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Safety and efficacy of fumonisin esterase from *Komagataella phaffii* DSM 32159 as a technological feed additive for pigs and poultry

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Abstract

Fumonisin esterase produced from a genetically modified strain of *Komagataella phaffi* is intended to degrade fumonisin mycotoxins contaminants in feeds for pigs and poultry. The production strain and its recombinant genes are not present in the final product. The applicant selected 300 U/kg feed to represent a likely upper limit. This concentration showed to be safe for piglets, chickens and turkeys for fattening and laying hens; the additive is thus safe for those categories. This conclusion is extended to all pigs, chickens reared for laying and turkeys reared for breeding and extrapolated to all other poultry species for growing and laying and to minor porcine species. No evidence of mutagenicity or genotoxicity was detected and no evidence of toxicity from a repeated-dose oral toxicity study; the residue assessment did not identify any concern. The use of the additive is, thus, considered safe for consumers. The additive is not toxic by inhalation and the respiratory exposure is likely to be low; however, a risk of sensitisation via the respiratory route cannot be excluded. The additive is non-irritant to skin and eyes and is not considered a dermal sensitiser. No risks for the environment are expected following the use of the additive in feeds under the proposed condition of use. The additive has the capacity to degrade fumonisin contaminants in feed of marketable quality when used at the minimum recommended dose of 10 U/kg complete feed, as shown in studies with chickens for fattening, laying hens and weaned piglets. Since the mode of action of the additive can be reasonably assumed to be the same in animal species for which the application is made, the Panel on Additives and Products or Substances used in Animal Feed **(**FEEDAP) considers the additive efficacious for all poultry and all pigs.

Keywords: technological additive, 'substances for reduction of the contamination of feed by mycotoxins', fumonisin esterase, *Komagataella phaffi*, pigs, poultry, sphinganine/sphingosine

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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 Biomin GmbH[2] for authorisation of the product fumonisin esterase (FUMzyme®), when used as a feed additive for all pigs and all avian species (category: technological additives; functional group: substances for reduction of the contamination of feed by mycotoxins).

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). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 2 March 2017.

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 fumonisin esterase (FUMzyme®), when used under the proposed conditions of use (see Section 3.1.7).

1.2. Additional information

The product under assessment is an enzyme-based additive intended to degrade fumonisin mycotoxins found as contaminants of feed. The safety and efficacy of the enzyme expressed in a different host from that used in the present application were the subjects of two previous opinions: the first, published in 2014, considered its use in feeds for pigs (EFSA FEEDAP Panel, 2014) and the second in 2016 its use in feed for all avian species (EFSA FEEDAP Panel, 2016). This additive is currently authorised for use in pigs[3] and avian species (1m03).[4]

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[5] in support of the authorisation request for the use of FUMzyme® 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[6] and the applicable EFSA guidance documents.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the fumonisin esterase (EC 3.1.1.87) (FUMzyme®) in animal feed. The Executive Summary of the EURL report can be found in Annex A.[7]

[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] Biomin GmbH, Erber Campus 1, 3131 Getzersdorf, Austria.
[3] Commission Implementing Regulation (EU) No 1115/2014 of 21 October 2014 concerning the authorisation of a preparation of fumonisin esterase produced by *Komagataella pastoris* (DSM 26643) as a feed additive for pigs. OJ L 302, 22.10.2014, p. 51.
[4] Commission Implementing Regulation (EU) 2017/913 of 29 May 2017 concerning the authorisation of a preparation of fumonisin esterase produced by *Komagataella pastoris* (DSM 26643) as a feed additive for all avian species. OJ L 139, 30.5.2017, p. 33.
[5] FEED dossier reference: FAD-2017-0005.
[6] 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.
[7] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0005_fumzyme.pdf

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of FUMzyme® is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on technological 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).

3. Assessment

The present application concerns an additive containing the enzyme fumonisin esterase as the single active agent intended for use as a technological additive (functional group substances for reduction of the contamination of feed by mycotoxins).

3.1. Characterisation

3.1.1. Characterisation of the active substance

The active substance, the enzyme fumonisin esterase (EC 3.1.1.87), partially degrades fumonisin B1 (FB1) and related fumonisins by cleavage of the diester bonds and release of the tricarballylic acid and is intended to reduce the toxicity of contaminated feed. The enzyme was identified in a *Sphingopyxis* sp., an α-Proteobacterium isolated from soil, and the genes encoding the activity used to transform a strain of the yeast *Komagataella phaffii* (formerly *Pichia pastoris*) which is then used as the production organism.[8]

3.1.2. Characterisation of production organism

The additive is produced with a genetically modified strain of *K. phaffii*, which is deposited in the German Collection of Microorganisms and Cell Cultures as *P. pastoris* with deposit number DSM 32159.[redacted] The strain was identified as *K. phaffii* by sequence analysis of the 26S/28S and ITS regions of the rRNA gene, and partial sequence of the EF-1α gene.[10]

3.1.2.1. Characteristics of the recipient or parental microorganism

The recipient strain is a genetically modified derivative of the parental strain *K. phaffii* CBS 7435 (NRRL Y-11430, ATCC 76273). The whole-genome sequence of this strain is published (Küberl et al., 2011). The strain was originally taxonomically classified as *P. pastoris*, a species which is considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA BIOHAZ Panel, 2017) when used for enzyme production. Recently, this strain was renamed as *K. phaffii* by molecular taxonomy analyses. The FEEDAP Panel considers that this reclassification does not have an impact in the safety status of the strain. [redacted]

3.1.2.2. Characteristics of the donor organism

The *fumD* [redacted] gene, coding for a FB1 carboxylesterase, is a codon-optimised synthetic sequence based on the *fumD* gene of the α-proteobacterium *Sphingopyxis* sp. MTA144.[12] This strain, which was isolated from soil, carries a gene cluster associated with fumonisin degradation as described by Heinl

[8] Technical Dossier/Section II/Annex II.19.

