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SCIENTIFIC OPINION

Safety and efficacy of a feed additive consisting of 6-phytase produced by *Aspergillus oryzae* DSM 33737 (HiPhorius™) for all poultry, all *Suidae* and all fin fish (DSM Nutritional Products Ltd)

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Abstract

Following a request from the European Commission, the EFSA 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 6-phytase produced by the genetically modified strain *Aspergillus oryzae* DSM 33737 (HiPhorius™ 10, 40, 20L and 50L) as a zootechnical feed additive for all poultry, all *Suidae* and all fin fish. The FEEDAP Panel concluded that the genetic modification of the production strain does not give rise to safety concerns. Based on the no observed adverse effect level identified in a subchronic oral toxicity study in rats, the additive was considered safe for all poultry, all *Suidae* and all fin fish at the proposed conditions of use. The Panel also concluded that the use of the product as a feed additive is of no concern for the consumers and the environment. The liquid formulations of the additive are not skin or eye irritants. The two solid ones are not skin irritants but are eye irritants. Owing to the lack of data, the Panel cannot conclude on the skin sensitisation of the final formulations of the additive. Due to the proteinaceous nature of the active substance (6-phytase), the additive is considered a respiratory sensitiser. The Panel concludes that the additive is efficacious when included in the diet of poultry for fattening or reared for laying/breeding, reproductive *Suidae*, and all fin fish. Due to the lack of sufficient data, the Panel could not conclude on the efficacy for laying and reproductive poultry and growing *Suidae*.

KEYWORDS

digestibility enhancers, efficacy, HiPhorius™, safety, zootechnical additives

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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 feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from DSM Nutritional Products Ltd (represented in the EU by DSM Nutritional Products Sp. zo.o.)[2] for the authorisation of the additive consisting of 6-phytase produced by *Aspergillus oryzae* DSM 33737 (HiPhorius™), when used as a feed additive for all poultry, all *Suidae* and all fin fish (category: zootechnical additive; 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 31 August 2022.

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 feed additive consisting of 6-phytase produced with *Aspergillus oryzae* DSM 33737 (HiPhorius™), when used under the proposed conditions of use (see **Section 3.1.6**).

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 6-phytase produced with *Aspergillus oryzae* DSM 33737 (HiPhorius™) as a feed additive. The dossier was received on 10/02/2022, and the general information and supporting documentation are available at https://open.efsa.europa.eu/questions/EFSA-Q-2022-00082.

In accordance with Article 38 of Regulation (EC) No 178/2002[4] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation, and of the Decision of EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[5] a non-confidential version of the dossier has been published on Open.EFSA.[6]

According to Article 32c(2) of Regulation (EC) No 178/2002 and to the Decision of EFSA's Executive Director laying down the practical arrangements for pre-submission phase and public consultations,[7] EFSA carried out a public consultation on the non-confidential version of the application from 10 July to 31 July 2023 for which no comments were received.

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, peer-reviewed scientific papers, other scientific reports and experts' (elicitation) knowledge, to deliver the present output.

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.[8]

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of 6-phytase produced with *Aspergillus oryzae* DSM 33737 (HiPhorius™) is in line with the principles laid down in Regulation (EC) No 429/2008[9] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c), Guidance on the

[1]Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2]DSM Nutritional Products Sp. z o.o.: Tarczyńska 113, Mszczonów, Poland – Poland.

[3]Dossier reference: FEED-2021-2299.

[4]Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–48.

[5]Decision available at: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements

[6]Available at: https://open.efsa.europa.eu/dossier/FEED-2021-2299

[7]Decision available at: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements

[8]Evaluation report received on 11/11/2022 and available on the EU Science Hub https://joint-research-centre.ec.europa.eu/publications/feed-2021-2299_en

[9]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.

assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3 | ASSESSMENT

The product under assessment contains 6-phytase activity (myo-inositol-hexakisphosphate 4-phosphohydrolase; EC 3.1.3.26) produced with a genetically modified strain of *Aspergillus oryzae* (DSM 33737), and it is intended for use as a zootechnical additive (functional group: digestibility enhancers) in feed for all poultry, all *Suidae* and all fin fish. The product will be hereafter referred to by its trade name, HiPhorius™.

3.1 | Characterisation

3.1.1 | Characterisation of the production organism

The 6-phytase present in the additive is obtained by fermentation with a genetically modified strain of *A. oryzae* which is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH with the deposition number DSM 33737.[10]

The taxonomic identification of the production strain was done by phylogenetic analysis using the coding region of three marker genes *caM*, *benA* and *rpb2* and the internal transcribed spacer (ITS) region of the rDNA gene; these sequences were compared with orthologous sequences from the reference strain *A. oryzae* RIB40 and the closely related species *Aspergillus miniscleotigenes*, *Aspergillus aflatoxiformans* and *Aspergillus flavus*.[11] It is noted that, although the whole genome sequence (WGS) of the production strain was available, the analysis did not consider a large set of core genes (e.g. BUSCO). The comparison confirmed the identification of DSM 33737 as *A. oryzae*.

3.1.1.1 | *Information related to the genetically modified microorganism*

The parental strain is *A. oryzae* A1560 (Institute for Fermentation of Osaka (IFO) 04177) [redacted].

The plasmids used to introduce the inserted sequences included the *Escherichia coli* [redacted] origin of replication and three selectable markers (*pyrG* (orotidine-5′-phosphate-decarboxylase) [redacted] and a fragment of the *niaD* gene (nitrate reductase) [redacted].

Description of the genetic modification

The *A. oryzae* production strain was constructed from the parental strain A1560 (IFO 4177) through a combination of genetic modification steps and classical mutagenesis. [redacted]

- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]

[10]Annex 2.2.2 DSM 33737 GMM Dossier 26042022.
[11]Annex 2.2.2 DSM 33737 GMM Dossier 26042022.

[redacted]

3.1.2 | Manufacturing process

The fermentation product containing 6-phytase activity is produced by a submerged batch-fed fermentation of the production strain *A. oryzae* DSM 33737.[12] The 6-phytase is recovered from the fermentation broth by steps involving flocculation, vacuum filtration, press filtration, centrifugation, ultrafiltration, evaporation and bacterial filtration. The resulting fermentation product ([redacted][13])[14] is blended with the rest of the ingredients and pH-adjusted to prepare the final liquid formulations (HiPhorius™ 20 L, HiPhorius™ 50 L), or it is granulated, dried and coated to obtain the solid formulations (HiPhorius™ 10 and HiPhorius™ 40). The applicant indicates that no antimicrobial compounds are used during the manufacture of the additive.

3.1.3 | Characterisation of the additive

HiPhorius™ is presented in four different formulations[15]:

- HiPhorius™ 10, solid, with a minimum enzyme activity of 10,000 FYT/g, containing [redacted] of enzyme concentrate ([redacted]), sodium sulfate [redacted], kaolin[16] [redacted], palm oil [redacted], cellulose [redacted], dextrin [redacted], water [redacted] and zinc sulfate [redacted].
- HiPhorius™ 40, solid, with a minimum enzyme activity of 40,000 FYT/g, containing [redacted] of enzyme concentrate ([redacted]), sodium sulfate [redacted], kaolin [redacted], palm oil [redacted], cellulose [redacted], wheat flour [redacted], dextrin [redacted], water [redacted], and zinc sulfate [redacted].
- HiPhorius™ 20 L, liquid, with a minimum enzyme activity of 20,000 FYT/g, containing [redacted] of enzyme concentrate ([redacted]), water [redacted], sorbitol [redacted], potassium sorbate [redacted], and sodium benzoate [redacted].
- HiPhorius™ 50L, liquid, with a minimum enzyme activity of 50,000 FYT/g, containing [redacted] of enzyme concentrate ([redacted]), water [redacted], sorbitol [redacted], potassium sorbate [redacted] and sodium benzoate [redacted].

