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Safety and efficacy of Nutrase P (6-phytase) for chickens for fattening, other poultry for fattening, reared for laying and ornamental birds

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Abstract

Nutrase P is available in powder, granulated, thermostable granulate and liquid forms. It is based on a 6-phytase produced by a genetically modified strain of *K. phaffii*. The production strain contains four copies of the ampicillin resistance gene and one copy of a bleomycin resistance gene. Although Nutrase PTS 10,000 (thermostable granulate) and Nutrase PG 10,000 (granulate) do not raise concern regarding the presence of viable cells of the production strain and its recombinant DNA, uncertainties remain on the presence of viable cells and DNA of the production strain in Nutrase PL 10,000 (liquid) and Nutrase PD 100,000 (powder). The FEEDAP Panel cannot conclude on the safety of the additive, in any form, for the target species for which application is made due to major limitations in the study in chickens for fattening provided. Considering the production strain and the results obtained in the genotoxicity studies, the Panel concludes that additive does not pose a toxicological safety concern regarding the consumers of the products obtained from animals receiving the additive. The additive, in its all formulations, is not a skin or eye irritant and is not dermal sensitiser. However, owing to the proteinaceous nature of the active substance it should be considered a respiratory sensitiser. The active substance of the additive is a protein and as such would not raise concerns for the environment. Considering that the production strain harbours AMR genes and there is uncertainties regarding the presence of viable cells and DNA of the production strain in Nutrase PL 10,000 (liquid) and Nutrase PD 100,000 (powder), the Panel cannot conclude on the safety of these two formulations of the additive for the target species, consumer, user and environment. Owing to the lack of data, the FEEDAP Panel cannot conclude on the efficacy of the additive.

Keywords: safety, efficacy, zootechnical additives, 6-phytase, poultry

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Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

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 Nutrex N.V.[2] for authorisation of the product Nutrase P (6-phytase), when used as a feed additive for chickens for fattening, other poultry for fattening, reared for laying and ornamental birds (category: zootechnical additives; functional group: digestibility enhancers).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 4 April 2019.

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 Nutrase P (6-phytase), when used under the proposed conditions of use (see Section 3.1.1).

1.2. Additional information

Nutrase P is a feed additive that contains 6-phytase. It is intended to be used as a zootechnical additive in feed for chickens for fattening, other poultry for fattening or reared for laying and ornamental birds. It has not been authorised in the European Union.

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 Nutrase P as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in Annex A.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of product is in line with the principles laid down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c) and Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b).

[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] Achterstenhoek 5, 2275 Lille, Belgium.
[3] FEED dossier reference: FAD-2019-0005.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports/fad-2019-0005?search&form-return
[5] 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.

3. Assessment

The additive Nutrase P contains 6-phytase (EC 3.1.3.26; phytase) that is available in solid (granulated and powder) and liquid formulations. This product is intended to be used in feed for chickens for fattening, other poultry for fattening, reared for laying and ornamental birds as a zootechnical additive (functional group: digestibility enhancers).

3.1. Characterisation

The formulations of the additive Nutrase P covered in this application are the following: Nutrase PL 10,000 (liquid – minimum 10,000 U/G), Nutrase PG 10,000 (granulate – 10,000 U/G), Nutrase PTS 10,000 (thermostable granulate – 10,000 U/G) and Nutrase PD 100,000 (powder – minimum 100,000 U/G).[6]

3.1.1. Characterisation of the production organism

The phytase present in the additive is produced by a genetically modified strain of the yeast *Komagataella phaffii* (originally identified as *Pichia pastoris*) that has been deposited in the China General microbiological Culture Collection Centre (CGMCC) with the deposit number 7.19.[7] The identity of the production strain as *K. phaffii* was confirmed after whole genome sequence (WGS) data-based analysis [redacted]

3.1.1.1. Information relating to the genetic modification

3.1.1.2. Characteristic of the recipient organism

The recipient strain [redacted]

3.1.1.3. Characteristics of the donor organism[9]

[redacted]

3.1.1.4. Description of the genetic modification

[redacted]

[6] During the assessment, it was clarified that the less concentrated formulations that are referred to in different sections of the Technical dossier are considered as premixtures consisting of the concentrated additive on a carrier. These formulations are: Nutrase PL 5,000 U/G, Nutrase PG 5,000 U/G and 20,000 U/G, Nutrase PTS 5,000 U/G, Nutrase PD, 5,000 U/G and 50,000 U/G, (supplementary information December 2019).

[7] Technical dossier/Section II/Annex 2.2.1.2 a.Conf.

