce antibiotics, the absence of the specific antibiotic(s) in the product should be confirmed by analysis. For products consisting of viable micro-organisms, the capacity of the strain to produce the antibiotic(s) should be excluded by analysis and preferably by molecular means to demonstrate the absence of the synthetic capacity.