The enzyme activity of eight batches of HiPhorius™ 10 (range: 14,800–16,300 FYT/g; average: 15,520 FYT/g), 40 (range: 44,700–51,600 FYT/g; average: 48,140 FYT/g), 20L (range: 24,440–25,420 FYT/g; average: 25,048 FYT/g) and 50L (range: 57,460–65,813 FYT/g; average: 62,019 FYT/g) showed compliance with the minimum specifications set by the applicant.[17]

The same batches from each formulation of the additive were analysed for microbiological contamination. In all batches and formulations, the results showed compliance with the internal specifications provided by the applicant for total viable counts (specs: < 50,000 colony forming units (CFU)/g; solid forms: 100–900 CFU/g; liquid forms: < 100 CFU/g), total coliforms (specs: < 30 CFU/g; solid forms: < 10 CFU/g; liquid forms: < 4 CFU/g), *Escherichia coli* and *Salmonella* spp. (no detection in 25 g). Further, three batches of each form of the additive showed Enterobacteriaceae, yeasts and filamentous fungi below the limit of detection (< 10 CFU/g).[18]

Three batches from each formulation were analysed for chemical impurities and the content of mycotoxins. In all batches of both liquid formulations, the content of lead, arsenic, mercury and cadmium fell below the respective limit of quantification (LOQ).[19] In all batches of the solid formulations, the content of mercury and cadmium fell below the LOQ. HiPhorius™ 10

[12]Manufacturing Process – HiPhorius.

[13]One Phytase Unit (FYT) is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute (concentration of 5.0 mM) at pH 5.5 and 37°C.

[14]RFI - Annex 4. TOS and Protein content.

[15]Annex 2.1.2 Quantitative Composition - H10 H20L H40 H50L.

[16]The Panel notes that the additive is under re-evaluation.

[17]Annex 2.1.11 HiPhorius 4 Forms batch-to-batch Impurities.

[18]RFI - Annex 2. Enterobac_Yeast_Fungi.

[19]LOQs (mg/kg): Arsenic: 0.3; Lead: 0.5 mg/kg; Mercury: 0.05; Cadmium: 0.05.

showed an average value of 2.69 mg/kg of lead and 1.37 mg/kg for arsenic, and HiPhorius™ 40 showed an average value of 2.89 mg/kg of lead and 1.44 mg/kg for arsenic.

The microbial contamination and the detected amounts of the above-described impurities do not raise safety concerns.

Culture supernatants from three fermentation batches were analysed for the presence of antimicrobial activity by a disc diffusion assay using the test strains *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 11229, *Bacillus cereus* ATCC 2, *Bacillus circulans* ATCC 4516, *Streptococcus pyogenes* ATCC 12344 and *Serratia marcescens* ATCC 14041. No antimicrobial activity was detected in any of the samples.[20] The applicant also investigated the presence of antimicrobial activity in three batches of each of the final formulations and the results showed the absence of antimicrobial activity in all tested batches.[21]

Some *Aspergillus* species are known to be capable of producing mycotoxins and other secondary metabolites. However, during the development of the production strain *A. oryzae* DSM 33737, the CPA and AFL gene clusters and genes involved in the synthesis of beta-lactams have been deleted, thereby eliminating the potential of the strain to produce them. In addition, a classical mutagenesis step has drastically reduced the potential of the strain to produce kojic acid. Three batches of all HiPhorius™ formulations were analysed for the presence of CPA, three batches of the liquid forms for the presence of β-nitropropionic acid (BNP) and four batches of the liquid concentrate used to formulate the final formulations of the additive ([redacted]) for the presence of BNP and CPA.[22] The results of all these analyses showed that CPA and BNP levels were below the level of detection (LOD) of the analytical method.

The presence of viable cells of the production strain was investigated in three batches of an intermediate liquid concentrate ([redacted].[23] [redacted]. No growth was detected in the samples tested while the positive controls grew as expected.

The presence of DNA from the production strain was tested in triplicate in 1 g of the same batches of the liquid concentrate analysed for the presence of viable cells. Samples of 1 g were analysed in triplicate.[24] [redacted]. DNA of the production strain was not detected in the samples tested.

3.1.4 | Physical properties of the additive

HiPhorius™ 10 and 40, the solid forms of the additive, are free-flowing beige particulate products with a loose/tapped density of 1100/1300 kg/m^3 and 1060/950 kg/m^3, respectively (average values of three batches). The dusting potential of three batches of each form of the additive was determined using the Stauber-Heubach method and showed values < 0.01 mg/m^3 (mg airborne dust per m^3 of air) in all cases. The particle size distribution of the additive was analysed by laser diffraction method on the same batches; the results showed that 100% (v/v) of the particles' diameter fell above 250 and 53 μm for HiPhorius™ 10 and 40, respectively.[25]

HiPhorius™ 20 L is a light brown liquid with a density of 1200 kg/m^3, pH 7.5 (1% solution in water) and a viscosity of 16 cP (25°C). HiPhorius™ 50L is a dark brown liquid with a density of 1200 kg/m^3, pH 7.0 (1% solution in water) and a viscosity of 40 cP (25°C).[26]

3.1.5 | Stability and homogeneity

The shelf-life of a minimum of three batches of each of the four final formulations of the additive was studied for 24 months when stored in air-tight glass containers at 10, 25 or 30°C (±2°C).[27] The average enzyme activity loss after 24 months for the solid formulations ranged between 0%–5%, 9%–22% and 24%–34% when stored at 10, 25 or 30°C, respectively. For the liquid forms, the average enzyme activity loss after 24 months ranged between 7%–9%, 32%–35% and 54%–55% when stored at 10, 25 or 30°C, respectively.

The stability of the solid forms of the additive (three batches for each form) in two commercial vitamin/mineral premixtures was studied when supplemented at a minimum target dose of 150,000 FYT/kg premix.[28] The supplemented premixes were stored in plastic bags at 25°C for up to 6 months. At the end of the storage period, the enzyme activity loss of both

[20]RFI - Annex 1. Antimicrobial activity.

[21]Annex 2.1.11 HiPhorius 4 Forms batch-to-batch Impurities.

[22]Annex 2.1.11 HiPhorius 4 Forms batch-to-batch Impurities; LOD (mg/kg): β-nitropropionic acid: 0.3647–0.5843; Cyclopiazonic acid: 0.003.

[23]Annex 2.2.2 DSM 33737 GMM Dossier 26042022.

[24]Annex 2.2.2 DSM 33737 GMM Dossier 26042022.

[25]Annex 2.1.15 Application properties and in feed stability of HiPhorius 10 and 40 NEW.

[26]Annex 2.1.16 Application properties and in feed stability of HiPhorius 20 (L) and 50 (L).

[27]RFI - Annex 6. 24 m per se stability HiPhorius.

[28]Annex 2.4.5 Application properties and in feed stability of HiPhorius 10 and 40 NEW.

premixes supplemented with the solid formulations of the additive ranged from 25%–33% in comparison with the starting one.

The stability of all forms of the additive (three batches per form) was evaluated in mash and pelleted feeds for chickens for fattening when supplemented at a minimum phytase activity of 1500 FYT/kg feed.[29] Samples were stored in plastic bags at room temperature for up to 3 months. The enzyme activity in the mash feeds at the end of the storage period ranged from 74%–93%, 104%–137%, 89%–92% and 87%–97% for HiPhorius™ 10, 40, 20 L and 50 L, respectively, in comparison with the starting one. For pelleted feeds, the enzyme activity ranged from 86%–90%, 97%–120%, 94%–96% and 91%–99% for HiPhorius™ 10, 40, 20 L and 50 L, respectively, in comparison with the starting one after pelleting.

The stability of the liquid forms of the additive (three batches per form) was evaluated in extruded feeds for aquaculture when supplemented by spraying at a minimum phytase activity of 1000 FYT/kg feed.[30] Samples were stored in plastic bags at room temperature for up to 3 months. The enzyme activity at the end of the storage period ranged from 79%–89% and 88%–97% for HiPhorius™ 20 L and 50 L, respectively, in comparison with the starting one.

