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Guidance for Industry: Studies to Evaluate the Utility of Anti-*Salmonella* Chemical Food Additives in Feeds

FINAL GUIDANCE

This final guidance is intended to provide advice on the standards upon studies to establish the utility of anti-*Salmonella* chemical food additives for achieving their intended technical effect of maintaining feeds *Salmonella*-negative should be based.

Electronic comments and suggestions regarding this document should be submitted via the Internet at: <http://www.accessdata.fda.gov/scripts/oc/dockets/edockethome.cfm>. Written comments and suggestions should be submitted to the Dockets Management Branch (HFA-305), Food and Drug Administration, 5630 Fishers Lane, Room 1061, Rockville, MD 20852. All comments should be identified with the Docket No. 94D-0147.

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Introduction

This final guidance represents our current thinking on this matter. It does not create or confer any rights for or on any person and does not operate to bind the Food and Drug Administration (FDA) or public. An alternative approach may be used if such approach satisfies the requirements of the applicable statutes and regulations.

Food-borne illnesses can have severe negative impacts on human and animal health, and can be fatal. The resultant economic losses can be quite significant. Food-borne illnesses occur worldwide and can be caused by microbes like *Salmonella*. In the United States, 76 million cases of food-borne illnesses are estimated to occur annually and to result in 325,000 hospitalizations and 5,000 deaths. Annual economic losses due to medical expenses, lost production, and loss of life have been estimated to be as much as \$35 billion (Ref. 3, 5, 10, 14, 16).

Salmonella are enteric bacteria that cause a significant proportion of food-borne illnesses. Expressions of the illnesses caused by *Salmonella* in humans and other animals range from mild to severe diarrhea to anorexia, fever, nervous and respiratory signs, abortion, depression, shock, and death. There are over 2,000 serotypes of *Salmonella*. Food animals are significant sources of *Salmonella* infection for humans, and feeds are important sources of *Salmonella* for food animals (Ref. 5, 7, 9, 11, 16).

Under section 402(a) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 342(a)) animal feed, feed ingredients, and pet food containing *Salmonella* are adulterated. Additionally, 21 CFR 500.35 provides that animal byproducts intended for use in animal feed that are contaminated with *Salmonella* bacteria will be regarded as adulterated. FDA's policy is that feeds must be *Salmonella*-negative as shown by a sampling plan. See, for example, CVM's action on a food additive petition published in the Federal Register of September 28, 1995 (60 FR 50098). It is also FDA's policy that the sampling plan should follow specifications in the 8th edition of FDA's Bacteriological Analytical Manual for fifteen 100 gram (g) samples, or more, to be randomly collected from foods for analysis for *Salmonella* (Ref. 2). Thus, a feed is said to be *Salmonella*-negative if fifteen 100 gram (g) samples randomly collected from it test negative for *Salmonella*.

Feeds can be maintained *Salmonella*-negative in several ways, including the addition of chemical substances to the feeds. Such chemical substances are deemed to be food additives under section 201(s) (21 USC 321(s)) of the Federal Food, Drug, and Cosmetic Act (the Act) unless they are generally recognized as safe (GRAS) for use against *Salmonella* in feed (Ref. 6). According to section 409(a) (21 USC 348(a)) of the Act, a food additive is deemed to be unsafe, unless it and its intended use conform to the terms of an exemption or existing regulation and/or (for food contact substances) an effective notification prescribing the conditions under which such additive may be safely used.

Companies or individuals (sponsors) intending to market a food additive for use against *Salmonella* in feeds may file, under section 409(b) of the Act, a food additive petition to establish the safety and utility of the food additive for such use. Details of the regulations governing the filing of food additive petitions and the data they must include can be found in 21 CFR 571.1 (Ref. 4). Briefly, a food additive petition must contain data establishing that the food additive is safe and will have the intended effect. The amount of food additive necessary to accomplish this effect must also be identified. 21 CFR 570.17 specifies the conditions under which a food additive or food containing a food additive intended for investigational use shall be deemed to be exempt from the requirements of section 409 of the Act.

Our experience with food additive petitions indicates a need for consistency in evaluating the ability of anti-*Salmonella* food additives to achieve their intended effect of maintaining feeds *Salmonella*-negative. Thus, the objective of this guidance is to help assure that appropriate studies are conducted by sponsors to evaluate the utility of anti-*Salmonella* food additives, and that uniform review and decision-making are accomplished by CVM. This should facilitate the approval process for such food additives.