[redacted]

[redacted]

3.1.2. Manufacturing process

The enzyme is produced by fermentation with the production strain *K. phaffii* [redacted]

3.1.3. Characterisation of the additive

The powder formulation (Nutrase PD) ensures a minimum phytase activity of 100,000 U[12] /g of product, the granulate (Nutrase PG), thermostable granulate (Nutrase PTS), and liquid (Nutrase PL) formulations ensure a minimum phytase activity of 10,000 U/g or mL of product.

The powder formulation (Nutrase PD, 100,000 U/G) contains the phytase concentrate (26%) and corn starch up to 100%; moisture content is up to 8%. The batch-to-batch variation of the formulation was studied in five batches and the mean value was 126,400 U/g product, ranging from 124,000 to 130,000 U/g.[13] Particle size distribution was measured by laser diffraction in three batches; particles below 100, 50 and 10 μm amounted to 82, 34, and 2.8%, respectively.[14] The dusting potential measured in three batches by the Stauber–Heubach ranged from 9.8 to 14.7 g/m^3 (average 12.6 g/m^3).[15] This formulation has a density of 500–600 kg/m^3.

The granular formulation (Nutrase PG, 10,000 U/G) contains the phytase concentrate (3%), pre-gelatinised starch (3%), sodium carboxy methyl cellulose (1%), water (8%) and corn starch up to 100%. The batch to batch variation was studied in five batches and the mean value was 13,440 U/g product, ranging from 13,000 U/g to 13,800 U/g.[13] Particle size distribution was measured by laser diffraction in three batches; particles below 100, 50 and 10 μm amounted to 10%, 9%, and 0.8%, respectively.[16] The dusting potential measured in three batches by the Stauber–Heubach ranged from 0.7 to 2.1 g/m^3 (average 1.3 g/m^3).[17] This formulation has a density of 600–700 kg/m^3.

The thermostable formulation (Nutrase PTS, 10,000 U/G) contains the phytase concentrate (3%), sodium carboxy methyl cellulose (1%), water (8%), magnesium sulphate (8%) and corn starch up to 100%. The batch to batch variation was studied in five batches and the mean value was 13,100 U/g product, ranging from 12,900 U/g to 13,500 U/g.[13] Particle size distribution was measured by laser diffraction in three batches; particles below 100, 50 and 10 μm amounted to 46%, 36%, and 22%, respectively.[18] The dusting potential measured in three batches by the Stauber–Heubach ranged from 52.8 to 62.3 g/m^3 (average 58.3 g/m^3).[19] This formulation has a density of 700–800 kg/m^3.

The liquid formulation of the additive (Nutrase PL 10,000 U/G) contains the phytase concentrate (11%), sodium chloride (14%), potassium sorbate (1%), sorbate (1%) and deionised water up to

[redacted]

[12] Unit, one unit is defined as the amount of enzyme that releases 1 μmol of inorganic phosphate from phytate per minute at pH 5.5 and 37°C.
[13] Technical dossier/Section II/Annex 2.1.3.a.
[14] Technical dossier/Section II/Annex 2.1.5.a.
[15] Technical dossier/Section II/Annex 2.1.5.d.
[16] Technical dossier/Section II/Annex 2.1.5.b.
[17] Technical dossier/Section II/Annex 2.1.5.e.
[18] Technical dossier/Section II/Annex 2.1.5.c.
[19] Technical dossier/Section II/Annex 2.1.5.f.

100%. The batch-to-batch variation of the formulation was studied in five batches and the mean value was 12,540 U/g product, ranging from 12,200 U/g to 12,800 U/G.[20] This formulation has a density of 1,100 kg/m^3.

Purity

The formulations of the additive Nutrase P covered in this application were analysed for chemical and microbiological contamination.

The analysis of heavy metals in three batches for each formulation included cadmium (Cd), lead (Pb) and mercury (Hg); arsenic (As) was also analysed.[21] The concentration of As, Cd, Pb and Hg in three batches of Nutrase PD 100,000 and Nutrase PL 10,000 were all < 0.05 mg/kg.[22] The average heavy metals concentrations in the three batches of Nutrase PG 10,000 were as follow: As 0.31 mg/kg (range: 0.30–0.32), Cd < 0.05, Pb 0.92 mg/kg (range: 0.86–0.97), Hg < 0.01 mg/kg.[23] The average heavy metals concentrations in the three batches of Nutrase PTS 10,000 were as follow: As 0.3 mg/kg (range: 0.30–0.31), Cd < 0.05, Pb 0.86 mg/kg (range: 0.84–0.90), Hg < 0.01 mg/kg.[24]