The stability of the solid forms of the additive (three batches per form) during pelleting was studied after mixing into mash feed for chickens for fattening to achieve a total phytase activity of 1500 FYT/kg feed.[31] The pelleting process included a mixing time of 2 min and a temperature of 80°C or 90°C. The enzyme activity in the pelleted feed ranged from 93%–103% and 91%–116% for HiPhorius™ 10 at 80 and 90°C, respectively, in comparison with the mash feed. For the HiPhorius™ 40, enzyme activity in the pelleted feed ranged from 79%–114% and 70%–93% at 80°C and 90°C, respectively, in comparison with the mash feed.

The capacity for homogeneous distribution of the HiPhorius™ 10 was studied in 10 subsamples of mash and pelleted feed for chickens for fattening.[32] The coefficient of variation was 20% and 14% for the mash and pelleted feeds, respectively.

The capacity for homogeneous distribution of the liquid forms of the additive was studied in 10 subsamples of mash and pelleted feed for chickens for fattening and in extruded feed for aquaculture.[33] For HiPhorius™ 20L, the coefficient of variation was < 1%, 7% and 7% for the mash, pelleted and extruded feeds, respectively. For HiPhorius™ 50L, the coefficient of variation was 4%, 5% and 7% for the mash, pelleted and extruded feeds, respectively.

3.1.6 | Conditions of use

The solid forms of the additive (HiPhorius™ 10 and 40) are intended for use in feed for all poultry and all *Suidae*, at a range between 200 and 4000 FYT/kg complete feed.

The liquid forms of the additive (HiPhorius™ 20L and 50L) are intended for use at a range between 200 and 4000 FYT/kg complete feed for all poultry and all *Suidae*, and between 1000 and 4000 FYT/kg complete feed for all fin fish.

3.2 | Safety

3.2.1 | Safety of the production organism

The production strain *A. oryzae* DSM 33737 differed from the parental strain (*A. oryzae* A1560) by expressing a synthetic and modified 6-phytase gene from *C. braakii* [redacted]. The introduced sequences raise no safety concerns. As a result of the genetic modification, *A. oryzae* DSM 33737 showed mutations in key genes for the production of beta-lactams, CPA and aflatoxins. Neither the production strain nor its DNA were detected in an intermediate product representative of all final forms of the additive. The product HiPhorius™ does not give rise to safety concerns regarding the genetically modified production strain.

3.2.2 | Toxicological studies

All the toxicological studies were performed with an unrefined intermediate enzymatic 6-phytase product ([redacted]) from which all the final HiPhorius™ formulations are obtained. The test item is considered representative of the final formulations of the additive.

[29] Annex 2.4.5 Application properties and in feed stability of HiPhorius 10 and 40 NEW and Annex 2.4.6 Application properties and in feed stability of HiPhorius 20 (L) and 50 (L).

[30] Annex 2.4.6 Application properties and in feed stability of HiPhorius 20 (L) and 50 (L).

[31] Annex 2.4.5 Application properties and in feed stability of HiPhorius 10 and 40 NEW.

[32] Annex 2.4.5 Application properties and in feed stability of HiPhorius 10 and 40 NEW.

[33] Annex 2.4.6 Application properties and in feed stability of HiPhorius 20 (L) and 50 (L).

3.2.2.1 | *Genotoxicity studies*

Bacterial reverse mutation assay

An Ames bacterial reverse mutation test was performed to assess the mutagenic potential of the test item.[34] The study was performed applying the treat-and-plate protocol in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 *uvrA* (pKM101) in accordance with OECD TG 471 (2020) and claimed to follow good laboratory practices (GLP). In the report, test item concentrations were expressed in terms of TOS, i.e. correction was made for a TOS content of 10.5%. At least five concentrations were tested ranging from 50 to 5000 μg TOS/mL in the absence and presence of metabolic activation. No toxicity and precipitation were reported. No increase in the number of revertant colonies was observed in any strain and experimental condition. The fermentation product 6-phytase from *Aspergillus oryzae* DSMZ 33737 did not induce gene mutations in bacteria under the experimental conditions applied in the study.

In vitro micronucleus test

The aneugenic and clastogenic potential of the test item was evaluated in an in vitro micronucleus assay in human peripheral blood lymphocytes.[35] The experimental protocol was in line with OECD guideline 487 and claimed to follow GLP. The maximum tested concentrations were established with a preliminary cytotoxicity experiment. Tests were conducted both in the presence and absence of a post-mitochondrial supernatant fraction (S9) obtained from the livers of rats treated with a combination of phenobarbital and β-naphthoflavone. Cells were stimulated for 48 h with phytohaemagglutinin (PHA) to produce exponentially growing cells and then treated for 3 h (followed by a 17-h recovery period) with 0, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 μg TOS/mL of the test item dissolved in water (purified by reverse osmosis) both in the absence and in the presence of S9-mix. In a parallel assay, cells were treated for 20 h in the absence of S9-mix with no recovery period. No reductions were observed in the cytokinesis-block proliferative index (CBPI) at any concentration tested. In the main study, cells were treated with 0, 1250, 2500 and 5000 μg of TOS/mL of the test item and two replicate cultures per treatment and 1000 binucleate cells per replicate (i.e. 2000 cells per dose) were scored for micronuclei. No evidence of chromosomal damage or aneuploidy was observed as frequencies of binucleated cells with micronuclei (BNMN) were not significantly different from concurrent controls and fell within historical control ranges for all treatments with the test item in the presence or absence of S9-mix metabolic activation. The positive controls performed as expected and induced a statistically significant increase in the frequency of BNMN cells. The test item did not induce chromosome damage (aneugenic or clastogenic effects) *in vitro* in mammalian cells under the experimental conditions employed in this study.

3.2.2.2 | *Subchronic oral toxicity study*

The toxic potential of the test item was evaluated by daily oral gavage administration to Han Wistar rats.[36] Groups of 10 RccHan™ WIST rats of each sex were given the test item diluted in water daily by gavage for 90 days at doses of 0, 110.3, 363.8 or 1102.5 mg TOS/kg body weight (bw) per day (equivalent to 35,700, 117,810 or 357,000 FYT/kg bw per day). The study was conducted according to OECD TG 408 and it was claimed to be GLP compliant. There were no treatment-related changes in any of the measurements or observations made during the study. Based upon the results of this study, the non-observed adverse effect level (NOAEL) was the maximum dose tested of 1102.5 mg TOS/kg bw per day, corresponding to 357,000 FYT/kg bw per day for the test item.

3.2.3 | Safety for the target species

No tolerance studies in relevant target species were submitted. In order to support the safety of the additive for the target species, the applicant referred to the subchronic oral toxicity study described above (see Section 3.2.2.4). The NOAEL identified (1102.5 mg TOS/kg bw per day; equivalent to 357,000 FYT/kg bw per day) was used to calculate the maximum safe level in chickens and turkeys for fattening, laying hens, piglets, pigs for fattening and lactating sows, salmon in accordance with the procedure described in the Guidance on the assessment of the safety for the target species (EFSA FEEDAP Panel, 2017b). The applicant also provided data to support default values for trout, seabream, seabass and tilapia[37] and calculated the maximum safe level in feed in accordance with the above-mentioned procedure. The results are shown in Table 1. The values obtained are higher than the maximum proposed use level of 4000 FYT/kg complete feed for the target species. Therefore, the Panel concludes that the additive is safe for all poultry, all *Suidae* and all fin fish at the maximum use level of 4000 FYT/kg complete feed.

[34] Annex 3.2.2 Ames study HiPhorius.

[35] Annex 3.2.3 Micronucleus HiPhorius.

[36] Annex 3.2.4 13-week subchronic rat study HiPhorius.

[37] Section 3.1 Safety for the target animals - HiPhorius – Final.

TABLE 1 Maximum safe level of HiPhorius™ in feed for the target species.

