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Safety and efficacy of 3-phytase FLF1000 as a feed additive for chickens for fattening and laying hens

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Abstract

The additive is a preparation of 3-phytase which is produced by a genetically modified strain of *Komagataella pastoris*. The production strain and its recombinant DNA were not detected in the final product. Therefore, the additive does not give rise to safety concerns with regard to the genetic modification of the production strain. Based on the results of the tolerance studies provided, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that the additive is safe for chickens for fattening and laying hens at the dose of 1,000 FTU/kg feed. However, a margin of safety could not be established for laying hens. The production strain belongs to a species considered to qualify for the qualified presumption of safety (QPS) approach to safety assessment when used for enzyme production. Since the identity of the strain was unambiguously established and the genetic modification raised no concerns, the FEEDAP Panel concludes that the use of the additive as a feed additive is of no concerns for consumers. The additive is not an irritant to eyes and skin, and it is not a dermal sensitiser. Owing to the proteinaceous nature of the active substance, the additive is a potential respiratory sensitiser, however, exposure may be limited. The use of the product as a feed additive does not pose risks to the environment. The Panel concluded that the additive has a potential to increase the availability of phytate phosphorus in chickens for fattening at 500 FTU/kg and in laying hens at 1,000 FTU/kg feed.

Keywords: zootechnical additives, digestibility enhancers, safety, efficacy, 3-phytase, chickens, laying hens

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Summary

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of 3-phytase FLF1000 (3-phytase) as a feed additive for chickens for fattening and laying hens.

The additive contains a 3-phytase (EC 3.1.3.8.) which is produced by a genetically modified strain of *Komagataella pastoris*. The identity of the strain was confirmed as *K. pastoris*. The viable production strain and its recombinant DNA were not detected in the final product. The additive does not give rise to safety concerns with regard to the genetic modification of the production strain.

Based on the results of the two studies provided, the FEEDAP Panel concluded that the additive is safe for chickens for fattening and laying hens at the dose of 1,000 FTU/kg feed. However, in the tolerance study in laying hens, the 10-fold dose group showed a tendency to have a lower body weight at the end of the study and had a significantly higher concentration of alkaline phosphatase in blood compared to the hens fed the negative control diet. Consequently, a margin of safety could not be established in laying hens.

The production strain belongs to a species considered to qualify for the qualified presumption of safety (QPS) approach to safety assessment when used for enzyme production. The identity of the strain was unambiguously established and the genetic modification raised no concerns. Therefore, the FEEDAP Panel concludes that the use of the product as a feed additive raises no concerns for consumers.

The additive is not an irritant to eyes and skin, and it is not a dermal sensitiser. Owing to the proteinaceous nature of the active substance, the additive is a potential respiratory sensitiser; however, exposure may be limited.

The use of the product as a feed additive does not pose risks to the environment.

The efficacy studies done in chickens for fattening showed that the addition of the additive to the feed increased phosphorus retention in three studies, in one at 250 FTU/kg and in two at 500 FTU/kg feed. Based on these results, the Panel concluded that the additive has a potential to be efficacious in chickens for fattening at 500 FTU/kg. In the case of the laying hens, the phosphorus retention was increased in two trials (in one at 1,000 FTU/kg feed and in the other one from the dose of 250 FTU/kg feed), and in a third trial, the daily egg mass production was higher from the dose of 250 FTU/kg feed as compared to the negative control. Therefore, the FEEDAP Panel concluded that the additive has a potential to be efficacious in laying hens at 1,000 FTU/kg feed.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Fertinagro Nutrientes SL[2] for authorisation of the product 3-phytase FLF1000 (3-phytase), when used as a feed additive for chickens for fattening and laying hens (category: zootechnical additives; functional group: digestibility enhancers).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 11 December 2015.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product 3-phytase FLF1000 (3-phytase), when used under the proposed conditions of use (see Section 3.1.5).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of 3-phytase FLF1000 as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[4] and the applicable EFSA guidance documents.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in Appendix A.[5]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of 3-phytase FLF1000 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c) and Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Fertinagro Nutrientes S.L. Pol. Ind. La Paz, parcel 185. 44195 Teruel, Spain.
[3] FEED dossier reference: FAD-2015-0026.
[4] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finirep-fad-2015-0026-preparation-3phytase.pdf

3. Assessment

3.1. Characterisation[6]

3.1.1. Characterisation of the active substance

The additive is a preparation of 3-phytase (EC 3.1.3.8.) which is produced by a genetically modified strain of the methylotrophic yeast *Komagataella pastoris* (formerly named as *Pichia pastoris*). The strain is deposited in the Spanish Type Culture Collection with the deposit number CECT 13094.[7] The identity of the strain was confirmed as *K. pastoris* by internal transcribed spacer (ITS) sequence analysis.[8]

K. pastoris is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach for safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2016), when used for enzyme production. The technical dossier contains detailed and sufficient information on the recipient microorganism, the origin and function of the different genetic elements introduced in the production strain, the genetic modification process and the genetic and phenotypic traits introduced.[9]

3.1.2. Manufacturing process

The 3-phytase is obtained by submerged aerobic fermentation of the production strain followed by a recovery and downstream processing.