[redacted]

[10] Technical Dossier/Supplementary Information/Annex (i).

[redacted]

[12] Technical Dossier/Section II/Annex II.27.

et al. (2010). The gene *fumD* codes for a carboxylesterase, which catalyses the hydrolysis of fumonisin B1 to aminopentol 1 (producing fully de-esterified fumonisin, HFB1).

3.1.2.3. Description of the genetic modification

3.1.3. Manufacturing process

Fumonisin esterase is produced by the submerged fermentation of the production strain. After fermentation, the biomass is separated by centrifugation, and the supernatant is then micro- and ultrafiltered. The final additive is prepared from the high-molecular weight material recovered from ultrafiltration mixed with 10% maltodextrin, which is then spray dried. The dried material is then blended with maltodextrin.

FUMzyme® is routinely quality controlled for the presence/absence of cultivable *K. phaffii*, following a specific procedure.[17] The absence of viable cells of the production strain is tested in 25 g after a resuscitation step in non-selective liquid medium at 37°C for 6–8 h, followed by plating 0.1 mL of the suspension onto selective medium and incubating at 30°C for 48–96 h.[18] No viable cells detected in three batches analysed in triplicate.[19] Recombinant DNA is also removed during downstream processing: no recombinant DNA was detected in 100 mg of three batches of the final product by real-time PCR,

[17] Technical dossier/Section II/Annex II.65.
[18] Technical dossier/Section II/Annex II.66.
[19] Technical Dossier/Supplementary Information/Annex (ii).

3.1.4. Characterisation of the additive

The additive typically contains approximately 8% of material derived from the fermentation and 90% of the added maltodextrin carrier and has a specified minimum content of 3,000 U esterase/g. One unit (U) is the enzymatic activity that releases 1 μmol propane-1,2,3-tricarboxylic acid per minute from 100 μM FB1 in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/mL bovine serum albumin at 30°C. Compliance with this minimum specification was demonstrated for five batches of the additive (mean 3,470 U/g, range 3,150–3,780 U/g additive).[21]

Routine monitoring for the presence of heavy metals and arsenic (As) in three different production batches of the additive showed that the concentrations of lead (Pb), cadmium (Cd), mercury (Hg) and As were in all cases below the respective limits of quantification (Pb < 0.5, Cd < 0.1, Hg < 0.01 and As < 0.5 mg/kg).[22]

Quality control parameters for microbial contaminants are set at a maximum of 30 colony-forming units (CFU)/g for total coliforms, a maximum of 100 CFU/g individually for yeasts and filamentous fungi and the absence of *Escherichia coli* and *Salmonella* spp. in 25 g product; certificates of analysis for three batches of product show compliance with these values.[23] A further three batches were examined for the presence of 11 mycotoxins commonly associated with cereals[24]; all values were below the limits of detection.[25]

Particle size analysis by laser diffraction (three batches) showed that approximately 60% of the final product had a particle size < 100 μm, 40% < 50 μm and ~ 5% of respirable size (< 10 μm).[26] The dusting potential of the same three batches determined according to the Stauber–Heubach method gave a mean value of 0.25 g/m^3.[27]

3.1.5. Stability and homogeneity

3.1.5.1. Shelf-life

The shelf-life of the product was established using five batches of product stored at room temperature or at 37°C for 18 months in sealed containers.[28] Samples were analysed for activity at six monthly intervals. There was essentially no loss of activity at room temperature after 18 months and only a small loss at the higher temperature (10–15%).

3.1.5.2. Stability in premixes and feed

A single batch of the additive was mixed with a commercial bentonite mineral feed at 20,000 U/kg and a commercial vitamin–mineral premix (containing choline chloride) to a concentration of 15,000 U/kg.[29] The samples were then stored for 6 months at room temperature. No loss of activity of any significance was seen after 6 months storage under these conditions.

Four batches of feed (in mash form) containing the additive were prepared, the first three based on a grower diet for pigs supplemented with 10, 15 or 20 U/kg feed and the fourth on a feed for chickens supplemented with 45 U/kg feed.[30] Activity was measured at the time of preparation and again after 6 months storage at 22 ± 2°C. Essentially no loss of activity was recorded.

3.1.5.3. Homogeneity

A single batch of additive was mixed with additional maltodextrin and then mixed with 99 kg piglet feed using a farm-scale feed mill.[31] The treated feed with an intended concentration of 450 U/kg feed was then distributed equally to 10 bags and, from each bag, 10 subsamples were taken and analysed for activity. The results showed a mean value of 477 U/kg feed with a coefficient of variation (CV) of 3.6%.