Animal species/category	Default values for daily feed intake (g DM per kg bw)	Maximum safe level in feed (FYT/kg complete feed)
Chickens for fattening	79	39,767
Turkeys for fattening	59	53,550
Laying hens	53	59,275
Piglets	44	71,400
Pigs for fattening	37	85,680
Lactating sows	30	104,125
Salmon	18	179,520
Trout*	31	101,342
Seabream*	27	116,355
Seabass*	21	149,600
Tilapia*	62	50,671

*For trout, seabream, seabass and tilapia, the applicant submitted data on the default values for the daily feed intake and body weight obtained from the Food and Agriculture Organization of the United Nations (https://www.fao.org/fileadmin/user_upload/affris/docs/Trout/English/table_5.htm/; https://www.fao.org/fileadmin/user_upload/affris/docs/Gilthead_Seabream/English/table_13.htm/; https://www.fao.org/fileadmin/user_upload/affris/docs/European_Seabass/English/table_13.htm/; https://www.fao.org/fileadmin/user_upload/affris/img/Niletilapia_table/table25-2.pdf).

3.2.4 | Safety for the consumer

The results obtained with a test item considered representative of the final formulations of the additive in the genotoxicity studies and the subchronic oral toxicity study do not indicate any reason for concern for consumer safety arising from the use of the product as a feed additive.

3.2.5 | Safety for the user

3.2.5.1 | *Effect on the respiratory system*

No specific studies were provided by the applicant regarding the effects of the additive on the respiratory system. Owing to the proteinaceous nature of the active substance, the additive is considered a respiratory sensitiser. The data on the dusting potential of the solid forms (< 0.01 mg/m^3) of the additive suggest that exposure via inhalation is unlikely.

3.2.5.2 | *Effect on eyes and skin*

The skin irritation potential of the two solid and the two liquid forms was assessed by the in vitro Episkin™ Reconstructed Human Epidermis according to OECD TG 439.[38] Based on the results obtained, both solid and liquid formulations are classified as non-irritant in accordance with the UN GHS 'No Category'.

The eye irritation potential of the two solid and the two liquid forms was assessed by means of the Bovine Corneal Opacity and Permeability Assay, according to OECD TG 437.[39] Based on the results obtained, the two liquid formulations are classified as non-irritant in accordance with the UN GHS 'No Category'. The two solid formulations are considered eye irritants. No information on skin sensitisation potential was provided; therefore, the FEEDAP Panel cannot conclude on the skin sensitisation potential of the four formulations of the additive.

3.2.5.3 | *Conclusions on safety for the user*

The final formulations of the additive are not skin irritants. The two liquid formulations of the additive are not eye irritants, while the two solid ones are to be considered eye irritants. The Panel cannot conclude on the skin sensitisation of the final formulations of the additive. Due to the proteinaceous nature of the active substance (6-phytase), the additive is considered a respiratory sensitiser. However, exposure by inhalation is considered unlikely.

3.2.6 | Safety for the environment

The production strain and its DNA were not detected in an intermediate product representative of the final forms of the additive. The additive does not raise safety concerns for the environment regarding the genetic modification of the

[38]Annex 8.2 Skin Irritation Report.

[39]Annex 8.3 Eye Irritation Report.

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

3.3 | Efficacy

The test items used in all the efficacy trials described below are based on the same intermediate enzymatic concentrate used to obtain the final formulations of HiPhorius™ but with a different qualitative or quantitative composition. The Panel considers that the test items used are representative of the additive under assessment.

3.3.1 | Efficacy in poultry

3.3.1.1 | *Efficacy for chickens for fattening*

Three short-term balance trials were performed aiming at assessing the effect of the 6-phytase on phosphorus retention and bone mineralisation in chickens for fattening. The design of the trials is summarised in Table 2, and the results are shown in Table 3.

TABLE 2 Trial design and analysed enzyme activity of the diets of the efficacy trials in chickens for fattening.

Trial	Total no of animals (animals/replicate) replicates/group	Breed (age) sex	Duration (adaptation/ collection)	Composition feed (form)	Groups (FYT/kg feed)	
					Intended	Analysed
1[40]	768 (8) 12	Cobb 500 (8 days) Male	9 days (5/4)	Maize, soybean meal (pellet)	0 187.5 375 750 1125 1500 1875 2250	37 182 328 640 1060 1347 1560 1931
2[41]	504 (8) 9	Cobb 500 (8 days) Male	9 days (5/4)	Maize, soybean meal (pellet)	0 187.5 375 750 1500 2000 PC	26 178 356 725 1425 1853 29
3[42]	384 (8) 12	Ross 308 (8 days) Male	9 days (6/3)	Maize, soybean meal (pellet)	0 187.5 375 750	< 20 202 302 603

In all trials, the birds were distributed in cages, which were randomly allocated to the different experimental groups. The basal diets were either not supplemented (control) or supplemented with the 6-phytase from the additive to provide levels from 187.5 to 2250 FYT/kg complete feed. The feed was analysed for the enzyme activity (see Table 2) and the content of Ca/P (Table 3). In trial 2, a positive control (PC) with a higher content of phosphorus (P) and calcium (Ca) was also considered. The experimental diets were offered ad libitum for 9 days. The mortality and health status of the animals were checked daily. The birds were caged, and the body weight and feed consumption were recorded on days 1 and 9 of the experiment per cage. The average body weight gain, feed intake and feed-to-gain ratio were calculated. Excreta were collected (total collection method) from days 6 to 9 (trials 1 and 2; 4 days) and 7 to 9 (trial 3; 3 days) and pooled per cage. Feed and excreta samples were analysed for dry matter and the mineral content (P, Ca and ash) to calculate the retention. On day 10, the right tibia from two birds per cage were collected to measure the mineral (P, Ca and ash) content. The experimental data were analysed with an analysis of variance (ANOVA), including the diet as a fixed effect. When a difference was observed, group means were compared with a Tukey test. The cage was used as the experimental unit. The significance level was set at 0.05.

The birds receiving the 6-phytase at 187.5 FYT/kg feed showed significantly higher P retention compared to the control diet in the three trials, and to the positive control diet in trial 2. The improvement of the P utilisation at the same level was also reflected in higher P bone content in trials 1 and 2.

[40]Annex 4-2 CAN-CB-2020-01 Trial 1 Broilers.

[41]Annex 4-4 CAN-CB-2020-06 Trial 2 Broilers.

[42]Annex 4–6 BE02-20 Trial 3 Broilers.

TABLE 3 Effect of the 6-phytase on the phosphorus retention and bone mineralisation of the chickens for fattening in the short-term trials.

Trial	Phytase (FYT/kg feed)	Ca/P (%)	P retention (%)	Bone content		Mortality and culling %
				Ash (% DM)	P (% DM)	
1	0	0.70/0.47	64.4^{d}	46.8^{e}	8.1^{e}	2.1
	187.5	0.68/0.46	71.5^{c}	50.1^{d}	8.6^{d}	1
	375	0.66/0.46	75.6^{b}	51.2^{cd}	8.9^{cd}	1
	750	0.71/0.47	80.5^{a}	52.4^{bc}	9.2^{bc}	0
	1125	0.69/0.46	82.5^{a}	52.9^{abc}	9.4^{ab}	1
	1500	0.69/0.46	83.3^{a}	53.6^{ab}	9.4^{ab}	2.1
	1875	0.69/0.47	82.0^{a}	54.7^{a}	9.7^{a}	0
	2250	0.69/0.47	81.9^{a}	53.7^{ab}	9.4^{ab}	2.1
2	0	0.69/0.51	59.2^{e}	45.7^{e}	7.6^{e}	4.2
	187.5	0.69/0.51	63.9^{d}	48.8^{de}	8.3^{d}	4.2
	375	0.66/0.49	67.5^{cd}	49.0^{cde}	8.4^{cd}	2.8
	750	0.65/0.48	71.9^{ab}	51.1^{abcd}	8.8^{abcd}	2.8
	1500	0.64/0.48	74.5^{a}	53.7^{a}	9.3^{a}	4.2
	2000	0.69/0.49	75.2^{a}	52.3^{abc}	9.1^{abc}	11.1
	PC	0.87/0.70	56.3^{e}	53.2^{ab}	9.2^{ab}	5.6
3	0	0.70/0.44	51.9^{d}	42.1^{c}	7.0^{c}	1
	187.5	0.70/0.44	60.8^{c}	44.3^{c}	7.5^{bc}	0
	375	0.71/0.43	68.8^{b}	47.2^{b}	8.0^{b}	1
	750	0.70/0.44	74.7^{a}	50.2^{a}	8.7^{a}	0

Abbreviation: PC, positive control.