After fermentation, the yeast cells are separated by centrifugation, homogenised and ultracentrifuged, and the resulting supernatant is mixed with the supernatant obtained after the initial cell separation step. The resulting product is then ultrafiltered, concentrated and formulated with sorbitol, sodium acetate trihydrate and potassium sorbate. No antibiotics are used in the manufacturing process.

3.1.3. Characterisation of the additive

The additive is available as a liquid formulation which contains enzyme protein (1.2 g/L), sorbitol (28–32%), sodium acetate trihydrate (0.4–0.6%), potassium sorbate (0.08–1.20%) and water (68–72%). This formulation ensures a minimum activity of 1,000 FTU/mL.[10] The study of the batch-to-batch variation in five batches showed a mean value of 1,255 FTU/mL (range: 1,237–1,292) with a coefficient of variation (CV) of 1.77%.[11] The formulation has a pH of 4.5,[12] bulk density (three batches) of 1.11–1.14 kg/L, viscosity of 2.68–3.80 cP and surface tension of 53.1–55.8 mN/m.[13]

Three batches of the additive were analysed for chemical and microbiological contamination.[14] The analyses of chemical contamination included arsenic (< 0.10 mg/kg), cadmium (< 0.10 mg/kg), lead (< 0.10 mg/kg), mercury (< 0.10 mg/kg), copper (2.5–3.8 mg/kg), zinc (2.3–5.6 mg/kg), total chromium (0.64–0.98 mg/kg), antimony (< 0.15 to 1 mg/kg) and barium (< 0.10 to 6.7 mg/kg). The following mycotoxins were also analysed: aflatoxin B1 (< 0.2 μg/kg), aflatoxin B2 (< 0.2 μg/kg), aflatoxin G1 (< 0.5 μg/kg), aflatoxin G2 (< 0.2 μg/kg), zearalenone (< 1 to 10 μg/kg) and fumonisin (< 1 μg/kg). Microbiological analysis included *Salmonella* spp. (absent in 25 g), *Escherichia coli* (< 3 CFU/g), coliforms (< 3 CFU/g), total aerobic plate counts (7.8×10^2 to 9.5×10^4 CFU/g at 22°C; < 10 to 1.4×10^3 CFU/g at 35°C), coagulase-positive staphylococci (< 10 CFU/g), yeasts (< 100 CFU/g) and moulds (< 100 CFU/g). The data showed high counts for aerobic microorganisms. After a modification of the manufacturing process, the counts in three batches were from 3.0×10^2 to 1.1×10^3 CFU/g at 22°C and from 7.0×10^2 to 1.4×10^3 CFU/g at 37°C.[15] No viable cells were detected in three batches (five 1 mL samples from each batch), of the additive by incubating in rich

[6] This section has been amended following the confidentiality claims made by the applicant.
[7] Technical dossier/Section II/Annex II.6.
[8] Technical dossier/Section II/Annex II.4.
[9] Technical dossier/Section II/Annex II.5.
[10] One FTU is the amount of 3-phytase which liberates 1 μmol of inorganic phosphate from phytate per minute pH 5.5 and 37°C.
[11] Technical dossier/Supplementary information August 2016/Annex 4.
[12] Technical dossier/Supplementary information August 2016/Annex 5.
[13] Technical dossier/Section II/Annex II.3.
[14] Technical dossier/Section II/Annex II.2.
[15] Technical dossier/Supplementary information August 2016/Annex 6.

non-selective medium at 30°C for 4 h, followed by plating in selective medium and cultivation at 30°C for 15 days.[16] No recombinant DNA was detected in three batches of the fermentation product, without added preservatives, analysed in triplicate by PCR.[17]

3.1.4. Stability and homogeneity

3.1.4.1. Shelf life

The shelf life of the additive was studied in samples from three batches (initial activities of 1,290, 1,190 and 1,340 FTU/mL, respectively) stored for 6 months at 25°C, 40°C or 4°C.[18] Recoveries of the initial enzyme activity after 6 months were higher than 61% (range 60.7–70.2%) for samples stored at 25°C and > 19% (19.7–24.3%) for samples stored at 40°C. In the samples stored at 4°C, no losses were observed. In the batches studied, compliance with the specifications would be found for storage times of 4 months at 25°C and for 1 month at 40°C.