The analysis of mycotoxins in three batches for each formulation included aflatoxin B1, B2, G1, G2 (< 1 μg/kg, limit of quantification (LOQ) = 1 μg/kg), HT-2 (< 30 μg/kg, LOQ = 10 μg/kg), T-2 Toxins (< 10 μg/kg, LOQ = 10 μg/kg), zearalenone (< 15 μg/kg, LOQ = 15 μg/kg), ochratoxin A (< 1 μg/kg, LOQ = 1 μg/kg), 3+15-acetyl-deoxynivalenol (< 20μ g/kg, LOQ = 20 μg/kg), cytochalasine E (< 2 μg/kg, LOQ = 2 μg/kg), deoxynivalenol (< 20 μg/kg, LOQ = 20 μg/kg), fumonisin B1 and B2 (< 20 μg/kg, LOQ = 20 μg/kg), and nivalenol (< 50 μg/kg, LOQ = 50 μg/kg).[25]

Microbial contamination was studied in three batches of each formulation included total coliform bacteria (< 10 colony forming units (CFU)/g, LOQ = 10/g), *Salmonella* spp. (absent in 25 g) and yeast and moulds (< 10 CFU/g, LOQ = 10/g).[26]

The sum of dioxins and dioxin like PCBs was measured in three batches of the four formulations and resulted to be 0.2 ng WHO-PCDD/F-PCB-TEQ/kg in the solid and liquid formulations.[27]

Presence of viable cells and DNA

The presence of viable cells [redacted]

The presence of [redacted]

The FEEDAP Panel noted that the presence of viable cells and DNA of the production strain was not tested in three of the final formulations (Nutrase PL 10,000 (liquid), Nutrase PD 100,000 (powder), and Nutrase PG 10,000 (granular)). However, considering the manufacturing process, the results of the analysis with the Nutrase PTS 10,000 can be extended to the Nutrase PG 10,000 (granular). The data from the Nutrase PTS may not be representative of the liquid formulation (Nutrase PL 10,000) since further processing steps are applied to obtain the Nutrase PTS. Similarly, the results on the Nutrase

[20] Technical dossier/Section II/Annex 2.1.3.b.
[21] The applicant provided the LOD and LOQ of the methods used for the measurements of As (LOD = 0.014 mg/kg, LOQ = 0.048 mg/kg), Cd (LOD = 0.012 mg/kg, LOQ = 0.040 mg/kg), Pb (LOD = 0.014 mg/kg, LOQ = 0.047 mg/kg), and Hg (LOD = 0.0019 mg/kg, LOQ = 0.0063 mg/kg). Technical dossier/Clarification received by e-mail June 2020.
[22] Technical dossier/Supplementary information December 2019/Annexes 2.3.2 a-c.
[23] Technical dossier/Supplementary information December 2019/Annexes 2.3.2 d-f.
[24] Technical dossier/Supplementary information December 2019/Annexes 2.3.2 g-i.
[25] Technical dossier/Supplementary information December 2019/Annexes 2.3.3.a-l.
[26] Technical dossier/Supplementary information December 2019/Annexes 2.3.1.a-c (PL 10,000), 2.3.1.d-f (PG 10,000), Annexes 2.3.1.g-I (PD 100,000), Annexes 2.3.1.j-l (PTS 10,000) and Technical dossier/clarification received by e-mail June 2020.
[27] Technical dossier/Section II/Annex. 2.1.4.c, 2.1.4.d.
[redacted]

PTS cannot be used to conclude on the powder formulation (Nutrase PD 100,000) since it contains 10 times more fermentation/concentrate than the one analysed.[30] Therefore, uncertainty remains on the absence of viable cells and DNA of the production strain in the formulations Nutrase PL 10,000 and Nutrase PD 100,000.

3.1.4. Stability and homogeneity

The shelf-life of the additive is claimed to be 18 months for the solid formulation and 6 months for the liquid when stored at 20°C in closed packages.

The shelf-life of the three solid formulations (Nutrase PD 5,000 U/G, Nutrase PT 10,000 U/G, Nutrase PTS 10,000 U/G) was studied in three batches of either formulation stored in closed containers at different temperatures (20 and 30°C) and time points (0, 3, 6, 12 and 18 months).[31] The enzyme activity in the solid formulation Nutrase PD 5,000 U/G was 80% of the initial activity (7,013 U/g) when stored at 20°C for 18 months and 77% of the initial activity when stored at 30°C for 18 months. The enzyme activity in the solid formulation Nutrase PT 10,000 U/G was 85% of the initial activity (13,310 U/g) when stored at 20°C for 18 months and 80% of the initial activity when stored at 30°C for 18 months. The enzyme activity in the solid formulation Nutrase PTS 10,000 U/G was 89% of the initial activity (13,598 U/g) when stored at 20°C for 18 months and 84% of the initial activity when stored at 30°C for 18 months.

The shelf-life of the liquid formulation was studied in three batches of Nutrase PL 10,000 U/G stored in closed containers at different temperatures (20 and 4°C) and time points (0, 1, 2, 3 and 6 months).[31] The enzyme activity in the liquid formulation was 89% of the initial activity (12,354 U/g) when stored at 20°C for 6 months and 96% of the initial when stored at 4°C for 6 months.