^{a,b,c,d,e}Values within the same trial and column with different superscripts are significantly different ($p < 0.05$).

3.3.1.2 | *Efficacy for laying hens*

The applicant submitted two long-term trials and one short-term digestibility trial in laying hens aiming at assessing the effect of the 6-phytase on the laying performance and phosphorus utilisation, respectively.

However, in one of the long-term trials,[43] the hens were reared individually during the whole experiment, which is not aligned with standard farming practices within the EU. Therefore, the zootechnical performance data recorded during the trial could not be considered further as evidence of the efficacy of the additive. As no appropriate data on the dietary P utilisation by the hens was provided, the trial was not considered further as evidence of efficacy.

In the other long-term trial (1),[44] 360 Lohmann Brown hens (25 weeks of age) were distributed in 90 cages (four hens per cage), which were randomly allocated to three groups (30 replicates per group). A basal diet based on maize, soybean meal and rapeseed meal was either not supplemented (control) or supplemented with the 6-phytase from the additive to provide 187.5 FYT/kg feed. The feed was analysed for the enzyme activity[45] and the content of Ca/P.[46] A PC diet with a higher content of P and Ca was also considered. The experimental feeds were offered ad libitum in mash form for 84 days. The mortality and general health of the animals were monitored daily. The hens were individually weighed at the start and end of the trial. Every 4 weeks, egg production, egg weight, number of broken eggs and feed consumption were recorded. Egg mass per hen, average daily feed intake and feed-to-egg mass ratio were calculated for the whole experimental period. At the end of the trial (day 85), 30 hens per treatment were killed, and the right tibia was collected and analysed for dry matter and the mineral content (ash, Ca and P). The experimental data were analysed with ANOVA or Kruskal–Wallis, depending on the normality of the data, with the diet as fixed effect. The experimental unit used for the laying performance was the cage, and the hen for bone parameters. When differences were observed, a mean comparison was performed with Dunnett's/Steel test. The significance level was set at 0.05.

The results are shown in Table 4. The mortality averaged 2.2% and did not differ between groups. The hens receiving the additive at 187.5 FYT/kg feed showed higher final body weight, laying rate, daily feed intake, daily egg mass, egg weight and tibia ash and phosphorus content. No differences were found between the 187.5 FYT/kg and PC groups in any parameter.

[43] Annex 4–10 H02-20 RD-65718 Trial 2 Laying Hens.

[44] Annex 4-8 SAU Report RD-0065719 Trial 1 Laying Hens.

[45] Phytase activity (FYT/kg feed) = < 50, 176 and < 50 for the control, 187.5 FYT/kg and PC groups, respectively.

[46] Ca/P (%) = 4.1/0.32, 3.8/0.31 and 3.9/0.41 for the control, 187.5 FYT/kg and PC groups, respectively.

TABLE 4 Effects of the 6-phytase on the performance and P utilisation of laying hens.

Phytase (FYT/kg)	Final body weight (kg)	Average daily Feed intake (g)	Laying rate (%)	Daily egg mass per hen (g/day)	Egg weight (g)	Feed-to-egg mass ratio	Bone content	
							Ash %	P %
0	1.62[b]	102[b]	90.1[b]	51.6[b]	56.8[b]	2.00	49.0[b]	5.4[b]
187.5	1.79[a]	113[a]	97.3[a]	57.1[a]	58.5[a]	1.99	50.3[a]	5.7[a]
PC	1.80[a]	113[a]	97.6[a]	57.7[a]	58.9[a]	1.97	50.6[a]	5.9[a]

Abbreviation: PC, positive control.

[a,b]Values within the same column with different superscript are significantly different ($p < 0.05$).

In the short-term trial (2),[47] 45 30-week-old Hyline W36 laying hens were individually caged and randomly allocated to three groups (15 replicates per group). A basal diet based on maize and soybean meal was either not supplemented (control) or supplemented with the 6-phytase from the additive to provide 187.5 or 375 FYT/kg feed. The feed was analysed for the enzyme activity[48] and the content of Ca/P.[49] The experimental diets were offered ad libitum in mash form for 7 days. Mortality and general health were monitored daily. Body weight was recorded at the beginning (day 1) and at the end of the trial (day 7). Feed intake and laying performance (laying rate, egg mass, feed-to-egg mass ratio) were monitored during the whole experimental period. From day 5 to 7, total excreta and all eggs from each cage were collected. The eggshell and content were separated and pooled independently. The feed, excreta, eggshell and egg content samples were analysed for dry matter and P content. The P utilisation was calculated. The data were analysed with ANOVA, considering the diet as fixed effect. Group means were compared with the Student–Newman–Keuls test. The significance level was set at 0.05. None of the hens died during the trial, but one was not included in the statistical analysis as it laid no eggs during the trial. The birds receiving the 6-phytase at 187.5 and 375 FYT/kg feed showed significantly higher P utilisation (51.8% and 47.6%) in comparison with the control diet (29.7%). There was no effect of the additive on the egg content of phosphorus.

3.3.1.3 | *Conclusions on the efficacy for poultry*

Based on the data provided, the Panel concludes that the additive is efficacious in chickens for fattening at the minimum use level of 200 FYT/kg feed. This conclusion can be extrapolated to all poultry for fattening and reared for laying/breeding. Due to the lack of sufficient data, the Panel is not in the position to conclude on the efficacy of the additive on laying hens, and, therefore, on other laying or reproductive poultry.

3.3.2 | Efficacy in *Suidae*

3.3.2.1 | *Efficacy for weaned piglets*

The applicant submitted two long-term trials and two short-term digestibility trials in weaned piglets aimed at assessing the effect of the 6-phytase on the growing performance or phosphorus retention. However, during a prolonged period of one of the long-term trials,[50] the content of copper in feed was above the maximum level authorised in the EU, and thus, the data obtained were not considered in the assessment of the efficacy. Besides, the other long-term trial[51] and one of the short-term trials[52] were performed during overlapping times in the same location using similar diets and, thus, were not considered independent among them. The performance and P utilisation parameters from both were considered as a single trial (referred to as Trial 1 in the description below).

Trial 1[53] assessed the effect of dietary supplementation with the additive on the zootechnical performance of the weaned piglets. In parallel, the trial included some animals under experimental conditions to collect faeces and urine to evaluate the effect on mineral retention. Two hundred hybrid[54] mixed-sex weaned piglets (28 days old) were blocked by initial body weight, distributed to 40 pens of five piglets each (two males and three females) and randomly allocated into five groups. Two basal diets (starter, from day 1 to 16; grower from day 17 to 42) based on maize and soybean meal were either not supplemented (control) or supplemented with the 6-phytase from the additive to provide 187.5, 375 or 750 FYT/kg complete feed. A PC with a higher content of P and calcium Ca was also considered. For the study of mineral retention, 30 male piglets (28 days old) were individually housed in metabolic cages and randomly allocated to three groups (10 replicates per group), a control (basal starter diet), 187.5 or 375 FYT/kg complete feed; the diets contained an external marker.

[47]Annex 4–12 RD-00063297 Trial 3 Univ Illinois layers.

[48]Phytase activity (FYT/kg feed) = 36, 164 and 361 for the control, 187.5 and 375 FYT/kg groups, respectively.

[49]Ca/P (%) = 3.03/0.35, 3.03/0.37 and 3.03/0.37 for the control, 187.5 and 375 FYT/kg groups, respectively.

[50]Annex 4–15 S09-19 RD- 00065715 Trial 2 Weaned Piglets.

[51]Annex 4–13 CAN-PP-2020-01 RD-65716 Trial 1 Weaned Piglets.