3.1.4.2. Stability in feed

The same batches of the additive were added to both complete feed for chickens for fattening and for laying hens (mash form, based on maize and soya bean meal) at 250 FTU/kg and 500 FTU/kg. Samples of the feed were stored in paper bags at 25°C or 35°C for 3 months.[19] Recovery in the feed for chickens for fattening stored at 25°C were 86% and 94% for supplementation at 250 and 500 FTU/kg, respectively; the mean recoveries at 35°C were 74% regardless of the dose. The feed for laying hens stored at 25°C showed no losses for the supplementation at 250 FTU/kg feed and a mean of 90% recovery at 500 FTU/kg; the corresponding figures for the same feed stored at 35°C were 68 and 62%, respectively.

3.1.4.3. Homogeneity

The six batches of the mash feed tested for stability in feed (supplemented at a dose of 500 FTU/kg) and three batches of pelleted feed for chickens for fattening were used to study the capacity of the additive to homogeneously distribute.[20] Ten subsamples of each batch were analysed for enzyme activity and the CV was calculated. The CV in the feed for chickens for fattening was ≤ 12.5% in mash feed and < 18% in the pelleted, the CV in the feed for laying hens was ≤ 14.1%.

3.1.5. Conditions of use

The additive is to be used in feed for chickens for fattening or laying hens, mash or pelleted (addition after pelleting). For chickens for fattening, it is recommended a minimum dose of 250 FTU/kg and a maximum dose of 1,000 FTU/kg. For laying hens, the recommended dose is 1,000 FTU/kg feed.

3.2. Safety

3.2.1. Safety of the genetic modification[21]

The species from which the production strain was derived is *K. pastoris,* which is considered by EFSA to be suitable for the QPS approach to safety assessment when used for enzyme production (EFSA BIOHAZ Panel, 2016).

Neither the viable production strain nor its recombinant DNA were detected in the final product. Therefore, the additive, manufactured by fermentation with *K. pastoris* CECT 13094, does not give rise to safety concerns with regard to the genetic modification of the production strain.

3.2.2. Safety for the target species

3.2.2.1. Safety for chickens for fattening

A total of 588 one-day-old male chickens for fattening (Ross) were penned in groups of 14 birds and allocated to six dietary treatments (representing seven replicates per treatment).[22] Two basal

[16] Technical dossier/Section II/Annex II.8.
[17] Technical dossier/Section II/Annex II.7 and Supplementary information August 2016/Annex 10.
[18] Technical dossier/Section II/Annex II.10.
[19] Technical dossier/Section II/Annex II.11.
[20] Technical dossier/Section II/Annex II.11 and supplementary information June 2016/Annex 7.
[21] This section has been amended following the confidentiality claims made by the applicant.
[22] Technical dossier/Section III/Annex III.1.

diets, starter and grower, based on maize and soya bean meal with a total phosphorus/calcium content of 0.58/0.59% and 0.52/0.70%, respectively, were either not supplemented (negative control) or supplemented with the additive to provide 250, 500, 1,000 (1× maximum recommended dose) or 10,000 (10×) FTU/kg feed. The enzyme activity was confirmed by analysis. A positive control diet was also considered (total phosphorus/calcium content of 0.65/0.73% and 0.66/0.98% for starter and grower diets, respectively). Diets were offered on *ad libitum* basis and in mash form for 42 days (21 days starter, 21 days grower). General health status and mortality of the birds were monitored throughout the study. Feed intake and body weight were recorded weekly and feed to gain ratio was calculated. On day 37, blood samples were collected from seven birds per treatment. Blood samples were analysed for biochemical[23] and haematological[24] parameters.

An analysis of variance (ANOVA) was carried out with the data. Mortality rate was compared using a chi square. For performance data, the room was included in the model as a blocking factor and the initial body weight was used as a covariate. Statistical significance level was set at $p < 0.05$ and group means were compared with LSD test and against the negative control with the Dunnett test.

A total of 34 birds died during the study, 18 of which died during the first week. Mortality rates were 9.5%, 5.9%, 9.5%, 5.9%, 7.1% and 2.4% for the negative control, 250, 500, 1,000, 10,000 FTU/kg feed and positive control, respectively. A higher numerical mortality was registered in the negative control, 500 and 10,000 FTU/kg groups which was explained by the death of animals during the first week. This high mortality in the first week (non-starter birds) is considered of no relevance. No significant differences were found in the feed intake (110 g/day) and the final body weight of the birds (3.3 kg). The feed to gain ratio was ~ 1.50, and a significant difference was found between the negative control and the 500 FTU/kg feed group (1.51 vs 1.43). For the parameters studied in blood, the only difference found was for the percentage of monocytes between the negative control and the group with 500 FTU/kg feed (1.75% vs 2.29%).