[21] Technical Dossier/Section II/Annex II.03.
[22] Technical Dossier/Section II/Annexes II.03, II.05 and II.06.
[23] Technical Dossier/Section II/Annexes II.08, II.09 and II.10.
[24] Technical Dossier/Section II/Annex II.07.
[25] Limits of detection (in μg/kg) are listed here. Deoxynivalenol: 20, zearalenone: 4, aflatoxin B1/B2/G1: 0.2, fumonisin B1/B2: 20, HT-2 toxin/T-2 toxin: 2.
[26] Technical Dossier/Section II/Annex II.12.
[27] Technical Dossier/Section II/Annexes II.13, II.14 and II.15.
[28] Technical Dossier/Section II/Annex II.67.
[29] Technical Dossier/Section II/Annex II.68.
[30] Technical Dossier/Section II/Annex II.69.
[31] Technical Dossier/Section II/Annex II.70.

3.1.6. Interference with the analysis of mycotoxins in feed

The possible interference of the additive in the determination of fumonisins in feedstuffs was examined.[32] The methanol/acetonitrile/water solution used for the initial extraction of fumonisins in the standard method was found to fully inhibit the action of fumonisin esterase and, therefore, no interference is expected. Given the specificity of the esterase, the analytical determination of other structural classes of mycotoxins would not be affected.

3.1.7. Conditions of use

The additive is intended for use with pigs and poultry at a minimum inclusion rate of 10 U/kg complete feed. No maximum incorporation rate is proposed. No withdrawal period is foreseen.

3.2. Safety

3.2.1. Safety aspects of the genetic modification

The original strain from which the production organism was derived belong to *K. phaffii*, formerly identified as *P. pastoris*, a species which is considered by EFSA to be suitable for the QPS approach to safety assessment when used for enzyme production (EFSA BIOHAZ Panel, 2017). The FEEDAP Panel considers that this reclassification does not have an impact in the safety status of the strain. The production strain carries several copies of the Zeocin™ resistance gene and the Geneticin™ resistance gene integrated into its chromosome.

Neither the viable production strain nor its recombinant DNA was found in the final product. Therefore, the product FUMzyme®, manufactured by fermentation with *K. phaffii* DSM 32159, does not give rise to safety concerns deriving from the genetic modification of the production strain.

3.2.2. Toxicological studies

The toxicity studies were conducted with a non-standardised production batch in which the proportion of the culture supernatant was greater than the standardised product resulting in higher concentrations of the enzyme (8,650 U/g).

3.2.2.1. Genotoxicity

Bacterial reverse mutation test

Two independent experiments were made in compliance with the Organisation for Economic Co-operation and Development (OECD) guideline 471, with the Salmonella Typhimurium strains TA1535, TA1537, TA98, TA100 and TA102 with and without metabolic activation.[33] Six doses were tested, starting at 31.6 μg and increasing to a top dose of 5,000 μg. No precipitation of the test item or toxic effects was seen. No increase in the number of revertant colonies was seen at any dose tested in the presence or absence of metabolic activation. The additive is, therefore, considered non-mutagenic under the conditions of the assay.

In vitro chromosome aberration test

This test was made according to the OECD guideline 473 using the Chinese hamster V79 cell line.[34] Chromosomes were prepared 21 h after the start of treatment with the test item and 150 metaphases per culture scored for structural chromosome aberrations. Treatment intervals were 4 h with and without metabolic activation in experiment 1 and 4 h with activation and 20 h without activation in experiment 2. The doses tested were 1,000, 1,500 and 2,000 μg/mL in both parts of the test. The appropriate positive controls were included (ethyl-methanesulfonate and cyclophosphamide) which behaved as expected. The test item did not induce structural chromosome aberrations in either experiment and no increase in the frequency of polyploid cells was observed in comparison with the negative control. The test item is thus considered non-clastogenic under the conditions of the test.

[32] Technical Dossier/Section II/Annex II.71.
[33] Technical Dossier/Section III/Annex III.34.
[34] Technical Dossier/Section III/Annex III.35.

In vivo mammalian erythrocyte micronucleus test

The test was made following OECD guideline 474 using mouse peripheral blood cells.[35] Blood samples were collected for analysis from 10 mice (strain NMRI; five males and five females) per treatment 44 and 66 h after a single administration of the test item. A maximum tolerated dose (MTD) of 2,000 mg/kg body weight (bw) was selected on the basis of a preliminary range finding experiment with intermediate doses of 1,000 and 400 mg/kg bw, representing 0.5 and 0.2 of the MTD. Animals given the two lower doses showed no clinical symptoms. Those given the top dose showed slight signs of systemic toxicity. For all doses, 10,000 polychromatic erythrocytes per animal were scored for the incidence of micronucleated immature cells. Negative controls were within the historical data and the positive control (cyclophosphamide) behaved as expected. No biologically relevant increase in micronucleated cells was seen at any dose, from which it was concluded that the test item is non-mutagenic with respect to clastogenicity and/or aneugenicity.

3.2.2.2. Repeated -dose 90-day oral toxicity study

A Good Laboratory Practice (GLP) compliant repeat dose study was made with equal numbers of male and female Wistar rats following the protocol for OECD guideline 408.[36] Animals were caged in groups of five animals of the same sex. Twenty animals (two cages of each sex per treatment) were assigned to each of a vehicular control and groups given daily 100, 1,000 and 2,000 mg/kg bw of the test item via the feed for 90 days. A further satellite group of 10 animals was also given the high dose for 90 days. Body weight was determined at the start and weekly thereafter and body condition monitored daily. Blood samples were taken on day 90 from the control and high-dose groups for haematology and clinical chemistry. A complete necropsy of all animals was made with histopathology for tissues from the high-dose group. Animals in the satellite group were observed for a further 28 post-treatment days before necropsy on day 118.