General Considerations

This guidance pertains only to studies designed to establish the utility of chemical food additives in maintaining feeds and/or feed ingredients *Salmonella*-negative. For the purpose of this guidance, the term “feed” will be used to mean complete feeds (those that can be fed as the sole ration) and/or feed ingredients that are recognized by the Association of American Feed Control Officials (AAFCO) for use in manufacturing complete feeds. Sponsors of anti-*Salmonella* food additives may propose the food additive for use only in complete feeds, only in feed ingredients, or in both.

Experiments designed for this purpose should be conducted in two main stages consisting of laboratory and field trials. Protocols should be written for the experiments and contain a clearly expressed statement of purpose. The protocols should also specify details of the materials, methods, and statistical tools that will be used in the trial. The experiments should be designed to use feeds that are usually consumed by the proposed target animals. The target animals should be specified. For food additives containing two or more chemicals, the composition of the food additive and a list of all substances used must be specified (21 CFR 571.1(c)(paragraph A of the petition form)).

Sponsors should submit protocols for review by CVM 90 days before the initiation of trials. Submitting protocols to CVM for review and comment before conducting the trials generally facilitates the approval process for a food additive.

Data should be collected and recorded in ways that enhance confidence in the integrity of data collected. Sponsors are referred to CVM's guidance number 58 entitled "Guidance

for Industry for Good Target Animal Study Practices: Clinical Investigators and Monitors" for detailed information about record keeping and record retention. A copy of the guidance can be requested from CVM's Communication Staff (HFV-12) at 301-594-1755. Copies can also be downloaded from CVM's site on the Internet at <http://www.fda.gov/cvm>.

To facilitate CVM's review of statistical procedures, raw data used by the sponsor in statistical analyses should be included in the petition for the food additive and should also be captured on a diskette, preferably in the form of an IBM compatible DOS ASCII file. The diskette should also be included in the food additive petition.

Laboratory Trials

General

The utility of a food additive against *Salmonella* can be influenced by several factors. The factors include the type and other characteristics of the medium in which the additive and *Salmonella* are made to interact, the mode of contamination of medium by *Salmonella*, type and concentration of *Salmonella*, concentration of the additive, and the duration of contact between *Salmonella* and the food additive. Pertinent medium characteristics include water activity, pH, temperature, and oxygen content (Ref. 18). It is possible that manipulation of these factors could, in the absence of uniform testing methods, result in over-estimation or under-estimation of the anti-*Salmonella* capabilities of food additives. To ensure that the data relied on by the FDA to approve anti-*Salmonella* food additives are relevant, the FDA believes that the additives should be subjected to a uniform testing method.

The goal of the uniform testing method is to determine, in a consistent manner, whether or not an anti-*Salmonella* chemical food additive is able to maintain feeds *Salmonella*-negative for a specific period of time, even if the feeds are repeatedly exposed to *Salmonella* during the period. Sponsors should conduct two laboratory experiments. The first should be a "dose titration" trial to determine the range of effective doses of the food additive. The second should consist of a "prevention of recontamination" trial to assess the ability of the minimum effective dose to maintain feeds *Salmonella*-negative despite repeated exposure of the food additive-treated feeds to the microorganism. Specific issues that should be addressed for each of the two trials follow:

Dose titration (dose determination) trial

CVM considers the "dose titration" trial to be pivotal. The main purpose of the trial is to determine the range of doses over which the food additive is effective against *Salmonella* in feeds. As much as it is possible, the range determined should include the minimum and maximum effective doses. Various concentrations of the food additive should be tested in feed against an inoculum containing a known concentration of a mixture of *Salmonella* serotypes.

The concentrations of food additive used should be multiples of the proposed effective dose. A minimum of six concentrations of the food additive in feed is recommended. One should be at zero, another at the proposed effective dose, and two each below and above the proposed effective dose (e.g., 0, 0.25X, 0.50X, X, 2.00X, and 4.00X; where X is the proposed effective dose). When using data from the trials to plot the dose response curve, the dependent variable would typically be the concentration of *Salmonella* in the sample of treated feed while the independent variable would typically be the concentration of food additive in the feed.

The feed used should be *Salmonella*-negative. A feed is considered to be *Salmonella*-negative if every one of 15 or more representative 100 g samples collected from it is shown by laboratory analysis to have no detectable levels of *Salmonella*. Samples of the feed, or 25 g subsamples randomly collected from each sample, may be analyzed for *Salmonella* individually or after combination into three or more pools consisting of a maximum of 3 samples (300 g) or 15 subsamples (375 g) per pool. Samples should be analyzed for *Salmonella* either by the methods described in the 8th edition of FDA's Bacteriological Analytical Manual (Ref. 1) or by other methods whose sensitivity and specificity are equal to or higher than that in the manual.