[52]Annex 4–19 CAN-PM-2020-02 RD-65717 Trial 4 Weaned Piglets.

[53]Annex 4–13 CAN-PP-2020-01 RD-65716 Trial 1 Weaned Piglets and Annex 4–19 CAN-PM-2020-02 RD-65717 Trial 4 Weaned Piglets.

[54](PIC 1050 × L337) × Landrace.

The feeds were analysed for the enzyme activities[55] and the content of Ca/P.[56] The experimental diets were offered ad libitum in pelleted form for 42 or 10 days for the performance study and mineral retention study, respectively. Mortality and health status were checked daily. The piglets were individually weighed at the start of the trial. Thereafter, body weight and feed consumption were recorded on a pen basis on days 16 and 42. The average body weight gain, feed intake and feed-to-gain ratio were calculated and corrected for mortality. For mineral retention, faecal and urine samples were individually collected daily from days 6 to 10 and pooled per replicate. Feed, urine and faecal samples were analysed for dry matter, external marker and the mineral content (P, Ca and ash) to calculate the retention. The experimental data were analysed with a generalised linear model, including the diet as fixed effects. When a difference between groups was observed, group means were compared with Dunnett's test. The significance level was set at 0.05.

The health status of the animals was good throughout the trial, and the mortality was low and not related to the dietary supplementation. The animals receiving the phytase from 187.5 FYT/kg showed improved zootechnical performance (higher final body weight, average daily gain and better feed-to-gain ratio) and higher P retention in comparison with the control (Table 5).

TABLE 5 Effects of the 6-phytase on the performance and phosphorus retention in weaned piglets in Trial 1.

Phytase (FYT/kg)	Daily feed intake (g)	Final body weight (kg)	Average daily weight gain (g)	Feed-to-gain ratio	Mortality and culling %	P retention %
0	587[b]	21.2[b]	296[b]	1.98[a]	1.0	25.1[a]
187.5	770[a]	26.8[a]	439[a]	1.76[b]	0	38.6[b]
375	871[a]	30.4[a]	526[a]	1.66[b]	0	45.6[b]
750	945[a]	33.1[a]	590[a]	1.60[b]	2.5	n/a
PC	993[a]	35.9[a]	657[a]	1.51[b]	2.5	n/a

Abbreviations: n/a, not analysed; PC, positive control.

[a,b]Values within the same column with different superscript are significantly different ($p < 0.05$).

In the short-term trial (trial 2[57]), 36 castrated male Redon × Landrace piglets (28 days old) were distributed in nine flat-deck cages (four animals per pen) and randomly allocated to three groups. The animals in each cage were housed together for 24 days. During the last 4 days of the experiment, animals were separated for individual faecal sampling. A basal diet based on maize and soybean meal was either not supplemented (control) or supplemented with the 6-phytase from the additive to provide levels from 187.5 or 375 FYT/kg complete feed. The enzyme activity was confirmed analytically.[58] The experimental diets contained an external marker for digestibility and were offered ad libitum in pelleted form for 28 days. Mortality and health status were checked daily. The zootechnical performance of the animals was monitored throughout the whole period. From day 25 to 28, faecal samples were individually collected and pooled per animal. At the end of the trial, all animals were killed, and the right femurs were collected. Feed and faecal samples were analysed for dry matter, external marker and the mineral content (P, Ca and ash) to calculate the apparent total tract digestibility. The femur samples were analysed for P, Ca and ash content. The experimental data were analysed with a generalised linear model, including the diet and pen as fixed effects. The animal was considered the experimental unit for the digestibility and mineral bone content. When differences between groups were observed, group means were compared with Tukey's test. The significance level was set at 0.05.

No piglet died during the trial. No adverse effects were observed on the zootechnical performance of the piglets during the experiment due to the inclusion of the additive in the feed. The animals receiving the 6-phytase from 187.5 FYT/kg feed showed a significantly higher P apparent total tract digestibility (27.5, 33.0 and 36.0% for the control, 187.5 and 375 FYT/kg groups, respectively) and femur P content (10.4, 10.7 and 10.8%) compared to control.

3.3.2.2 | *Efficacy for sows*

Four short-term trials were performed aimed at assessing the effect of the 6-phytase contained in the additive on phosphorus utilisation and bone mineralisation in gestating (trials 1 and 2) and lactating (trials 3 and 4) sows. All trials followed a similar experimental design (see Table 6). Results are shown in Table 7.

[55]Phytase activity (FYT/kg feed): performance trial = < 50, 171, 396 and 806 for the controls, 187.5, 375 and 750 FYT/kg groups, respectively; digestibility trial = 68, 197 and 378 for the control, 187.5 and 375 FYT/kg groups, respectively.

[56]Ca/P (%): performance trial = 0.81/0.43 and 0.72/0.42 for the starter and grower control diets; and 0.81/0.72 and 0.72/0.64 for the starter and grower PC diets. Digestibility trial = 0.76/0.43, 0.76/0.43 and 0.77/0.43 for the control, 187.5 and 375 FYT/kg groups, respectively.

[57]Annex 4–17 S08_19 RD-65714 Trial 3 Weaned Piglets.

[58]Phytase activity (FYT/kg feed): < 20, 219 and 394 for the control, 187.5 and 375 FYT/kg groups, respectively.

TABLE 6 Trial design of the efficacy trials performed in sows.

Trial	Number of animals (animals/replicate) replicates/group	Breed (parity)	Phase (start)	Duration (adaptation/ collection)	Composition feed (form)	Groups (FYT/kg feed)
1[59]	45 (1) 15	PIC L1050 × L337 (primiparous)	Gestation (day 94)	10 days (5/5)	Maize, wheat barn, soybean meal (pellet)	0 187.5 375
2[60]	48 (1) 16	Large White × Landrace (1–8)	Gestation (day 95)	15 days (10/5)	Maize, sugar beet pulp, soybean meal (mash)	0 187.5 375
3[61]	45 (1) 15	PIC L1050 × L337 (primiparous)	Lactation (day 6)	10 days (5/5)	Maize, soybean, sunflower meal (mash)	0 187.5 375
4[62]	49 (1) 16/17	Large White × Landrace (1–8)	Lactation (day 18)	22 days (17/5)	Maize, soybean meal, wheat barn (pellet)	0 187.5 375

In all trials, the sows were individually housed and randomly allocated into three groups based on the diet supplementation. In trials 2 and 4, sows entered in the trial in three different batches and were blocked by parity. The basal diet was either not supplemented (control) or supplemented with the 6-phytase from the additive at 187.5 or 375 FYT/kg feed. The enzyme activity was confirmed analytically (see Table 7). The experimental feeds included an external marker and were offered following a restricted regime based on the physiological status of the animals. The sows' health status was checked daily. The zootechnical performance of the sows and the litter was monitored throughout the whole period. Faecal samples were collected and pooled per sow. Feed and faecal samples were analysed for dry matter, external marker and the mineral content (P, Ca and ash) to calculate the apparent total tract digestibility. The experimental data were analysed with a generalised linear model, including the diet (all trials), batch and parity (trials 2 and 4) as fixed effects. The animal was considered the experimental unit. When differences between groups were observed, group means were compared with Tukey's test. The significance level was set at 0.05.

No sows died during the trials. No adverse effects were observed on the zootechnical performance of the piglets during the experiment due to the inclusion of the additive in the feed. In the four trials, the sows receiving the 6-phytase from 187.5 FYT/kg feed showed higher P apparent total tract digestibility in comparison with the control.

TABLE 7 Analysed enzyme activity of the diets and effect of the 6-phytase on the apparent total tract digestibility (ATTD) of P in sows.

Trial	Phytase activity (FYT/kg feed)		Ca/Total P (%)	ATTD P (%)
	Intended	Analysed		
1	0	< 150	0.81/0.49	15.3[c]
	187.5	220	0.86/0.50	28.1[b]
	375	408	0.76/0.49	33.8[a]
2	0	< 60	0.82/0.56	41.9[b]
	187.5	204	0.92/0.51	49.3[a]
	375	410	1.02/0.56	51.2[a]
3	0	< 150	0.72/0.43	23.6[c]
	187.5	195	0.70/0.44	43.9[b]
	375	331	0.70/0.44	50.2[a]
4	0	< 60	0.99/0.48	35.0[c]
	187.5	194	1.07/0.49	40.5[b]
	375	375	1.04/0.47	45.3[a]

[a,b]Values within the trial and within the same column with different superscript are significantly different ($p < 0.05$).