No mortalities, behavioural changes or adverse clinical signs were noted during the study. Growth of males and females was not significantly different between the treatment groups. No statistically significant changes in clinical chemistry results were seen in either sex or in haematology results for males. The only significant difference observed was a change in the relative volume of lymphocytes in female rats given the top dose compared to the control group. This change is reported as being within the historical range for the colony. There were no significant differences in relative or absolute organ weights in male rats, but significant differences were seen in the absolute weight of uterus and ovaries and relative weight of ovaries. These changes were not dose related and were within historical values and were attributed by the study authors to animals at different stages of their oestrus cycle.

3.2.2.3. Metabolites resulting from the degradation of fumonisins

Metabolites resulting from the complete or partial de-esterification of fumonisins were assessed for safety in the previous opinion on the use of a fumonisin esterase in pigs and found to be less toxic than the parent mycotoxin (EFSA FEEDAP Panel, 2014). Since the action of the esterase on any contaminating fumonisins will be essentially the same in the pig and poultry digestive tracts and independent of the source of the enzyme, the previous assessment and its conclusion apply equally to the present application.

3.2.3. Safety for the target species

The recipient strain *K. phaffii* used for the production of the enzyme is a species considered by EFSA to be suitable for the QPS approach to the assessment of safety. Since the genetic modifications give no cause for concern and since the enzyme itself has been assessed for safety in the context of previous opinions, there is little reason to consider further target animal safety. However, the applicant has chosen to provide four additional tolerance studies with weaned piglets, chickens and turkeys for fattening and laying hens.

3.2.3.1. Safety for weaned piglets

A total of 96 weaned piglets (mixed sex O-HYB-F1 with mean start weight of approximately 10 kg) were assigned to one of three groups: a control group and two test groups provided with the additive.

[35] Technical Dossier/Section III/Annex III.36.
[36] Technical Dossier/Section III/Annex III.37.

Each treatment consisted of four replicate pens of eight animals.[37] Since no recommended or maximum inclusion level of the additive is proposed, the applicant selected 300 U/kg feed to represent the likely maximum inclusion rate for the ×1 group and an overdose group given 100-fold this value (30,000 U/kg feed). Concentrations in feed were confirmed by analysis; feed showed analytically to be free of contaminating fumonisins. All animals were given a basal diet based on wheat, barley and soybean in mash form for 42 days. Pigs were weighed at the start and at days 14, 28 and 42 and average weight gain calculated; feed intake was measured for the corresponding periods. Feed intake per animal and day and feed to gain calculated. The pen was used throughout as the experimental unit. After a test for normal distribution (Shapiro–Wilk test), data were analysed by the analysis of variance (ANOVA) or by the non-parametric Kruskal–Wallis test.

No mortalities were recorded. No significant differences were found in any of the measured or calculated parameters. Final weight was approximately 31 kg, weight gain 21 kg, feed intake 0.9 kg per animal per day and feed to gain ratio 1.84.

3.2.3.2. Safety for chickens for fattening

A total of 600 one-day-old chicks (Ross 308, mixed sex) were distributed to the same three treatment groups described above.[38] Each treatment was allocated 10 replicate pens each with 20 birds per pen. Birds were given a basal starter and grower diets based on maize and soybean in mash form which was shown to be essentially free of contaminating fumonisins. The duration of the study was 35 days. Birds were weighed at the start of the study, after 14 days and at the end. Feed consumption was measured over the corresponding periods, and from these data, weight gain and feed to gain ratio were calculated. The statistical analysis applied was that described for the study in weaned piglets, using the pen as experimental unit. Data analysis included tests for homogeneity of variances (Levene's test). Where variances were not homogeneous, ANOVA was replaced by a Welch test. This was used in the analysis of feed to gain values.

No veterinary intervention was required during the study. Mortality was not reported. There were no significant differences in final weight of birds (range: 1.71–1.80 kg) or feed intake (3.0 kg). Feed to gain ratio appeared to be marginally improved in the two treated groups compared to control birds (1.87 vs. 1.72 and 1.73, $p = 0.097$).

3.2.3.3. Safety for turkeys for fattening

A randomised block design was used involving a total of 360 female Hybrid Converter turkey poults allocated to the same treatments groups as the previously described tolerance studies (0, ×1 and ×100).[39] However, analysis showed that the recovered enzyme activity was approximately 50% higher than the intended level (i.e. 0, × 1.5 and × 150). Birds were allocated to 10 replicate pens of 12 female animals per treatment group and fed a basal maize–soybean diet free of fumonisins for 42 days. Birds were individually weighed at the start and on days 14, 28 and 42 and feed intake per pen measured for the corresponding periods. Weight gain and feed to gain ratio were calculated from these data. All data were subjected to an ANOVA, with least significant difference used to compare treatment means when a significant difference was identified by ANOVA. The pen was the experimental unit.

Five birds died during the trial, but remaining birds were healthy and no veterinary intervention was required. There were no significant differences in any measured production parameter when control birds were compared to those in the treatment groups. Final body weight was 2.1 kg, feed intake ~ 3.2 kg and feed to gain was within the range 1.55–1.60.