The stability of the phytase in a vitamin and mineral premixture[32] was studied in three batches of the solid formulations added to provide 100,000 U/kg premixture.[33] Samples were stored for 6 months at room temperature (packed in plastic bags). The enzyme activity after 6 months was 80, 77, 121% of the initial enzyme activity in the samples of Nutrase PD, Nutrase PG and Nutrase PTS, respectively.

The stability of the phytase in feed was evaluated in three batches of the solid/liquid formulations when added to a complete feed for chickens for fattening.[34] Nutrase PD,[35] Nutrase PG[36] and Nutrase PTS[37] were added to a mash feed, Nutrase PD,[38] Nutrase PG[39] and Nutrase PTS,[40] Nutrase PL[41] were added to pelleted feed. The expected level of phytase was 500 FTU/kg feed. The mash feed supplemented with the solid formulation was pelleted in order to study the effect of the temperature (65°C Nutrase PD, Nutrase PG, Nutrase PTS and 85°C Nutrase PTS).[42] The heat treatment lead to a loss of activity in Nutrase PD and Nutrase PG, with recovery after pelleting at 65°C of 58% and 72%, respectively. Nutrase PTS showed no losses of activity after treatment at 65°C, while a recovery of 72% was observed at a pelleting temperature of 85°C. Samples of the mash and pelleted feed were stored for 3 months at room temperature (in plastic bags). The stability of Nutrase PD, Nutrase PG and Nutrase PTS in mash feed showed recovery values of 93, 96, and 110%; the stability of Nutrase PD, Nutrase PG, Nutrase PTS and Nutrase PL in pelleted feed showed no changes in the initial enzyme activity.

The capacity of the phytase to homogeneously distribute in feed was studied in 10 subsamples of pelleted feeds. Samples of Nutrase PD,[43] Nutrase PG[44] Nutrase PTS[45] and Nutrase PL[46] showed a Coefficient of variation (CV) of 6.4, 6.3, 7.9 and 6.6%, respectively.

[30] Technical dossier/Clarification received via e-mail February 2020.
[31] Technical dossier/Section II/Annex 2.4.1.a.
[32] Technical dossier/Section II/Annex 2.4.1.b.
[33] Technical dossier/Section II/Annex 2.4.1.c, Annex 2.4.1.d, Annex 2.4.1.e.
[34] Technical dossier/Section II/Annex 2.4.1.f.
[35] Technical dossier/Section II/Annex 2.4.1.g.
[36] Technical dossier/Section II/Annex 2.4.1.h.
[37] Technical dossier/Section II/Annex 2.4.1.i.
[38] Technical dossier/Section II/Annex 2.4.1.j.
[39] Technical dossier/Section II/Annex 2.4.1.k.
[40] Technical dossier/Section II/Annex 2.4.1.l.
[41] Technical dossier/Section II/Annex 2.4.1.m.
[42] Technical dossier/Section II/Annex 2.4.1.n.
[43] Technical dossier/Section II/Annex 2.4.2.a.
[44] Technical dossier/Section II/Annex 2.4.2.b.
[45] Technical dossier/Section II/Annex 2.4.2.c.
[46] Technical dossier/Section II/Annex 2.4.2.d.

3.1.5. Conditions of use

The additive is intended to be used in feed for chickens for fattening, other poultry for fattening, reared for laying and ornamental birds at a minimum enzyme activity of 250 FTU/kg feed.

3.2. Safety

3.2.1. Safety of the genetic modification

The recipient strain from which the production organism was derived belongs to *K. phaffii*, a species considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment when used for enzyme production (EFSA, 2007; EFSA BIOHAZ Panel, 2020). The production strain was well identified [redacted] Neither this gene nor its phytase activity is considered of concern. The production strain contains four copies of an ampicillin resistance gene and one copy of a bleomycin resistance gene.

Viable cells of the production strain and recombinant DNA were not detected in the Nutrase PTS 10,000 (thermostable granulate) formulation of the additive. This conclusion applies also to the Nutrase PG 10,000 (granulate). Therefore, these two formulations do not pose any safety concern with regard to the genetic modification of the production strain.

Since the genetic modification introduces antimicrobial resistance (AMR) genes in the production strain and uncertainty remains on the absence of viable cells and DNA of the production strain in the Nutrase PL 10,000 (liquid) and Nutrase PD 100,000 (powder) formulations, the FEEDAP Panel cannot conclude on the safety for the target species, consumers, users and the environment of these two formulations with regard to the genetic modification of the production strain.[30]

3.2.2. Toxicological studies

Toxicological studies are not required for fermentation products produced by a genetically modified microorganism for which the recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment and for which the genetic modification raises no concerns. The genetic modification introduced an ampicillin and bleomycin resistance genes; however, this is not expected to have an impact on the toxicological profile of the production strain. Therefore, from this point of view the production strain is presumed as safe. However, the applicant submitted the below genotoxicity studies to support the safety of the additive.