[59]Annex 4–21 CAN-SW-2019-08 RD-65707 Trial 1 Gestating sows.

[60]Annex 4–23 IRTA trial TR-62_Trial 2 Gestating Sows.

[61]Annex 4–25 CAN-SW-2019-09 RD-65708 Trial 3 Lactating Sows.

[62]Annex 4–27 IRTA trial TR-63_Trial 4 Lactating Sows.

3.3.2.3 | *Conclusions on the efficacy for* Suidae

Based on the data provided, the Panel concludes that the additive is efficacious on gestating and lactating sows at the proposed minimum use level of 200 FYT/kg feed. This conclusion can be extrapolated to all reproductive *Suidae*. Due to the lack of sufficient data, the Panel is not in the position to conclude on the efficacy of the additive on weaned piglets, and, therefore, on growing *Suidae*.

3.3.3 | Efficacy in fin fish

The applicant submitted three trials in rainbow trout (*Oncorhynchus mykiss*), and another three in three additional fin fish species (gilthead seabream – *Sparus aurata*, European seabass – *Dicentrarchus labrax* and Nile tilapia – *Oreochromis niloticus*) to support the efficacy of the additive.

3.3.3.1 | *Efficacy in rainbow trout*

Three trials aimed at assessing the effect of the 6-phytase contained in the additive on the zootechnical performance and whole-body phosphorus content of rainbow trout (*Oncorhynchus mykiss*) were submitted. A summary of the experimental design is included in Table 8, and the main results of the performance and P retention are in Table 9.

In all trials, fish were distributed in tanks (250 L in trials 1 and 2; 500 L in trial 3), which were randomly allocated to the different groups. The basal diets were either not supplemented (control) or supplemented with the 6-phytase from the additive to provide 1000 (all trials), 2000 or 3000 (trials 1 and 3) FYT/kg feed. In trials 2 and 3, a PC with higher levels of P and Ca was also considered. The 6-phytase activity was analytically confirmed in the feed (see Table 8). The experimental diets were offered for 91 (trial 1), 102 (trial 2) and 106 (trial 3) days. In trials 1 and 2, animals were fed in a restricted way following a defined regime based on the physiological stage or body weight of the fish. In trial 3, the feed was offered ad libitum.

TABLE 8 Trial design and enzyme activity of the diets of the efficacy trials performed in rainbow trout.

Trial	Total no of animals (animals/replicate) replicates/group	Body weight (sex) duration	Composition feed (form)	Groups (FYT/kg feed)	
				Intended	Analysed (average)
1[63]	240 (20) 3	46.3 g (♀) 91 days	Soy protein concentrate, fish meal (13.2%), wheat gluten (3 mm/4 mm pellets)	0 1000 2000 3000	< 50 966 1730 2686
2[64]	240 (20) 4	57.5 g (♀) 96 days	Soy protein concentrate, fish meal (13.2%), wheat gluten (3 mm/4 mm pellets)	0 1000 PC	< 50 949 < 50
3[65]	360 (30) 3	29.2 g (♀) 106 days	Soy protein concentrate, fish meal (13.2%), wheat gluten (2 mm/3 mm pellets)	0 1000 2000 3000 PC	< 50 893 1860 2639 < 50

In the three trials, the survival and health status of fish were monitored daily. Fish were individually weighed at the start of the trial. Thereafter, body weight was measured on tank basis every 2 weeks until the end of the trial (91 days on trial 1; and day 96 for trial 2), and on days 29, 73 and 106 (trial 3); average body weight was calculated. Feed intake was monitored daily. The specific growth rate (SGR,[66] three trials) and feed-to-gain ratio (trials 1 and 2) were calculated for the whole period. At the end of each trial, five (trials 1 and 2) or 10 (trial 3) fish per tank were killed, sampled and pooled per tank. Feed and whole-body fish samples were analysed for the content of dry matter and mineral content (P and ash; Ca only in trials 1 and 2), and the whole-body retention was calculated. In all trials, the experimental data were analysed with an ANOVA, including the diet as fixed effect. The experimental unit was the tank. When differences were found, group means were compared with the Student–Newman–Keuls test. The significance level was set at 0.05.

[63] Annex 4–29 PHY201 RD-64132 Trial 1 Trout April 2022.
[64] Annex 4–30 PHY202 RD-65968 Trial 2 Trout April 2022.
[65] Annex 4–31 EXT12 RD-65969 Trial 3 Trout April 2022.
[66] SGR (%BW/day) = 100 × (ln (FBW/IBW))/total number of days.

TABLE 9 Effects of the 6-phytase on the performance and whole-body P retention in rainbow trout.

Trial	Groups (FYT/kg complete feed)	Ca/P in diet (%)	Feed intake[1] (g)	Final body weight (g)	Specific growth rate (%BW/day)	Feed-to-gain ratio[2]	Survival (%)	P whole-body retention (%)
1[67]	0	0.85/0.76	–	334^{c}	2.17^{b}	0.87^{a}	100	32.8
	1000	0.85/0.76	–	357^{b}	2.25^{a}	0.84^{b}	98.3	50.4
	2000	0.84/0.76	–	374^{a}	2.30^{a}	0.82^{b}	100	44.6
	3000	0.85/0.76	–	376^{a}	2.30^{a}	0.81^{b}	98.3	51.1
2[68]	0	0.87/0.75	–	424	2.08	0.86^{a}	100	38.3
	1000	0.88/0.75	–	444	2.13	0.84^{b}	98.8	50.3
	PC	1.10/1.00	–	449	2.14	0.82^{c}	98.8	39.1
3[69]	0	0.87/0.75	7143	238	1.99	–	91.1	21.2^{b}
	1000	0.87/0.76	7501	255	2.06	–	91.1	39.9^{a}
	2000	0.89/0.77	7498	256	2.07	–	91.1	33.4^{a}
	3000	0.87/0.75	7378	257	2.07		90.0	37.8^{a}

^{a,b,c}Values within the trial and within the same column with different superscript are significantly different ($p < 0.05$).

[1]In trials 1 and 2, feed was provided in a restricted way. All groups received the same amount of feed.

[2]In trial 3, data on feed-to-gain ratio were not provided.

No differences in survival rates were observed among treatments in any trial. In trials 1 and 2, fish receiving the 6-phytase at 1000 FYT/kg feed showed improved zootechnical performance compared to the control (higher final body weight, SGR and better feed-to-gain ratio in trial 1; and better feed-to-gain ratio in trial 2). No effects on the performance were observed in trial 3.

In trial 3, the whole-body P retention was higher in the fish receiving the 6-phytase at 1000 FYT/kg feed compared to the control. No statistical difference was found in the whole-body retention at any supplemented level compared to the control in trials 1 and 2.

3.3.3.2 | *Efficacy for other fin fish*

One trial in gilthead seabream, one in European seabass and one in Nile tilapia were submitted aimed at assessing the effect of the 6-phytase on the zootechnical performance and whole-body phosphorus retention. A summary of the experimental design is included in Table 10, and the main results of the performance and P retention are in Table 11.

TABLE 10 Trial design and enzyme activity of the diets of the efficacy trials performed in other fin fish.