The Panel concludes that the additive is safe for chickens for fattening at the maximum recommended dose.

3.2.2.2. Safety for laying hens

A total of 288 22-week-old laying hens (Lohmann Brown) were caged in 72 cages in groups of four and were allocated to six dietary treatments (representing 12 replicates per treatment).[25] A basal diet based on maize and soya bean meal, with a total phosphorus/calcium content of 0.37/2.97% was either not supplemented (negative control) or supplemented with the additive to provide 250, 500, 1,000 (1x) or 10,000 (10×) FTU/kg feed. The enzyme activity was confirmed by analysis. A positive control diet was also considered (total phosphorus/calcium content of 0.61/4.07%). Diets were offered *ad libitum* in mash form for 60 days. General health status and mortality of the hens were monitored throughout the study. Feed intake and body weight were recorded on days 1, 42, 53 and 59 of the study; average daily feed intake was calculated. Blood samples were collected and analysed for biochemical[23] and haematological[24] parameters. An ANOVA was carried out with the data obtained and Dunnett test was used to compare the group means against the negative control. Statistical significance level was set at $p < 0.05$.

Mortality was low; four hens died (one hen in each of the groups treated with the phytase). Daily feed intake was 108, 108, 111, 105, 107 and 109 g/day for the negative control, 250, 500, 1,000 and 10,000 FTU/kg, and positive control groups, respectively. The corresponding values for egg production were 97.5%, 97.5%, 97.9%, 98.0%, 96.9% and 97.3%; for egg mass output were 60.6, 60.4, 59.7, 59.8, 58.8 and 59.3 g/day and for feed to egg mass ratio were 1.80, 1.80, 1.83, 1.76, 1.82 and 1.85, respectively. None of these parameters was affected by the diet. The initial body weight of the hens was not different between treatments but at the end hens receiving the 10× showed a tendency ($p = 0.07$) to lower body weight compared to the negative control (1,854 vs 1,802 g for the negative control group and 10×, respectively).

Haematological parameters showed an effect of the diet on the mean corpuscular haemoglobin content and concentration. Hens in the groups fed with 250 and 10,000 FTU/kg feed had lower values than the negative control. Since this decrease was not related to the dose this modification is of no

[23] Including: glucose, calcium, phosphorus, alkaline phosphatase, alanine transaminase, aspartate transaminase, total bilirubin, amylase, gamma-glutamyl transpeptidase, urea, creatinine, albumin and total protein content.
[24] Including: haematocrit, haemoglobin, total white blood cells and differential counts, red blood cells, platelets and mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were calculated.
[25] Technical dossier/Section III/Annex III.2 and Supplementary information August 2016/Annex 12.

concern. The following blood chemistry parameters showed statistical differences: glucose, phosphorus, alkaline phosphatase, gamma-glutamyl transferase and uric acid concentration. Some of the differences identified were found in only one of the supplemented groups and therefore were not dose related (e.g. glucose) or were found between the positive and the negative controls (gamma-glutamyl transferase and phosphorus) and consequently are considered of no concern. Values of uric acid were lower compared to the negative control in all diets (including the positive control), but the decrease was not phytase-dose related and the values in the phytase-supplemented groups were similar to the positive control. Therefore, this reduction in the uric acid is considered of no concern. However, alkaline phosphatase concentration in the hens fed the 10× diet (2,522 U/L) was significantly higher compared to the negative control (678 U/L) and to any other treatment. The other groups supplemented with the phytase showed no statistically different values compared to the controls but there was a numerical increase with the dose (729, 976 and 812 U/L for 250, 500 and 1,000 FTU/kg feed). This result may indicate a negative effect of the phytase when administered at 10-fold the recommended dose.

The hens fed 10-fold the recommended dose tended to have a lower body weight at the end of the study and had a significantly higher concentration of alkaline phosphatase in blood compared to the hens fed the negative control diet. Hens fed the recommended dose (1,000 FTU/kg feed) showed no negative effects as compared to the negative control diet. Therefore, the FEEDAP Panel considers that the additive is safe at the maximum dose (1,000 FTU/kg feed), but a margin of safety cannot be established.

3.2.2.3. Conclusions on safety for the target species

The FEEDAP Panel concludes that the additive is safe for chickens for fattening and laying hens at the dose of 1,000 FTU/kg feed. A margin of safety for the additive in laying hens cannot be established.

3.2.3. Safety for the consumer

Toxicological studies are not required if the fermentation product is produced by a genetically modified microorganism that is considered by EFSA to qualify for the QPS approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2016) and the genetic modification raises no concerns. The enzyme is produced by a genetically modified strain of *K. pastoris*, this species is considered to qualify for the QPS approach to safety assessment when used for enzyme production. The identity of the strain was unambiguously established and the genetic modification raised no concerns. Therefore, the FEEDAP Panel concludes that the use of the product as a feed additive raises no concerns for consumers.