Bacterial reverse mutation test

The liquid formulation of the additive[47] was tested for the induction of gene mutations in bacteria using *Salmonella* Typhimurium strains TA97a, TA98, TA100, TA102 and TA1535 (Annex 3.2.2.2.a).[48] The experimental protocol was in line with Organisation for Economic Co-operation and Development (OECD) guideline 471 (1997). The test item was tested at five different concentrations ranging from 50 to 5,040 μg/plate in two independent experiments applying the plate incorporation and pre-incubation methods in the presence and absence of the metabolic activation. Appropriate positive and negative controls were evaluated concurrently. All positive control chemicals induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9-mix. No precipitate and toxicity were observed in any experimental condition. No increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains with or without S9-mix. The Panel concluded that the test item did not induce gene mutations in bacteria under the experimental conditions employed in this study.

In vitro mammalian chromosome aberration test

An *in vitro* chromosome aberration test[49] was performed according to OECD Test Guideline 473 (OECD, 1997) and following good laboratory practice (GLP) to evaluate the potential to induce chromosome damage in human peripheral blood lymphocytes applying a short treatment (4 h + 18 h

[47] Highly concentrated product from fermentation (stabilised with 3% sorbitol, 10% sodium chloride, 2% sodium citrate and 0.3% potassium sorbate). Enzyme activity 20,000 U/mL (nominal), 21,600 (measured – Technical dossier/Section III/Annex 3.2.2.2.b).
[48] Technical dossier/Section III/Annex 3.2.2.2.a.
[49] Technical dossier/Section III/Annex 3.2.2.2.c.

of recovery) in the presence and absence of metabolic activation and a long treatment (24 h + 0 recovery) in the absence of metabolic activation. The same liquid formulation tested in the bacterial reverse mutation test was used. Based on the limit of solubility (5 g/L) SMIZYME 6-Phytase was tested at eight concentrations ranging from 36.1 to 2,505.6 ug/mL. No precipitate and cytotoxicity were observed at all the concentrations tested and the three concentrations selected for the analysis of chromosome damage, namely 1,000.6, 1,500.9, 2,501.5 μg/mL with metabolic activation and 799.9, 1,396.5, 2,505.6 μg/mL without metabolic activation. Appropriate positive and negative control chemicals were used, and the results obtained confirmed that the experimental system was sensitive and valid. No statistically significant increase in the frequency of structural chromosome aberrations was observed after short treatment in the absence and presence of metabolic activation. After long treatment, an increase of chromosome breaks was observed only at the lowest concentration. The increase was statistically significant ($p = 0.01$), above the range of the negative historical controls, but not dose-related. The Panel noted that at this experimental point not homogeneous results were reported from the two scorers which carried out the analysis; in addition, the Panel considered that the lack of induction of aberrations at the mid and high concentrations could not be due to cell cycle delay interfering with the expression of chromosome damage (Ritter et al., 2002, Int J Rad Biol, 78: 191) since no changes in mitosis progression, as measured by mitotic index, were induced by the test item at any concentration tested. On this basis, the Panel considered not biologically relevant the observed increase and concluded that the test item did not induce structural chromosome aberrations in the conditions employed in this study. However, the Panel observed that the analysis of numerical chromosome aberrations was not reported and considered the dataset limited since the requirement of the 'Guidance on the assessment of the safety of feed additives for the consumer' (EFSA FEEDAP Panel, 2017a–c) to assess the three genotoxic endpoints, namely gene mutations, structural and numerical chromosome aberrations, was not fulfilled.

3.2.3. Safety for the target species

In order to support the safety of the additive for the target species, the applicant submitted a tolerance study in chickens for fattening.[50] The study was conducted with 200 one-day-old male birds (Ross 308) which were under study for 43 days. The mean feed intake of the birds during the study was 87 g/day and the mean final body weight was 1,920 g. The feed intake and the final body weight were a 30 and a 40%, respectively, lower than the expected for the animals used. This low performance may be due to the diets used in the study which presented a low energy and protein/ amino acid content. Therefore, the study cannot be considered further in the assessment as it would not represent the most sensitive status of the animals, reducing the sensitivity of the test. Therefore, the FEEDAP Panel cannot conclude on the safety of the product, in any formulation, for any of the target species for which application is made.

Moreover, the Panel also notes that uncertainty remains on the safety of the formulations Nutrase PL 10,000 and Nutrase PD 100,000 as regards to the absence of viable cells of the production strain and its DNA. The production strain contains antimicrobial resistance genes which, if present in these two formulations, would represent a safety concern for the target species.