Species	Total no of animals (animals/replicate) replicates/group	Bodyweight (sex) duration	Tank size salinity	Composition feed (form)	Groups (FYT/kg feed)	
					Intended	Analysed
Seabream[70]	740 (37) 4	55.3 g (ND) 94	500 L 35.5 µg/L	Soybean meal, corn gluten meal, sunflower meal, fish meal (7.50%) (3 mm pellets)	0 500 1000 2000 PC	< 50 541 933 1630 < 50
Seabass[71]	760 (38) 4	57.6 g (ND) 94	500 L 35.5 µg/L	Soybean meal, corn gluten meal, rapeseed meal, fish meal (7.50%) (3 mm pellets)	0 500 1000 2000 PC	< 50 541 930 1545 < 50
Tilapia[72]	600 (30) 4	39.5 g (♂) 93	350 L 0 µg/L	Soybean meal, corn gluten meal, rapeseed meal, fish meal (2.50%) (3 mm pellets)	0 500 1000 2000 PC	175 570 897 1463 < 50

Abbreviations: ND, not determined; PC, positive control.

[67]Annex 4–29 PHY201 RD-64132 Trial 1 Trout April 2022.

[68]Annex 4–30 PHY202 RD-65968 Trial 2 Trout April 2022.

[69]Annex 4–31 EXT12 RD-65969 Trial 3 Trout April 2022.

[70]Annex 4-34 PHYBREAM RD-65552 Seabream.

[71]Annex 4–33 PHYBASS RD-65551 Seabass

[72]Annex 4–32 PHYTIL RD-65550_Tilapia.

In all trials, fish were distributed in tanks, which were randomly allocated to four groups based on the level of the additive supplementation. The basal diets were either not supplemented (control) or supplemented with the 6-phytase included in the additive to provide 500, 1000 or 2000 FYT/kg feed. The feed was analysed for the enzyme activity (see Table 10) and the content of Ca/P (see Table 11). The experimental diets were offered ad libitum (three meals per day) in pelleted form for 93 (tilapia) or 94 (seabream and seabass) days.

In the three trials, the survival and health status of the animals were monitored daily. Fish were individually weighed at start of the trial. Thereafter, average tank body weight was recorded on days 27, 66 and 94 (seabream), 48 and 94 (seabass) or 30, 64 and 93 days (tilapia). The feed intake was recorded daily. The SGR and feed-to-gain ratio were calculated for the whole period. At the end of each trial, six fish per tank were sampled and pooled per tank. Feed and whole-body fish samples were analysed for the content of dry matter and mineral content (P and Ca), and the whole-body retention was calculated. The experimental data were analysed with an ANOVA, using the diet as fixed effect. The tank was the experimental unit. When differences were observed, group means were compared with the Student–Newman–Keuls test. The significance level was set at 0.05. A summary of the main results is shown in Table 10.

The survival rate in all trials was above 99% for all groups. In the three trials, the dietary supplementation with the additive at the use level of 1000 FYT/kg feed resulted in improved zootechnical performance (higher final body weight, SGR and better feed-to-gain ratio) and whole-body P retention compared to the control. No differences in performance were observed between the supplemented groups and the PC. The whole-body P retention was higher in the fish receiving the 6-phytase at 1000 FYT/kg feed compared to the positive control.

TABLE 11 Effects of HiPhorius™ on the performance and phosphorous retention in seabream, seabass and tilapia.

Trial	(Groups FYT/kg complete feed)	Ca/P in diet (%)	Feed intake (%BW/day)	Final body weight (g)	Specific growth rate (%BW/day)	Feed-to-gain ratio	Survival (%)	Whole-body P retention (%)
Seabream	0	0.63/0.78	1.21^{a}	143^{c}	1.01^{c}	1.29^{a}	100	45.9^{b}
	500	0.59/0.79	1.17^{ab}	148^{b}	1.05^{b}	1.21^{b}	100	53.5^{b}
	1000	0.59/0.79	1.14^{b}	156^{a}	1.11^{a}	1.12^{c}	100	62.6^{a}
	2000	0.59/0.78	1.15^{ab}	157^{a}	1.10^{a}	1.14^{bc}	100	59.9^{a}
	PC	0.85/1.12	1.18^{ab}	158^{a}	1.11^{a}	1.16^{bc}	100	48.0^{b}
Seabass	0	0.66/0.70	1.21	142^{c}	0.96^{c}	1.35^{a}	99.3	40.3^{d}
	500	0.67/0.69	1.18	149^{b}	1.01^{b}	1.25^{b}	100	44.0^{c}
	1000	0.66/0.68	1.19	153^{a}	1.04^{a}	1.24^{b}	100	51.8^{b}
	2000	0.64/0.69	1.17	157^{a}	1.06^{a}	1.19^{b}	100	62.0^{a}
	PC	0.86/0.93	1.19	153^{a}	1.04^{a}	1.24^{b}	100	46.3^{c}
Tilapia	0	0.77/0.96	1.79	144^{c}	1.39^{c}	1.45^{a}	99.2	26.2^{e}
	500	0.78/0.94	1.78	163^{b}	1.52^{b}	1.36^{b}	99.2	35.5^{d}
	1000	0.75/0.96	1.78	175^{a}	1.60^{a}	1.31^{bc}	99.2	45.2^{b}
	2000	0.77/0.95	1.75	184^{a}	1.66^{a}	1.26^{c}	100	50.7^{a}
	PC	1.07/1.22	1.82	175^{a}	1.60^{a}	1.34^{bc}	100	38.7^{c}

Abbreviations: BW, body weight; PC, positive control.

$^{a\text{-}e}$Values within the trial and within the same column with different superscript are significantly different ($p < 0.05$).

3.3.3.3 | *Conclusion on the efficacy for fin fish*

Based on the data provided, the Panel concludes that the additive has the potential to be efficacious in all fin fish at the minimum proposed use level of 1000 FYT/kg feed.

3.3.4 | Conclusions on efficacy

The Panel concludes that the additive has the potential to be efficacious in all poultry for fattening and reared for laying/breeding and all reproductive *Suidae* at the minimum proposed use level of 200 FYT/kg complete feed and in all fin fish at 1000 FYT/kg complete feed. Due to the lack of sufficient data, the Panel cannot conclude on laying and reproductive poultry, and on *Suidae* for fattening or reared for reproduction.

3.3.5 | 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[73] and good manufacturing practice.

[73]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.

4 | CONCLUSIONS

The additive does not raise safety concerns with regard to the genetic modification of the production strain. The production strain and recombinant DNA were not detected in an intermediate product representative of the final formulations.

HiPhorius™ is safe for all poultry, all *Suidae* and all fin fish at the highest proposed use level of 4000 FYT/kg complete feed.

The use of HiPhorius™ in animal nutrition is of no concern for consumer safety and for the environment.

The final formulations of the additive are not skin irritants. The two liquid formulations of the additive are not eye irritants, while the two solid ones are eye irritants. The Panel cannot conclude on the skin sensitisation of the final formulations of the additive. Due to the proteinaceous nature of the active substance, the additive is considered a respiratory sensitiser.

The Panel concludes that the additive has the potential to be efficacious in all poultry for fattening and reared for laying/breeding and all reproductive *Suidae* at the minimum proposed use level of 200 FYT/kg complete feed and in all fin fish at 1000 FYT/kg complete feed. Due to the lack of sufficient data, the Panel cannot conclude on laying and reproductive poultry and on *Suidae* for fattening or reared for reproduction growing *Suidae* (suckling/weaned, for fattening or reared for reproduction).

ABBREVIATIONS

ANOVA	Analysis of variance
ATTD	Apparent total tract digestibility
BNMN	binucleated cells with micronuclei
BNP	β-nitropropionic acid
BUSCO	Benchmarking Universal Single-Copy Orthologs
BW	Body weight
CBPI	Cytokinesis-block proliferation index
CFU	Colony forming units
CPA	Cyclopiazonic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practices
IFO	Institute for Fermentation Osaka
ITS	Internal transcribed spacer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of Detection
LOQ	Limit of Quantification
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PC	Positive control
PCR	Polymerase chain reaction
PHA	phytohaemagglutinin
rDNA	Recombinant Deoxyribonucleic acid
SGR	Specific Growth Rate
TOS	total organic solids
UN GHS	Globally Harmonised System of Classification and Labelling of Chemicals
UTR	Untranslated region
WGS	Whole Genome Sequence
YPG	Yeast Extract–Peptone–Dextrose

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

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