3.2.4. Safety for the user

3.2.4.1. Effects on the respiratory system

No specific study was provided. Owing to the proteinaceous nature of the active substance, the additive is considered as a potential respiratory sensitiser. The inhalation exposure is likely to be limited due to the method of handling the product.

3.2.4.2. Effects on skin and eyes

The irritant properties of the additive to skin and eyes were evaluated according to Organisation for Economic Co-operation and Development (OECD) test guidelines (TG) 404 and 405, respectively, in New Zealand White rabbits.[26] The test items were classified as not irritants.

The skin sensitising potential of the additive was evaluated in the guinea pig following OECD TG 406.[27] After induction (intradermic injection with 20% and topical application with 100% test item), the challenge phase consisted in a single topical application of 100% and 50% test item under occlusive dressing for 24 h. No cutaneous reaction attributable to allergy was reported after the challenge phase. Therefore, the additive is not a dermal sensitiser.

3.2.4.3. Conclusions on safety for the user

The additive is not irritant to eyes and skin and is not a dermal sensitiser. Owing to the proteinaceous nature of the active substance, the additive is a potential respiratory sensitiser, however, exposure may be limited.

[26] Technical dossier/Section III/Annex III.3 and III.4 and Supplementary information August 2016/Annex 13.
[27] Technical dossier/Section III/Annex III.5 and Supplementary information August 2016/Annex 13.

3.2.5. Safety for the environment

Neither the production strain nor its recombinant DNA was detected in the product. Therefore, the additive does not trigger an environmental safety concern associated with the genetic modification of the production strain.

The active substance of the additive is a protein and as such will be degraded/inactivated during the passage through the digestive tract of animals. Therefore, no risks to the environment are expected and no further environmental risk assessment is required.

3.3. Efficacy

3.3.1. Efficacy for chickens for fattening

Five trials were submitted, three were short-term trials and two were long-term trials which included measurements on the phosphorus retention.

3.3.1.1. Short-term trials

A total of 96 21-day-old male chickens for fattening (Ross) were penned in groups of eight birds and allocated to six dietary treatments.[28] After 7 days, the birds were caged in pairs in 48 cages (representing eight replicates per treatment) for 10 days. A phosphorus balance study was performed on days 15–17 of study (36–38 days of life). The treatments were obtained from a basal diet based on maize, wheat and soya bean meal and with a total phosphorus/calcium content of 0.50/0.68% that was either not supplemented (negative control) or supplemented with the additive to provide 250, 500, 1,000 or 10,000 FTU/kg feed. The enzyme activity was confirmed by analysis. A positive control diet was also considered (total phosphorus/calcium content of 0.60/0.91%). Diets were offered *ad libitum* in mash form. General health status of the birds was monitored throughout the study. Feed intake and body weight were recorded before and during the balance study. A balance for phosphorus was performed following total collection of excreta and measurement of feed intake for 3 days. At the end of the study (38 days of life), one animal per cage was killed, the tibia bone was collected, defatted and analysed for mineral composition. An ANOVA was performed with the data and group means were compared to control with Dunnett test. During the study, one animal from the group receiving 10,000 FTU/kg feed died. Phosphorus retention (%) was 38.8, 46.3, 46.6, 45.3, 40.6 and 34.2 for negative control, 250, 500, 1,000, 10,000 and the positive control, respectively. Values obtained in the groups receiving 250 and 500 FTU/kg feed were significantly higher than those obtained in the negative control group. Tibia phosphorus content (% in dry matter (DM)) was 9.3, 9.2, 9.1, 9.0, 8.9 and 9.2 for negative control, 250, 500, 1,000, 10,000 and the positive control, respectively, with no differences between treatments.

The other two short-term trials were carried out in the same place and shared the same experimental design.[29] In each trial, a total of 32 18-day-old male chickens for fattening (Ross 308) were caged in groups of two and allocated to two dietary treatments. The treatments were obtained from basal diets based on maize, wheat and soya bean meal and with a total phosphorus/calcium content of 0.50/0.68% in one trial and 0.57/0.74% in the other that were either not supplemented (negative control) or supplemented with the additive to provide 500 FTU/kg feed. The enzyme activities were confirmed by analysis. Diets were offered *ad libitum* in mash form. General health status of the birds was monitored throughout the study. Feed intake and body weight were recorded before and during the balance study. A balance for phosphorus was performed following total collection of excreta for 4 days (31–35 days of life). An ANOVA was performed with the data. Results showed statistically significant increases in the phosphorus retention in the birds fed the phytase at 500 FTU/kg feed in the two trials (49% vs 54% in one trial and 51% vs 56% in the other).

