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SCIENTIFIC OPINION

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Safety and efficacy of a feed additive consisting of endo-1,4-beta xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-beta-1,4-glucanase produced by *Trichoderma citrinoviride* DSM 33578 (Huvezym® neXo 100 G/L) for all poultry species, ornamental birds and piglets (weaned and suckling) (Huvepharma EOOD)

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Fašmon Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa, Ruud Woutersen, Gabriele Aquilina, Luca Tosti, Andrey Yurkov, Montserrat Anguita, Jaume Galobart, Jordi Ortuño, Fabiola Pizzo, Jordi Tarrés-Call and Elisa Pettenati

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 the product Huvezym® neXo 100 G/L containing an endo-1,4-beta xylanase, an endo-1,4-beta-glucanase and a xyloglucan-specific-endo-beta-1,4-glucanase produced by a non-genetically modified strain of *Trichoderma citrinoviride* (DSM 33578) as a zootechnical additive for feed in all poultry species, ornamental birds and piglets (weaned and suckling). The information regarding the production strain did not allow to confirm its taxonomic identification. The batches used for the characterisation of the final formulations showed compliance with the minimum specifications of the additive in terms of enzyme activities but showed ratios between the enzymes lower than the ones specified for the additive. The FEEDAP Panel considered that the below-described conclusions would apply to the final formulations of the additive as per specifications with xylanase:glucanase, xylanase: xyloglucanase and glucanase:xyloglucanase ratios of 15, 15 and 1, respectively. Based on the data available, the Panel concluded that the additive is safe for the target species, consumers and the environment. Huvezym® neXo 100 G (granulated form) is neither skin corrosive nor eye irritant but should be considered a potential skin sensitiser. Huvezym® neXo 100 L (liquid) is neither skin corrosive nor sensitising and it is not an eye irritant. Due to lack of data, no conclusions can be drawn on the skin irritation of the final formulations of the additive. Due to the proteinaceous nature of the active substances, the additive is considered a respiratory sensitiser. The FEEDAP Panel concluded that the additive has the potential to be efficacious in chickens for fattening, chickens reared for laying and breeding, and all growing poultry and ornamental birds at the minimum intended level of 1,500 EPU, 100 CU and 100 XGU/kg complete feed. Owing to the lack of sufficient data, the Panel could not conclude on the efficacy of the additive for laying hens and weaned piglets.

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Keywords: zootechnical additives, digestibility enhancers, Huvezym® neXo 100 G/L, *Trichoderma citrinoviride*, safety, efficacy

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Correspondence: feedap@efsa.europa.eu

Panel members: Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Fašmon Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa and Ruud Woutersen.

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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 Huvepharma EOOD[2] for the authorisation of the additive consisting of endo-1,4-beta xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-beta-1,4-glucanase produced by *Trichoderma citrinoviride* DSM 33578 (Huvezym® neXo 100 G/L), when used as a feed additive for all poultry species, ornamental birds and piglets (weaned and suckling) (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). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 29 July 2021.

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 endo-1,4-beta xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-beta-1,4-glucanase produced by *T. citrinoviride* DSM 33578 (Huvezym® neXo 100 G/L), when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

The additive is not authorised as a feed additive 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 endo-1,4-beta xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-beta-1,4-glucanase produced by *T. citrinoviride* DSM 33578 (Huvezym® neXo 100 G/L) 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 substances 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 endo-1,4-beta xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-beta-1,4-glucanase produced by *T. citrinoviride* DSM 33578 (Huvezym® neXo 100 G/L) is in line with the principles laid down in Regulation (EC) No 429/2008[5] 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 FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP

[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] Huvepharma EOOD, 3A Nikolay Haytov str, 1,113, Sofia (Bulgaria).
[3] FEED dossier reference: FAD-2021-0036.
[4] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2021-0036_en.
[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.