3.2.3.4. Safety for laying hens

Three groups of 260 laying hens (NovoBrown) were established with each group housed in 10 paired cages holding 26 hens.[40] Treatments consisted of a control group given a basal diet of wheat and sunflower cake in mash form and two treatment groups supplemented with either 300 (× 1) or 30,000 (× 100) U enzyme activity (confirmed by analysis). Supplementation started when hens were 133 days of age with a 7-day acclimatisation period followed by an experimental period from 140 days of age to 198 days of age (56 days observation). Mortality, feed consumption and egg production were monitored throughout, body weight was measured on days 140, 168 and 198 and average egg

[37] Technical Dossier/Section III/Annex III.01.
[38] Technical Dossier/Section III/Annex III.13.
[39] Technical Dossier/Section III/Annex III.28.
[40] Technical Dossier/Section III/Annex III.22.

size measured every 2 weeks. All eggs produced over a 24 h period were collected at days 140, 168 and 198 and examined for quality parameters (individual egg weight, shell strength, Haugh units). All normally distributed data were subject to ANOVA, with the paired cage as the experimental unit for production parameters and the egg for quality parameters; otherwise, a non-parametric Kruskal–Wallis test was used.

Overall mortality was low (0.6%). No significant differences were seen for any of the measured parameters between the control group and either of the test groups. The laying rate and average egg weight were normal for the beginning of lay (91% and 55 g). Cumulative egg numbers averaged 52.5 and cumulative egg mass 2.86 kg. Egg quality parameters were not affected by treatment.

3.2.3.5. Conclusions on safety for the target species

A value of 300 U/kg complete feed was selected by the applicant to represent a maximum use level. This was the rate described as a recommended maximum use level in the two previous applications for the same activity made by the same producer.

A 100-fold overdose of this maximum application rate was tolerated by weaned piglets, chickens and turkeys for fattening and by laying hens and so the selected maximum rate of 300 U/kg feed can be considered safe for these species/categories, with a wide margin of safety.

This conclusion can also be directly applied to all pigs, chickens reared for laying and turkeys reared for breeding and extrapolated to all other poultry species for growing and laying and to minor porcine species at the same maximum application rate of 300 U/kg feed without a need for further studies.

3.2.4. Safety for the consumer

The production organism is considered suitable for the QPS approach to safety assessment. Taking into account the nature of the genetic modification and the extensive purification undertaken to exclude DNA fragments from the final additive, the production or retention of toxic metabolites produced during fermentation is considered improbable. This was confirmed by the results of two *in vitro* and one *in vivo* tests for genotoxicity and a subchronic oral toxicity study. No evidence of mutagenicity or genotoxicity was detected and no evidence of toxicity found in the oral toxicity study.

Metabolites resulting from the complete or partial de-esterification of fumonisins have been previously assessed for safety and found to be less toxic than the parent mycotoxin. The action of fumonisin esterase on any contaminating fumonisins will be independent of source and essentially the same in pig and poultry digestive tracts and will act to reduce any toxic load. Consequently, the use of the additive in pigs and poultry will not introduce hazards for consumers.

3.2.5. Safety for the user

3.2.5.1. Effects on the respiratory system

An acute inhalation toxicity study in rats was made following the OECD guideline 403.[41] This involved a single nose-only application followed by observation over the next 14 days. Rats recovered from an initial irregular respiration within 24 h; thereafter, no adverse findings were observed. The LC_{50} derived from for this test was > 5.17 mg/L, the highest concentration tested.

Although exposure to the additive via respiration is likely to be low – the additive has a low-dusting potential with approximately 5% of particles having a diameter of respirable size (< 10 μm) – given the results of the skin sensitisation test (see Section 3.2.5.2.) and the proteinaceous nature of the additive, sensitisation via respiratory route cannot be excluded.

3.2.5.2. Effects on skin and eyes

An acute dermal toxicity study was made with the additive following OECD guideline 402.[42] A single limit dose of 2,000 mg/kg bw was applied over a 24 h period followed by 14 days observation. No local dermal or general clinical signs were observed. The LD_{50} derived from this test was > 2,000 mg/kg bw. The acute study was followed by skin and eye irritation studies following OECD guidelines 404 and 405. All adverse dermal responses were fully reversed within 1 h of patch removal and all effects on the eye reversed within 24 h. The additive is considered non-irritant to skin and eyes.

[41] Technical Dossier/Section III/Annex III.42.
[42] Technical Dossier/Section III/Annex III.33.

The capacity to induce dermal sensitisation was tested in a maximisation test with guinea pigs (OECD guideline 406).[43] The test item caused mild erythema in 2/10 animals in the test group (0/10 in the control group) identified as sensitisation at the tested concentration. According to the EC criteria on classification, labelling and packaging of substances and mixtures (Guidelines in Regulation (EC) No 1272/2008)[44] labelling as a sensitiser is not necessary, as the percentage of sensitised animals was less than 30%.

3.2.5.3. Conclusions on safety for the user

The additive is not toxic by inhalation and the respiratory exposure is likely to be low; however, a risk of sensitisation via the respiratory route cannot be excluded. The additive is non-irritant to skin and eyes and is not considered as a dermal sensitiser.