3.3.1.2. Long-term trials

The first long-term trial is the tolerance trial already described in the chapter on the safety for the target species (see Section 3.2.3), measurements of the performance and of the phosphorus retention were carried out. On day 28 of the study, two to three animals per pen were selected (average weight) and were caged in groups of two in 48 cages (representing eight replicates per treatment). After 6 days of adaptation, a balance for phosphorus was done following total collection of excreta for

[28] Technical dossier/Section IV/Annex IV.1 and Supplementary information August 2016/Annex 14.
[29] Technical dossier/Supplementary information August 2016/Annex 15 and 16.

3 days (days 34 and 37 of life). On day 37, one animal per cage was killed and the left tibia bone was collected, defatted and analysed for mineral content. An ANOVA was done with the data and group means were compared against the negative control with the Dunnett test. The results are presented in Table 1. Although the absolute weight of the tibia (DM), of the ash and of the P of the tibia were significantly improved by the additive from 250 FTU/kg feed, the tibia P content, as % of the DM, was not affected. The only significant effect was seen in the feed to gain ratio of birds fed 500 FTU/kg feed, which showed an improvement compared to the negative control. Since that was the only effect found and taking into account the limitations in the study design (animals in cages), the Panel considers these data as supportive of the efficacy.

Table 1: Effects of the additive on the performance of birds (42 days old), phosphorus retention (%) and tibia phosphorus content (%) (37 days old)

Group	Daily feed intake (g)	Final body weight (g)	Feed to gain ratio	Mortality (%)	P retention (%)	Tibia P content (% DM)
Negative control	114	3,363	1.51	9.5	42.1	9.5
250	112	3,318	1.51	5.9	40.2	9.7
500	108	3,333	1.43*	9.5	46.9	9.7
1,000	110	3,327	1.47	5.9	45.7	9.7
10,000	111	3,327	1.47	7.1	41.6	9.7
Positive control	114	3,296	1.51	2.4	36.2	9.7

DM: dry matter.
*Significantly different to the negative control diet ($p < 0.05$).

The second long-term trial was performed with a total of 160 one-day-old male chickens for fattening (Cobb) that were caged in groups of four in a total of 40 cages and distributed to five treatments (representing eight replicates per treatment). Two basal diets, starter and grower, based on maize, wheat and soya bean meal and with a total phosphorus/calcium content of 0.63/0.77% and 0.60/0.85%, respectively, were either not supplemented (negative control) or supplemented with the liquid additive to provide 250, 500 or 1,000 FTU/kg feed. The enzyme activity was confirmed by analysis. A positive control diet was also considered (total phosphorus/calcium content of 0.80/1.03% and 0.80/1.03% for starter and grower diets, respectively). Diets were offered *ad libitum* in mash form for 35 days (21 days starter, 14 days grower) and contained titanium dioxide as an external marker. The general health status of the birds was monitored throughout the study. Feed intake and body weight were recorded weekly; feed to gain ratio was calculated. On the last day, all birds were killed and the tibia bones of three birds per cage were collected, defatted and analysed for ash content. Ileal contents from the same animals were sampled for the determination of the digestibility of phosphorus. An ANOVA was carried out with the data and group means were compared with LSD method. Results are presented in Table 2. Mortality was not reported. The data showed no significant differences between the groups supplemented with the 3-phytase under assessment and the negative control.

Table 2: Effects of the additive on the performance of birds, tibia phosphorus content and ileal phosphorus digestibility

Group	Daily feed intake (g)	Final body weight (g)	Feed to gain ratio	Ileal P digestibility (%)	Tibia ash content (% DM)
Negative control	85.6^{bc}	$2{,}054^{ab}$	1.49	49.9	51.5
250	82.8^{c}	$2{,}030^{b}$	1.46	54.2	51.4
500	83.5^{bc}	$1{,}997^{b}$	1.49	53.4	51.5
1,000	87.3^{ab}	$2{,}108^{a}$	1.48	55.7	51.6
Positive control	90.5^{a}	$2{,}139^{a}$	1.51	50.3	52.2

DM: dry matter.
a,b,c: Values within a column with different superscript are significantly different ($p < 0.05$).

The addition of the enzyme resulted in a higher P retention in three studies, in one at 250 FTU/kg and in two at 500 FTU/kg feed. Based on these results, the Panel concludes that the additive has a potential to be efficacious in chickens for fattening at 500 FTU/kg.

3.3.2. Efficacy for laying hens

Four short-term trials and one long-term trial were provided. Two of the short-term trials were not considered because the phosphorus balance was conducted following the method of total collection of excreta and the collection was done for only 2 days.[30] The Panel considers that the collection should last for at least 3 days (Bourdillon et al., 1990).