Because the anti-*Salmonella* effects of chemical food additives can be influenced by the presence of other microbes in the food additive-treated feeds, it is recommended that the general microbial load of the experimental feeds should be ascertained by conducting the following additional analyses on the samples:

- a) Aerobic plate count (Ref. 13)
- b) *Escherichia coli* count (Ref. 8)
- c) Fungal spore count (Ref. 17)

The *Salmonella*-negative feed should be divided into three or more replicates. Each replicate should be divided into seven experimental lots of feed. All seven lots should be separately treated with appropriate amounts of the food additive, assayed to determine the concentration of food additive that is actually present, and then inoculated with *Salmonella*. One lot should serve as a negative control (containing neither *Salmonella* nor the food additive), and another as a positive control (containing *Salmonella* but not the food additive). Each lot should then be subdivided into twelve or more units. The units should be randomly assigned to one of four groups containing equal number of units. There should be three or more units per group. Throughout the trial, the positive and negative controls should be handled exactly like the other experimental units of feed.

The method used in inoculating experimental feeds with *Salmonella* can influence the outcome of the dose titration study. Three methods are currently available. Each involves inoculation with one of the following:

- a) Materials naturally contaminated with *Salmonella*

- b) Materials contaminated with *Salmonella* in a way that simulates natural contamination (simulated naturally-contaminated materials)
- c) Broth cultures of *Salmonella*

Contamination or recontamination of feeds with *Salmonella* can occur directly through several avenues including infected or contaminated wild birds, rats, mice, cockroaches, and indirectly through contaminated inanimate objects like dust (Ref. 18). Thus, it appears logical to use naturally-contaminated materials (e.g., feces) from these sources as inoculi for experimental feeds. However, CVM believes that the use of this natural method of contamination would be incompatible with the goal of uniform testing because the concentration of *Salmonella* in such materials varies widely.

Broth cultures can be manipulated to provide inoculi containing fairly uniform amounts of *Salmonella*, and their use in inoculation will significantly reduce the variability in *Salmonella* concentration associated with the natural method. However, the direct addition of such inoculi to experimental feeds may result in an over-estimation of the ability of the food additive to achieve its intended effect, because *Salmonella* present in broth culture-inoculated feeds has been shown to be less resistant to insults than that present in naturally-contaminated feeds (Ref. 15).

The use of a method involving inoculation with simulated naturally-contaminated materials will overcome, to a large extent, the variability in *Salmonella* concentration and the reduction in *Salmonella* resistance associated with the natural and broth culture methods. Therefore, this method involving the inoculation of feeds with simulated naturally-contaminated materials is recommended.

A technique for producing simulated naturally-contaminated meat and bone meal containing stable populations of *Salmonella* has been described by Liu and his co-workers (Ref. 12). They also showed that the *Salmonella* present in feeds inoculated with the meal was more resistant to insults than that present in feeds directly inoculated with broth cultures of *Salmonella*. Briefly, in the Liu et al technique, a sterile suspension of meat and bone meal inoculated with *Salmonella* was incubated for 48 hr at 37 °C. After incubation, the suspension was centrifuged and the sediment dried, ground to a fine powder and stored at 4 °C pending its use as the inoculum for experimental feeds. The population of *Salmonella* in the meal was reported to remain relatively stable over an 11-month period. To produce simulated naturally-contaminated materials for use as inoculi for experimental feeds in the dose titration study, sponsors should use the Liu et al technique or any other similar one that is shown to equally or more effectively ensure the stability of *Salmonella* even during cold storage.

The level of *Salmonella* in the inoculum should be high enough to ensure a final concentration of 10⁴ colony-forming units (CFUs) or greater of *Salmonella* per gram of experimental feed. The inoculum should contain equal amounts of American Type

Culture Collection (ATCC) cultures of *Salmonella typhimurium*, *Salmonella senftenberg*, *Salmonella montevideo*, and *Salmonella enteritidis*. The identity of the serotype in each ATCC culture should be independently confirmed prior to its use in the trial. *S. typhimurium* is being recommended because of its universal nature, *S. senftenberg* because it has been reported to be one of the more resistant serotypes (Ref. 12), *S. montevideo* because it is reported to be the serotype most frequently isolated from feeds and is said to have a relatively high resistance (Ref. 10), and *S. enteritidis* because it is a major public health concern. Other *Salmonella* serotypes that are of animal or public health significance could be included by the sponsor, if desired.