3.2.6. Safety for the environment

Neither the viable production strain nor its recombinant DNA was found in the final product. Therefore, the product FUMzyme®, manufactured by fermentation with *K. phaffii* DSM 32159, does not give rise to environmental safety concern deriving from the genetic modification of the production strain.

The active substance of the additive, fumonisin esterase, is a protein and as such will be largely degraded/inactivated during the passage through the digestive tract of animals. In addition, any hydrolysis of fumonisins in the digestive tract would simply anticipate that which would occur naturally in soils. Therefore, no risks for the environment are expected following the use of the additive in feeds under the proposed conditions of use.

3.3. Efficacy

The European Commission has set guidance values for the presence of fumonisins in complementary and complete feedingstuffs of 5 mg/kg for pigs and 20 mg/kg for poultry (Commission Recommendation 2006/576/EC[45]). These advisory limits are set at a level intended to protect the animal from any adverse effects. Consequently, efficacy studies which respect these limits would not be expected to demonstrate an improvement in performance. Although performance characteristics were monitored in all of the efficacy studies, the FEEDAP Panel uses such data only to confirm that animals on trial behaved as expected.

In the previous applications assessed by the FEEDAP Panel with this enzyme, a minimum efficacious supplementation rate of 15 U/kg feed was established for pigs and poultry (EFSA FEEDAP Panel, 2014, 2016). The applicant has now provided additional efficacy studies with a reduced minimum supplementation rate of 10 U/kg feed as proposed in the conditions of use (see also Section 3.1.7).

3.3.1. Efficacy for chickens for fattening

A total of 480 mixed sex Ross 308 chicks were distributed to one of three treatment groups.[46] Eight floor pens of 20 birds were used for each treatment group: a control group fed a basal mash diet based on maize and soybean for a 21-day period (day 14–35 of life), a second group fed the same diet deliberately contaminated with fumonisins B1/B2 (FB2) at an intended concentration of 15 mg/kg feed and a test group fed the same contaminated feed supplemented with the additive at 10 U/kg feed. The concentration of fumonisins and enzyme was confirmed by analysis. Performance characteristics were measured and a pooled faecal sample collected from each pen at the end of the study for analysis of fumonisins and their metabolites.

Data were checked for normal distribution (Shapiro–Wilks test). Results of the faecal analysis were found to be non-normally distributed. Since neither fumonisins or enzyme was to be expected in the control group, a paired comparison was made between samples from birds given the contaminated feed and those given the contaminated treated feed using a Mann–Whitney U test. Results of the faecal analysis are shown in Table 1.

[43] Technical Dossier/Section III/Annex III.45.
[44] Regulation (EC) No 1272/2008 of the European Parliament and of the council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, OJ L 353, 31.12.2008, p.1
[45] Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. OJ L 229, 23.8.2006, p. 7.
[46] Technical Dossier/Section IV/Annex IV.12.

Table 1: Effect of fumonisin esterase on the concentration of fumonisin B1 and its metabolites (ng/g) in faeces of chickens for fattening after 21 days

Parameter[(a)]	Control group	Contaminated group (a)	Treated contaminated group (b)	p value a vs. b
FB1	346	41,593	15,330	< 0.001
pHFB1a	23	319	832	< 0.001
pHFB1b	26	423	2,600	< 0.001
HFB1	99	84	5,341	< 0.001

(a): FB1: fumonisin B1; pHFB1a, pHFB1b: partially hydrolysed fumonisin; HFB1: fully de-esterified fumonisin.

Inclusion of the additive significantly reduced the concentration of FB1 in faeces with a concomitant increase in partially hydrolysed fumonisin (pHFB1a and b) and fully de-esterified fumonisin (HFB1).

3.3.2. Efficacy for laying hens

A 14-day trial with 120 laying hens (Lohmann Brown, 25 weeks of age at start) was designed with three experimental groups: a control group fed a basal diet, a group given the basal diet deliberately contaminated with FB1/FB2 and a test group receiving the contaminated diet supplemented with the enzyme at 10 U kg feed.[47] The contaminated feed contained approximately 8 mg fumonisin per kg feed. A total of 30 pens each holding four hens were used with 10 pens randomly allocated to a treatment. The basal diet was based on wheat, maize and soybean and was provided *ad libitum* in mash form. Birds were weighed at the start and end of the study and eggs counted and weighed daily. A single-pooled faecal sample was collected from each pen at the start, on day 7 and on day 14. Performance characteristics were statistically treated as in the trial described above. The results for faecal FB1 content was analysed using a Kruskal–Wallis pairwise test and the metabolites using a Mann–Whitney U test as above. Results of the faecal analysis are shown in Table 2.

Table 2: Effect of fumonisin esterase on the concentration of fumonisin B1 and its metabolites (ng/g) in faeces of laying hens after seven and 14 days

Parameter[(a)]	Control group	Contaminated group (a)	Treated contaminated group (b)	p value a vs. b
After 7 days				
FB1	120	11,075	4,291	0.136
pHFB1a	7	2,043	560	0.050
pHFB1b	8	2,831	1,205	0.008
HFB1	21	989	3,370	0.003
After 14 days				
FB1	132	9,507	4,807	0.108
pHFB1a	7	1,275	609	0.029
pHFB1b	8	2,158	1,318	0.019
HFB1	21	787	3,550	< 0.001

(a): FB1: fumonisin B1; pHFB1a, pHFB1b: partially hydrolysed fumonisin; HFB1: fully de-esterified fumonisin.