3.3.2.1. Short-term trials

The first short-term trial was performed in the tolerance trial described in the chapter on the safety for the target species (see Section 3.2.3). Hens, 22-week-old, received the experimental diets for 3 weeks and then a balance trial was performed. Feed intake was measured and excreta were collected for 2 days. Titanium dioxide was used as an external marker. At the end of the tolerance trial, 31 weeks of life, one hen per cage (12 hens per treatment) was killed and the left tibia bone was collected, defatted and analysed for phosphorus content. An ANOVA was carried out with the data obtained and group means were compared against the control with Dunnett test. No differences were found in the daily feed intake, egg production, daily egg mass production and the feed to egg ratio between the diets supplemented with the phytase and the negative control diet. Phosphorus retention was significantly higher in the group fed 1,000 FTU/kg feed as compared to the negative control diet (38.5% vs 26.6%). Tibia phosphorus content showed no differences between treatments.

In the second short-term trial, a total of 175 23-week-old laying hens (Lohmann Brown) were randomly caged in groups of five animals and allocated to five dietary treatments (representing seven replicates per treatment).[31] A basal diet based on maize and soya bean meal and with a total phosphorus/calcium content of 0.39/3.95% was either not supplemented (control) or supplemented with the additive to provide 250, 500 or 1,000 FTU/kg feed. The enzyme activity was confirmed by analysis. A positive control diet was also considered (0.65/3.95% phosphorus/calcium). Diets were offered *ad libitum* in mash form for 4 weeks. Diets contained titanium dioxide as an external marker. Hens were weighed at the beginning and at the end of the study. Total number of eggs produced was registered and the eggs laid in the last 2 days of each week were weighed per pen and used to estimate the egg weight per cage laid per week. In the last week of the experiment, excreta samples were collected for 3 days and analysed for phosphorus retention. An ANOVA was performed with the data and the group means were compared with a Dunnett test. Phosphorus retention was 33, 44, 44, 49 and 52% for the control diet, 250, 500, 1,000 FTU/kg feed and the positive control, respectively. The supplementation of the diet with the additive resulted in significant differences compared to the control diet from 250 FTU/kg feed onwards. Feed intake, egg production, egg weight and feed to gain ratio showed no significant differences between treatments.

3.3.2.2. Long-term trial

The long-term trial provided had the same experimental design considering the animals involved, replicates and treatments (see above).[32] Hens were under study from week 22 of life to week 46 of life. Hens were weighed at the beginning and at the end of the study. Total number of eggs produced was registered throughout the study and the eggs laid in the last 2 days of each week were weighed per pen and used to calculate the egg mass per cage laid per week. The number of broken, dirty and shell-less eggs was registered. A total of 12 eggs per cage were used to measure the Haugh units, yolk colour, shell strength and shell thickness. At the end of the study, two hens per cage were killed and the tibia bones were collected to analyse phosphorus. Two eggs per replicate were collected in week 46 to measure the phosphorus content. An ANOVA was performed with the data and the group means were compared against the negative control with Dunnett test.

Results are presented in Table 3. No mortality occurred. The supplementation with 3-phytase from 250 FTU/kg feed increased the daily egg mass, compared to the negative control. None of the other parameters were affected.

[30] Technical dossier/Section IV/Annex IV.5 and IV.6.
[31] Technical dossier/Supplementary information August 2016/Annex 17.
[32] Technical dossier/Supplementary information August 2016/Annex 18.

Table 3: Effect of the additive on the laying performance of laying hens and phosphorus in the tibia bone and eggs

Group	Daily feed intake (g)	Egg production (%)	Egg weight (g)	Daily egg mass	Feed to egg mass	P in tibia ash (%)	P in egg ash (%)
Negative control	114	92.4	60.1	55.5	2.05	16.9	53.7
250	119	96.0	62.2	59.8*	2.00	16.9	53.7
500	118	95.1	61.7	59.1*	2.01	16.9	53.3
1,000	116	95.7	62.0	59.3*	1.96	16.9	54.4
Positive control	116	94.6	61.5	58.2	1.99	17.1	54.9

*Values are significantly different to the value in the negative control diet ($p < 0.05$).

In two short-term trials in laying hens, the supplementation with 3-phytase increased phosphorus retention, one at 1,000 and another one from 250 FTU/kg. In a long-term trial, the 3-phytase from 250 FTU/kg increased the daily egg mass production of the laying hens. The FEEDAP Panel concludes that the additive has a potential to be efficacious in laying hens at 1,000 FTU/kg feed.