3.2.4. Safety for the consumer

The enzyme is produced by a genetically modified strain of *K. phaffii*; this species is considered to qualify for the QPS approach to safety assessment when used for enzyme production. The identity of the strain was established, and the genetic modification of the production strain raises no concerns as regards to the toxicological profile of the production strain. The genotoxicity studies would support the safety of the production strain. Therefore, the Panel considers that the formulation Nutrase PTS 10,000 and Nutrase PG 10,000 rise no concerns for the consumer of the products obtained from the animals fed with these two formulations. However, the Panel also notes that uncertainty remains on the safety of the formulations Nutrase PL 10,000 and Nutrase PD 100,000 as regards to the absence of viable cells of the production strain and its DNA. Furthermore, the production strain contains antimicrobial resistance genes which, if present in these two formulations, would represent a safety concern for the consumers.

[50] Technical dossier/Section III/Annex 3.1.1.a, Annex 3.1.1.b, Annex 3.1.1.c Annex 3.1.1.d.

3.2.5. Safety for user

3.2.5.1. Effects on the respiratory system

No specific tests were submitted; however, based on the proteinaceous nature of the active substance of the additive, it is considered as a respiratory sensitiser. The Panel also notes that the solid formulations have a high dusting potential (highest measured value 62.3 g/m^3), which makes exposure of users by inhalation very likely.

3.2.5.2. Effects on the skin and eyes

The skin and eye irritation potential of a highly concentrated (non-commercial) liquid concentrate from fermentation was tested in *in vivo* valid studies performed according to OECD guideline 404 and 405, which showed that it is not irritant to skin or eyes.[51]

The same test item was tested for skin sensitisation following the OECD guideline 429.[52] The test item tested at 100% did not induce skin sensitisation. According to the classification, labelling and packaging of substances and mixtures criteria (European Chemical Agency, 2017) the test item is classified as non-dermal sensitiser.

The FEEDAP Panel notes that the studies were performed not with the final formulations of the additive but with a concentrate form of 6-phytase. However, considering the nature of the other components of the final formulations of the additive, the conclusions reached with the concentrate would apply to the final formulations.

3.2.5.3. Conclusions on safety for the user

The different formulations of the additive Nutrase P are not a skin or eye irritants and are not dermal sensitisers. However, owing to the proteinaceous nature of the active substance they should be considered a respiratory sensitiser.

Owing to the uncertainty regarding the absence of viable cells and DNA of the production strain in the Nutrase PL 10,000 (liquid) and Nutrase PD 1,00,000 (powder) formulations and the fact that the genetic modification introduces AMR genes in the production strain, the FEEDAP Panel cannot conclude on the safety for the user of these two formulations.

3.2.6. Safety for the environment

The active substance present in the additive is a protein and as such will be degraded/inactivated during the passage through the digestive tract of animals. Therefore, no risks to the environment are expected from the active compound.

The production strain harbours AMR genes. No viable cells and their recombinant DNA were detected in the Nutrase PTS 10,000 (thermostable granulate) formulation of the additive. Therefore, this formulation of the additive can be considered safe for the environment. This conclusion is applicable also to the Nutrase PG 10,000 (granulate). Due to the lack of data, uncertainty remains on the absence of viable cells and DNA of the production strain in the Nutrase PL 10,000 (liquid) and Nutrase PD 100,000 (powder) formulations. Therefore, the FEEDAP Panel cannot conclude on the safety for the environment of these two formulations.

3.3. Efficacy

The applicant provided three studies, one short-term trial and two long-term trials. The short-term trial was not considered further in the assessment due to the high mortality registered in some of the groups (up to 12.7% in the control group for a 21-day study).[53] The two long-term trials were designed to study the effect of the phytase on the performance of the chickens as well as on the utilisation and retention of phosphorus from the diets.

In the first long-term trial,[54] a total of 216 one-day-old female chickens (Ross 308) were caged in groups of six birds and allocated to six dietary treatments (representing six replicates per treatment). Two basal diets, starter and grower, based on maize and soybean meal was either not supplemented (control) or supplemented with Nutrase PTS to provide 125, 250, 500 or 1,500 FTU/kg feed. A positive

[51] Technical dossier/Section III/Annexes 3.3.1.2.a, 3.3.1.2.b.
[52] Technical dossier/Section III/Annex 3.3.1.2.c.
[53] Technical dossier/Section IV/Annex 4.2.1.a to 4.2.1.e and supplementary information December 2019.
[54] Technical dossier/Section IV/Annex 4.3.1.a to 4.3.1.e and supplementary information December 2019 Annexes 3.3.a to 3.3.d.