Essentially the same pattern of results was seen with the laying hen as with the chickens. Although the reduction in FB1 seen after both 7 and 14 days did not reach significance, the concentration of the fully de-esterified fumonisin was significantly increased at both time points.

3.3.3. Efficacy for weaned piglets

Three studies could be assessed, involving only a limited number of animals are described, two made within the European Union (EU) and the third elsewhere.

In the first study, a total of 18 piglets (28 days of age, start weight 7.6 kg, O-HYB-F10, nine of each sex) was distributed between six pens of three animals.[48] Two pens were assigned to each of three treatments: a control group fed a diet consisting of a mash feed based on wheat–soy–barley, a

[47] Technical Dossier/Section IV/Annex IV.01.
[48] Technical Dossier/Section IV/Annex IV.22.

group given a mash feed naturally contaminated with 3.5 mg/kg FB1 and FB2 and a third group given the contaminated feed supplemented with 6 U of the fumonisin esterase/kg feed. The study lasted 42 days. Blood samples were taken from each animal at the end of the study and analysed for sphinganine/sphingosine ratio (Sa/So), considered a sensitive marker of fumonisin toxicity. A single faecal sample was also collected from each pig at the end of the study following a 'spontaneous defecation'. However, since the pen is considered the experimental unit, a statistical analysis of the faecal data could not be made. The results of the Sa/So ratio are shown in Table 3.

Table 3: Effect of fumonisin esterase on the serum sphinganine/sphingosine ratio (Sa/So) and the concentration of fumonisin B1 and its metabolites (μg/g) in faeces of weaned piglets

Parameter[(a)]	Control group	Contaminated group (a)	Treated contaminated group (b)	p value a vs. b
		Study 1		
Sa/So ratio (42 days)	0.15	0.28	0.16	0.005
		Study 2		
Sa/So ratio (28 days)	–	0.27	0.19	0.038
Sa/So ratio (42 days)	–	0.27	0.22	0.076
FB1 (42 days)	–	5.76	0.37	0.010
pHFB1a (42 days)	–	1.01	0.40	0.210
pHFB1b (42 days)	–	0.69	0.47	0.487
HFB1 (42 days)	–	0.11	2.60	0.029
		Study 3		
Sa/So ratio (14 days)	0.14	0.16	0.13	0.045
Sa/So ratio (28 days)	0.14	0.25	0.14	0.003
Sa/So ratio (41 days)	0.13	0.21	0.14	0.021

(a): Sa/So: sphinganine/sphingosine ratio; pHFB1a, pHFB1b: partially hydrolysed fumonisin; HFB1: fully de-esterified fumonisin.

The second study involved eight individually penned piglets (20 days of age, start weight ~ 6 kg, four males and four females).[49] Four piglets were given a maize and soybean mash feed naturally contaminated with 2 mg FB1 and FB2/kg feed. The second group of four were given the same feed supplemented with the additive at 10 U/kg feed. The study lasted 42 days. Blood and faecal samples were collected from all animals on days 14, 28 and 42. Blood was analysed for serum Sa/So ratio and faecal samples for fumonisin and its metabolites. Results were examined for normality (Shapiro–Wilks test) and the means of those found normally distributed compared using a Student t-test and those not normally distributed were compared using a Mann–Whitney U test. Results are summarised in Table 3.

The third study was a near duplicate of the first (described above) and was made at the same location.[50] Again 18 mixed sex weaned piglets were used distributed to the same number of pens (six) with two pens used for each of the three treatments. Animals were 6 weeks of age at the start with a mean weight of 11.5 kg. In this case, the intended level of contamination with FB1 and FB2 was 5 mg/kg feed with the test group supplemented with 10 enzyme U/kg feed. The study lasted 41 days. Blood and faecal samples were collected on days 14, 28 and 41. Blood was analysed for serum Sa/So ratio and faecal samples for fumonisin and its metabolites. As previously, since the number of replicates did not allow a statistical analysis, the faecal data were not considered. Sa/So ratio data were compared using Mann–Whitney U test. Results are shown in Table 3.

The Sa/So ratio was significantly reduced in the treated group in all three studies with values similar to those of the control group given the feed essentially free of fumonisins (where this was measured, studies 1 and 3). In the single study with sufficient replication to allow a statistical consideration of the faecal concentration of fumonisins and metabolites, the pattern of effect on the fumonisin-contaminated feed by the enzyme was essentially that seen in poultry. The FB1 concentration in faeces was significantly reduced while that of the de-esterified fumonisin (HFB1) was significantly increased.

[49] Technical Dossier/Section IV/Annex IV.33.
[50] Technical Dossier/Section IV/Annex IV.42.

3.3.4. Enzyme specificity

A number on *in vitro* studies made with FB1 considered in the previous opinions of the FEEDAP Panel showed that the action of fumonisin esterase resulted in partial or complete de-esterification, releasing tricarballylic acid and the fully or partially de-esterified aminopentol backbone (EFSA FEEDAP Panel, 2014, 2016). Although FB1 and FB2 are the most prevalent fumonisins, a total of eight are recognised differing only by the number and position of hydroxyl groups on the aminopentol backbone. However, since all fumonisins possess tricarballylic substituents on carbons 6 and 7 and since the presence of the hydroxyls are insufficiently bulky to affect enzyme binding or catalytic activity, the enzyme will be equally effective in its action against all recognised fumonisins.