Because the antimicrobial activity of organic acids and other chemicals appears to be time-dependent, the dose titration trial should be designed to demonstrate the nature of that time-dependency when the chemicals are used as antimicrobial food additives. The design should also allow a determination of shortest interval of time it takes for the additive to exert its effect against *Salmonella*. The preferred method for achieving this is the generation of a series of dose titration curves using data obtained from the analyses of groups of units of feed at 1, 4, 8, 24 and 48 hours after inoculation of the feeds with *Salmonella*. The units of feed used are obtained, as described earlier, by subdividing each lot of experimental feed into 12 or more equal portions.

Each unit is assigned to one of four groups containing three or more units. The amount of inoculated feed constituting each individual unit should be enough to ensure its adequacy for quantitative and qualitative analyses for *Salmonella*. The number of samples to be collected from each unit for *Salmonella* analysis should be determined by a statistical model that instills 99% confidence that *Salmonella* will be detected if present. The analysis of all of the inoculated feed in each unit, or the collection and analysis of 60 or more representative 100 g samples from each, should be compatible with the model. In the latter case, the samples, or 25 g subsamples randomly collected from each sample, may be analyzed for *Salmonella* individually or composited for analysis into three or more pools consisting of a maximum of 3 samples (300 g) or 15 subsamples (375 g) per pool.

Temperature, pH, water activity and other pertinent experimental conditions should be specified. Because of the pivotal nature of the dose titration studies, sponsors are strongly encouraged to submit the results of the study to CVM for review and comment before initiating either the “prevention of recontamination” study or the field trial.

Study of ability to maintain feeds *Salmonella*-negative

The purpose of the second laboratory trial is to demonstrate the ability of the minimum effective level of the anti-*Salmonella* chemical food additive to maintain feeds *Salmonella*-negative for a specific period of time, even if the feeds are repeatedly exposed to *Salmonella* during the period. For complete feeds or feed ingredients that, shortly after manufacture, are made directly available to animals or used to produce complete feeds, that time should be a minimum of 14 days. For complete feeds or feed ingredients that

are intended to be bagged, or stored in some other manner for sometime prior to use, a period such as 90 days is suggested.

In this trial, the level of food additive determined in the dose titration trial to be the minimum effective dose should be added to *Salmonella*-negative feed, and challenged with *Salmonella* contained in a simulated naturally-contaminated material as described earlier under the dose titration study. The challenge with *Salmonella* should be conducted during day 1 of the experiment and again at one or more other days afterwards, including the last day of the time period for which the sponsor intends to claim the food additive as being effective. Sixty or more representative 100 g samples each should be collected from the treated feed and its controls immediately before and again at two time intervals after each challenge with *Salmonella* and analyzed qualitatively for *Salmonella*. The samples, or 25 g subsamples randomly collected from each sample, may be analyzed individually or composited into three or more pools consisting of a maximum of 3 samples (300 g) or 15 subsamples (375 g) per pool. The first time interval for the collection of samples should be that at which the minimum dose of the food additive was shown to be effective in the dose titration trial, and the second one 24 hours afterwards.

The quantity of feed used in the study should be enough to ensure its adequacy for the analysis for *Salmonella*. As with the dose titration trial, temperature, pH, water activity, and other pertinent experimental conditions should be specified. The trial should also include a control and a negative control, and contain an adequate number of replicates. The duration of the experiment and the number of challenges with *Salmonella* should be consistent with claims the sponsor intends to make for the food additive.

Feeds used in both laboratory trials should consist of specified quantities of complete feeds (e.g., broiler starter) and/or feed ingredients (e.g., meat and bone meal). They should either be obtained commercially or self-produced. Details of the composition of complete feeds (including all added drugs and additives) and the techniques used for their manufacturing or processing should be specified. The amount of feed assigned to each experimental group should be enough to permit the collection of all required samples.

Field Trials

The dose of food additive determined during the dose titration trial to be the dose that will achieve the intended effect should be further evaluated in field trials. The purpose of field trials is to determine if the food additive could be safely used to achieve its intended technical effect of maintaining feeds *Salmonella*-negative under actual conditions of use. Such conditions of use would include those associated with the preparation, storage and consumption of food additive-treated feeds.