control was also considered. Enzyme activities were confirmed by analysis (analysed values 100, 106, 378, 580 and 1,640 U/kg feed for control, 125, 250, 500 and 1,500 groups, respectively). Diets were offered *ad libitum* in the pelleted form up to day 35 of life. Mortality was checked every day. Feed intake was measured throughout the study and body weight was measured on days 0, 14, and 35 (cage basis) and feed to gain ratio calculated. Water intake was measured per pen during two time periods (day 15–17 and day 29–31). In order to study the utilisation/retention of phosphorus, total excreta were collected quantitatively during five consecutive days starting on day 20 and analysed for phosphorus and calcium content. On day 35, the left tibia bone was collected from eight birds per treatment and analysed for dry matter, phosphorus and calcium content. The data was analysed with an analysis of variance (ANOVA) and mean groups compared with Bonferroni, with the exception of bone parameters that were compared with Least Significant difference (LSD) test. Significance level was set at 0.05. The results are presented in Table 1.

In the second long-term trial,[55] a total of 432 one-day-old female chickens (Ross 308) were placed in pens in groups of 18 birds and allocated to three dietary treatments (representing eight replicates per treatment). Two basal diets, starter and grower, based on maize, wheat and soybean meal were either not supplemented (control) or supplemented with Nutrase PTS to provide 250 or 500 U/kg feed. Enzyme activities were confirmed by analysis (analysed values 265, 518 and 834 for control, 250 and 500 groups). Diets were offered *ad libitum* up to 41 days of life (starter as crumble, grower as pellets). Titanium dioxide was added to the diets as an external marker. Mortality was checked every day. Feed intake was measured throughout the study and body weight was recorded on days 1, 14, and 41 and feed to gain ratio calculated. In order to study the utilisation/retention of phosphorus, excreta samples were collected during five consecutive days starting on day 20. On day 41, left tibia bones were collected from two birds per pen (16 per treatment, 48 in total) and analysed for dry matter, ash, phosphorus and calcium. One-way ANOVA was performed with the data and group means were compared with Tukey's test. Significance level was set at 0.05. The results are presented in Table 1.

[55] Technical dossier/Section IV/Annex 4.3.2.a to 4.3.2.g.

Table 1: Effect of Nutrase PTS on the performance and mortality in chickens for fattening, phosphorus retention and bone mineralisation

Trial	Diets			Performance and mortality					P retention (%)	Bone parameters[1]	
	Group (U/kg feed)	Total P starter/ grower (g/kg feed)	Total calcium starter/grower (g/kg feed)	Daily feed intake (g/day)	Final body weight (g)	Body weight gain (g/day)	Feed to gain ratio	Mortality (n)		ash	P
1	Positive control	8.5/6.3	9.1/6.9	88.7	2,165	60.7	1.46	4	51.1^{c}	43.2^{ab}	8.4
	Control	6.9/4.9	7.8/5.7	90.6	2,171	60.9	1.49	0	59.4^{b}	41.4^{b}	8.3
	125	6.4/4.9	7.5/5.7	90.8	2,173	60.9	1.49	0	60.7^{b}	41.7^{ab}	8.5
	250	6.5/4.8	7.6/5.6	90.6	2,168	60.8	1.49	1	62.5^{ba}	41.5^{b}	8.3
	500	6.5/4.8	7.5/5.6	91.0	2,169	60.8	1.49	1	62.8^{ba}	43.5^{a}	8.6
	1,500	6.4/4.9	7.7/5.6	91.4	2,218	62.2	1.47	1	65.1^{a}	43.4^{ab}	8.6
2	Control	6.2/5.0	8.1/7.5	97.5	2,544^{b}	59.5^{b}	1.64	5	53.6^{c}	16.9^{b}	3.0^{b}
	250	6.5/4.9	9.1/6.9	101.4	2,684^{a}	62.8^{a}	1.62	10	57.3^{b}	21.6^{ab}	3.8^{ab}
	500	6.5/4.9	9.3/7.2	99.2	2,619^{ab}	61.2^{ab}	1.62	5	61.8^{a}	22.5^{a}	4.0^{a}

1: Values in trial 1 are reported as % of the dry matter (DM), while in trial 2 are reported as % of the bone.
a,b,c: For a given trial and within a column values with different superscript are significantly different.

The results showed that the animals that received the phytase at 250 U/kg had a higher final body weight and body weight gain compared to those in the control group in trial 2. The phosphorus retention was higher in birds receiving the phytase compared to control at the level of 250 U/kg and 500 U/kg in trial 2 and at 1,500 U/kg feed in trial 1. Effects on bone mineralisation were found in both trials at 500 U/kg feed. With the current data, the FEEDAP Panel cannot conclude on the efficacy of the additive and notes that in order to conclude at 250 U/kg two more studies showing significant effect at that dose would be required.