3.3.2.3. Conclusions on efficacy for the target species

The FEEDAP Panel concludes that the additive has a potential to be efficacious in improving the availability of phytate phosphorus in chickens for fattening at 500 FTU/kg feed and in laying hens at 1,000 FTU/kg feed.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[33] and Good Manufacturing Practice.

4. Conclusions

The production strain and its recombinant DNA were not detected in the additive. Therefore, the final product does not pose any safety concern associated with the genetic modification of the production strain.

The additive is safe for chickens for fattening and laying hens at the dose of 1,000 FTU/kg feed, but in laying hens a margin of safety cannot be established.

The use of 3-phytase FLF1000 as feed additive does not give rise to concerns for consumers.

The additive is not an irritant to eyes and skin and it is not a dermal sensitiser, but it is a potential respiratory sensitiser.

The FEEDAP Panel concludes that the additive has a potential to be efficacious in improving the availability of phytate phosphorus of the diets in chickens for fattening at 500 FTU/kg feed and in laying hens at 1,000 FTU/kg feed.

Documentation provided to EFSA

1) 3-Phytase FLF1000 as a feed additive for chickens for fattening and laying hens. July 2015. Submitted by Fertinagro Nutrientes S.L.
2) 3-Phytase FLF1000 as a feed additive for chickens for fattening and laying hens. Supplementary information. August 2016. Submitted by Fertinagro Nutrientes S.L.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for 3-phytase FLF1000.
4) Comments from Member States.

[33] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

References

Bourdillon A, Carré B, Conan L, Francesch M, Fuentes M, Huyghebaert G, Janssen WM, Leclercq B, Lessire M, McNab J, Rigoni M and Wiseman J, 1990. European reference method of in vivo determination of metabolisable energy in poultry: reproducibility, effect of age, comparison with predicted values. British Poultry Science, 31, 567–576.

EFSA (European food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007;5(12):587, 16 pp. doi:10.2903/j.efsa.2007.587

EFSA (European Food Safety Authority), 2008, revised in 2009. Technical Guidance of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) for assessing the safety of feed additives for the environment. EFSA Journal 2008;6(10):842, 28 pp. doi:10.2903/j.efsa.2008.842

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2016. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 4: suitability of taxonomic units notified to EFSA until March 2016. EFSA Journal 2016;14(7):4522, 37 pp. doi:10.2903/j.efsa.2016.4522

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: tolerance and efficacy studies in target animals. EFSA Journal 2011;9(5):2175, 15 pp. doi:10.2903/j.efsa.2011.2175

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for zootechnical additives. EFSA Journal 2012;10(1):2536, 19 pp. doi:10.2903/j.efsa.2012.2536

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance for establishing the safety of additives for the consumer. EFSA Journal 2012;10(1):2537, 12 pp. doi:10.2903/j.efsa.2012.2537

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. doi:10.2903/j.efsa.2012.2539

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2011. Scientific Opinion on Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use. EFSA Journal 2011;9(6):2193, 54 pp. doi:10.2903/j.efsa.2011.2193

Abbreviations

ANOVA	analysis of variance
CFU	colony-forming unit
CV	coefficient of variation
DM	dry matter
EURL	European Union Reference Laboratory
FEEDAP Panel	EFSA Panel on Additives and Products or Substances used in Animal Feed
ITS	internal transcribed spacer
LSD	least significant difference
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
QPS	qualified presumption of safety

Appendix A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for 3-phytase FLF1000.

In the current application authorisation is sought under article 4(1) of the Regulation (EC) No 1831/2003 for a Preparation of 3-phytase (FLF1000) under the category/functional groups 4a and 4c "zootechnical additives"/"digestibility enhancers" and "substances which favourable affect the environment". Specifically, authorisation is sought for the use of the feed additive for chickens for fattening and laying hens.

According to the Applicant, FLF1000 is an aqueous solution containing 3-phytase as active agent. The 3-phytase enzymatic activity is expressed in FTU units, where "one FTU is the amount of enzyme which releases one micromole of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37°C".

The product is intended to be marketed as liquid formulations having a guaranteed minimum phytase activity of 1000 FTU/ml. FLF1000 is intended for use in direct applications in mash feed or in pelleted feed after pelleting to obtain a minimum activity of 500 and 1000 FTU/kg feedingstuffs for chicken for fattening and for laying hens respectively.

For the quantification of phytase activity in feedingstuffs the Applicant applied the colorimetric EN ISO 30024 standard method. Furthermore the Applicant also applied the ISO standard with minor experimental modifications to the analysis of the feed additive (FLF1000) and obtained similar method performance characteristics. Based on the performance characteristics obtained, the EURL recommends for official control the colorimetric methods mentioned above for the quantification of phytase activity in the feed additive and feedingstuffs.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.