The experiments should be conducted at feed-mills or farms with histories of recurrent contamination by *Salmonella*. Experimental and control groups should be located on the same premises or farms and subjected to identical system of handling or management except for the addition of the food additive to the experimental group. The feeds used

should be *Salmonella*-negative. Their detailed composition should be specified to include all added drugs and additives. The quantities of feeds used, and the conditions under which they are manufactured or produced, stored, transported and consumed, should be the same as those expected if the food additive is approved for use.

Samples should be collected for *Salmonella* analysis from the experimental and control groups of feeds before and after the addition of food additive, and sufficiently often enough to substantiate the claim for the food additive. The times of sample collection should be specified. For trials involving feed ingredients, like meat and bone meal, samples should be collected from treated and untreated ingredients after their manufacture, during their storage, and immediately prior to their use in manufacturing complete feeds. For trials involving complete feeds, like broiler or hog starter rations, samples should be collected from treated and untreated feeds after their manufacture at the feed-mill, during their storage, and during the period the feeds are made directly available for consumption by the target animals.

The number of samples to be collected at each location or sampling interval should be determined by a statistical model that instills 95% confidence that *Salmonella* will be captured among samples collected even if the concentration of the microorganism in the feed was as low as 1 CFU/250 g. The collection and analysis of 30 or more representative 100 g samples should be compatible with this model. The samples, or 25 g subsamples from each, may be analyzed for *Salmonella* individually or after being composited into three or more pools consisting of a maximum of 3 samples (300 g) or 15 subsamples (375 g) per pool.

CVM has additional recommendations for field investigations involving anti-*Salmonella* chemical food additives intended for use in the feeds of aquatic animals. Sponsors of such food additives are referred to CVM guidance number 53 entitled "Guidance for the Evaluation of the Utility of Food Additives in Diets Fed to Aquatic Animals." A copy of the guidance can be requested from CVM's Communication Staff at 301-594-1755. Copies can also be downloaded from CVM's site on the Internet at <http://www.fda.gov/cvm>.

Because food additives are approved for use on a nationwide basis, trials should be conducted in geographically different locations and during two or more different seasons of the year, one of which should be the summer.

Collection and Analysis of Samples

The sampling techniques employed in both the laboratory and field trials under this type of study are critical to the validity of results obtained. Samples should be aseptically collected without unduly disturbing the experimental feeds or target animals consuming the feeds. FDA's Bacteriological Analytical Manual contains pertinent information about sample handling (Ref. 2).

The time of sample collection should be recorded and scheduled so that storage of samples prior to analysis is avoided as much as possible. If it is necessary to store the samples, the conditions under which the samples are stored (e.g., temperature) should be specified and shown not to be detrimental to either the survival of *Salmonella* or the maintenance of the integrity of nutrients. The duration of storage should also be specified.

Microbiological analysis for *Salmonella* should be initiated within 1 hour after sample collection and steps taken to ensure that pH and other critical parameters of the growth media that can influence growth of the microorganism are not modified by presence of the food additive. In situations like field trials where it may not be feasible to initiate analysis within one hour of sample collection, the activity of food additive in samples used for *Salmonella* analysis should be neutralized or arrested immediately after sample collection. The compound or technique used to neutralize or arrest activity should be one that has been shown not to have any negative impact on the survival of *Salmonella*. If this is not achievable, sample analysis should be initiated at the earliest possible time after sample collection and the length of time between the collection of samples and their analysis for *Salmonella* should be specified.

Laboratory analyses should be conducted "blind." It is recommended that the experimental feeds should be prepared and coded by one person or team, and samples collected from the feeds analyzed by a different person or team unaware of the concentration of the food additive in the samples or the coding technique used to identify samples. If it is necessary to transport samples, they should be packaged to ensure the maintenance of their microbiological integrity during transportation, and sent by the fastest means possible.

The concentration of *Salmonella* present in samples collected from feeds used in the dose titration trial should be determined either by the standard dilution and pour-plate technique, or the most probable number method. Because the sensitivity of the standard dilution and pour-plate technique is low when used in isolating *Salmonella* from feeds containing viability-impaired and/or very small numbers of *Salmonella*, the samples analyzed by this technique should also be analyzed qualitatively for *Salmonella* using a procedure that includes pre-enrichment and enrichment.

Samples of feed collected for *Salmonella* analysis during both the "prevention of recontamination" and field trials should be analyzed qualitatively to determine the presence or absence of the microorganism.

CVM recommends the use of methods described in the FDA's Bacteriological Analytical Manual (Ref. 1). Sponsors may use other analytical techniques, but those techniques should be demonstrated to be at least as sensitive and specific for *Salmonella*.

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