3.3.1. Conclusions on the efficacy

The FEEDAP Panel cannot conclude on the efficacy of the additive in chickens for fattening, and, as a consequence, for any of the other target species for which application is made.

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[56] and Good Manufacturing Practice.

4. Conclusions

The production strain contains four copies of the ampicillin resistance gene and one copy of a bleomycin resistance gene. Although Nutrase PTS 10,000 (thermostable granulate) and Nutrase PG 10,000 (granulate) do not raise concern regarding the presence of viable cells of the production strain and its recombinant DNA, uncertainties remain on the absence of viable cells and DNA of the production strain in Nutrase PL 10,000 (liquid) and Nutrase PD 100,000 (powder).

The FEEDAP Panel cannot conclude on the safety of the additive, in any form, for the target species for which application is made due to major limitations in the study in chickens for fattening provided.

Considering the production strain and the results obtained in the genotoxicity studies, the Panel concludes that the additive does not pose a toxicological safety concern regarding the consumers of the products obtained from animals receiving the additive.

The additive, in all its formulations, is not a skin or eye irritant and is not a dermal sensitiser. However, owing to the proteinaceous nature of the active substance it should be considered a respiratory sensitiser.

The active substance of the additive is a protein and as such would not raise concerns for the environment.

Considering that the production strain harbours AMR genes and there is uncertainties regarding the absence of viable cells and DNA of the production strain in Nutrase PL 10,000 (liquid) and Nutrase PD 100,000 (powder), the Panel cannot conclude on the safety of these two formulations of the additive for the target species, consumer, user and environment.

Owing to the lack of data, the FEEDAP Panel cannot conclude on the efficacy of the additive.

5. Documentation as provided to EFSA/Chronology

Date	Event
5/02/2019	Dossier received by EFSA. Nutrase P (6-phytase) for chickens for fattening, other poultry for fattening, reared for laying and ornamental birds. Submitted by Nutrex N.V
19/02/2019	Reception mandate from the European Commission
04/04/2019	Application validated by EFSA – Start of the scientific assessment
23/05/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: methods*
18/06/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: Characterisation*
04/07/2019	Comments received from Member States
09/08/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: safety and efficacy*
20/12/2019	Reception of supplementary information from the applicant
10/02/20	Request of clarification from the applicant via e-mail
12/02/20	Reception of clarification from the applicant - Scientific assessment re-started
23/03/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
12/06/2020	Request of clarification from the applicant via e-mail

[56] 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.

Date	Event
19/06/2020 24/06/2020	Reception of clarification from the applicant
30/09/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ANOVA analysis of variance
AMR antimicrobial resistance

CFU	colony forming unit
CGMCC	China General microbiological Culture Collection Centre
CV	coefficient of variation
DM	dry matter
EURL	European Union Reference Laboratory
FCR	feed conversion ratio
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GLP	good laboratory practice
LSD	Least Significant Difference
LOD	limit of detection
LOQ	limit of quantification
OECD	Organisation for Economic Co-operation and Development
PCB	polychlorinated biphenyls
PCDD/F	polychlorinated dibenzo-*p*-dioxins and dibenzofurans
QPS	Qualified presumption of safety
TEQ	toxic equivalent
WGS	whole genome sequence
WHO	World Health Organization

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method of Analysis for Nutrase (6-phytase)

In the current application, authorisation of a preparation of 6-phytase (EC 3.1.3.26) is sought under Article 4(1) for avian species under the category/functional group 4 (a) "zootechnical additives"/ "digestibility enhancers".

According to the Applicant, the active agent is 6-phytase. The phytase activity is expressed in phytase units (FTU). One FTU unit, as described in EN ISO 30024, is defined as "the amount of enzyme that releases 1 μmol of inorganic phosphate from sodium phytate per minute under reaction conditions of pH 5.5 and 37 °C".

The product is intended to be marketed as solid (Nutrase PD, Nutrase PG and Nutrase PTS) and liquid (Nutrase PL) formulations with a guaranteed minimum 6-phytase activity of 100000 FTU/g for the Nutrase PD and of 10000 FTU/g for the remaining formulations. It is intended to be included through premixtures or directly in feedingstuffs to obtain a minimum recommended activity of 250 FTU/kg feedingstuffs.

For the quantification of the phytase activity the Applicant submitted the ring-trial validated colorimetric standard method ISO 30024 (for the feed additive, premixtures and feedingstuffs). However as the scope of the ISO 30024 is limited to feedingstuffs the EURL requested to the Applicant to apply the ring-trial validated VDLUFA 27.1.4 and VDLUFA 27.1.3 methods to representative feed additive and premixture samples.

Based on the performance characteristics available the EURL recommends for official control the colorimetric ring-trial validated methods mentioned above for the quantification of the phytase 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.