3.3.5. Conclusions on efficacy

The additive has the capacity to degrade fumonisin contaminants in feed of marketable quality when used at the minimum recommended dose of 10 U/kg complete feed, as shown in studies with chickens for fattening, laying hens and weaned piglets. This conclusion is based on a reduced fumonisin faecal excretion accompanied by an increased excretion of de-esterified fumonisin (in one study each in chickens for fattening, laying hens and piglets) and the smaller Sa/So ratio in serum (in three studies in piglets). Since the mode of action of the additive can be reasonably assumed to be the same in animal species for which the application is made, the FEEDAP Panel considers the additive as efficacious for all poultry and all pigs.

The conclusion on efficacy was drawn considering also the established efficacy assessed in previous applications of the same enzyme.

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[51] and Good Manufacturing Practice.

4. Conclusions

Neither the viable production strain nor its recombinant DNA was found in the final product. Therefore, the product FUMzyme®, manufactured by fermentation with *K. phaffii* DSM 32159, does not give rise to safety concern deriving from the genetic modification of the production strain.

An application rate of 300 U/kg feed is safe for weaned piglets, chickens and turkeys for fattening and laying hens with a wide margin of safety. This conclusion can also be directly applied to all pigs, chickens reared for laying and turkeys reared for breeding and extrapolated to all other poultry species for growing and laying and to minor porcine species at the same maximum application rate of 300 U/kg feed without a need for further studies.

The use of the additive in pigs and in poultry under the conditions specified will not introduce hazards for consumers.

The additive is not toxic by inhalation and the respiratory exposure is likely to be low; however, a risk of sensitisation via the respiratory route cannot be excluded. The additive is non-irritant to skin and eyes and is not considered as a dermal sensitiser.

No risks for the environment are expected following the use of the additive in feeds under the proposed conditions of use.

The additive has the capability to degrade fumonisins in feed of marketable quality fed to poultry and pigs when used at the minimum recommended dose of 10 U/kg complete feed.

Documentation provided to EFSA

1) FUMzyme® all pigs and all avian species. January 2017. Submitted by Biomin GmbH
2) FUMzyme® all pigs and all avian species. Supplementary information. August 2017. Submitted by Biomin GmbH.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for fumonisin esterase (EC 3.1.1.87) (FUMzyme®).
4) Comments from Member States.

[51] 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.

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Abbreviations

ANOVA	analysis of variance
As	Arsenic
ATCC	American Type Culture Collection
BIOHAZ	EFSA Panel on Biological Hazards
bw	Body weight
CBS	Centraalbureau voor Schimmelcultures
Cd	Cadmium
CFU	Colony Forming Units
CV	Coefficient of variation
DNA	deoxyribonucleic acid
DSM	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen
EURL	European Union Reference Laboratory
FB1	fumonisin B1
FB2	fumonisin B2
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practices
GM	Genetically Modified
HFB1	fully de-esterified fumonisin
Hg	Mercury
MTD	maximum tolerated dose

No	Number
NRRL	Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research
OECD	Organisation for Economic Co-operation and Development
Pb	Lead
PCR	polymerase chain reaction
pHFB1	partially de-esterified fumonisin
QPS	Qualified Presumption of Safety
rRNA	Ribosomal ribo nucleic acid
Sa	sphinganine
So	sphingosine
U	Unit of activity

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for fumonisin esterase (EC 3.1.1.87) (FUMzyme®)

In the current application, authorisation is sought under article 4(1) for *FUMzyme®* under the category/functional group 1(m) 'technological additives'/'substances for reduction of the contamination of feed by mycotoxins', according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all pigs and all avian species. *FUMzyme®* is used for its ability to degrade fumonisin B1. The enzyme detoxifies this mycotoxin by cleavage of the toxin's diester bonds and removal of the propane-1,2,3-tricarboxylic acid side chains. According to the applicant, the active substance in *FUMzyme®* is *fumonisin esterase* (EC 3.1.1.87) which is added to a maltodextrin carrier to result in a minimum guaranteed enzyme activity of 3,000 U/g. The applicant defined the enzyme activity unit (U) as follows:

One unit (U) is the enzymatic activity that releases 1 μmol propane-1,2,3-tricarboxylic acid per minute from 100 μM fumonisin B1 in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/mL bovine serum albumin at 30°C.

FUMzyme® is intended to be used in *premixtures* and *feedingstuffs*, with a proposed enzyme activity ranging from 10^{52} to 300 U/kg *feedingstuffs*.

For the quantification of the *fumonisin esterase* activity in the *feed additive* and *feedingstuffs*, the applicant proposed a single laboratory validated and further verified method based on high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The method is based on the quantification of the propane-1,2,3-tricarboxylic acid released from the action of the enzyme on fumonisin B1.

For the quantification of the enzyme activities in *premixtures*, the applicant suggested to dilute *premixture* samples with animal feed (according to the recommended inclusion rate) and analyse them applying the method for *feedingstuffs* mentioned above.

Based on the experimental evidence available the EURL recommends for official control the HPLC-MS/MS method submitted by the applicant for the quantification of the *fumonisin esterase* activity in the *feed additive*, *premixtures* 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

[52] This figure has been corrected by EFSA, as there was identified a typo error.