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Panel, 2017c), Guidance on the 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

This opinion assesses the safety and efficacy of the product that contains endo-1,4-β-xylanase (xylanase, EC 3.2.1.8), endo-1,4-β-glucanase (glucanase, EC 3.2.1.4) and xyloglucan-specific-endo-beta-1,4-glucanase (xyloglucanase, EC 3.2.1.151) produced by *T. citrinoviride* DSM 33578 (Huvezym® neXo 100 G/L) as a zootechnical additive (functional group: digestibility enhancers) for all poultry species, ornamental birds and piglets (weaned and suckling). The additive under assessment will be hereafter referred to as Huvezym® neXo 100 G or Huvezym® neXo 100 L.

3.1. Characterisation

3.1.1. Characterisation of the production microorganism

The additive contains three enzyme activities (xylanase, glucanase and xyloglucanase) produced by a non-genetically modified strain of *T. citrinoviride* which is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) with the accession number DSM 33578.[6]

The taxonomic identification analyses provided allocated the production strain with a well-characterised *T. citrinoviride* strain .[7] .[8] However, the Panel notes that the sequence ( ) and parameters used are not appropriate for *Trichoderma* identification. Based on the data available, the Panel cannot conclude on the identification of the production strain.

The applicant submitted two sets of data to exclude the capacity of the production strain to produce antimicrobials. .[9] No antimicrobial activity was detected.

The applicant conducted a literature search to investigate the potential toxigenicity and ability of the production strain *T. citrinoviride* DSM 33578 to produce metabolites harmful to humans and/or animals.[10] The search was performed in NCBI and ScienceDirect and using as search terms '*Trichoderma citrinoviride*' in combination with 'toxic', 'pathogen' and 'virulence'. The search identified 10 relevant publications involving the species *T. citrinoviride* either in pathogenicity (Kuhls et al., 1999) or toxigenicity (i.e., production of compounds with antifeedant activity against aphids (Evidente et al., 2008, 2009), peptaibols (Maddau et al., 2009; Sadykova et al., 2015a,b; Castagnoli et al., 2018; Marik et al., 2019), terpens and other compounds with antimicrobial and cytotoxic activity (Hu et al., 2014; Liang et al., 2016a,b; Liu et al., 2020)). Based on the information retrieved from the literature search, the whole genome sequence (WGS) data of the production strain *T. citrinoviride* DSM 33578 was interrogated . The bioinformatic analyses showed no clusters coding for compounds of

[6] Technical dossier/Section II/Annexes_Sect.II/Annex_II_15.
[7] Technical dossier/Section II/Annexes_Sect.II/Annex_II_16 and Annex_II_58.
[8] Technical dossier/Section II/Annexes_Sect.II/Annex_II_17 and Supplementary information May 2022/Annexes/RTQ_02.
[9] Technical dossier/Section II/Annexes_Sect.II/Annex_II_25 and Supplementary information May 2022/Annexes/RTQ_07.
[10] Technical dossier/Section II/Annexes_Sect.II/Annex_II_24.

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foreseeable concern. Moreover, the presence of gliotoxin and alamethicin was not detected in the fermentation product used to formulate the additive [redacted][11] No peptaibols were found in three batches of the fermentation product, [redacted].

3.1.2. Manufacturing process

[redacted][12] [redacted][13] [redacted][14] [redacted][15]

The applicant stated that no antimicrobials are used during the manufacturing process.[16]

3.1.3. Characterisation of the additive

The additive is available in two different formulations, granulated form (Huvezym® neXo 100 G) and liquid form (Huvezym® neXo 100 L).

Both formulations have a guaranteed minimum activity per gram of product of 15,000 xylanase units (EPU), 1,000 glucanase units (CU) and 1,000 xyloglucanase units (XGU). This results in ratios of xylanase:glucanase, xylanase:xyloglucanase and glucanase:xyloglucanase of 15, 15 and 1, respectively.

The batch-to-batch variation was studied in six batches[17] of each formulation and the mean values per gram of product were 21,917 EPU (ranging from 21,700 to 22,200 EPU/g), 3,320 CU (ranging from 3,190 to 3,520 CU/g), 3,178 XGU (ranging from 3,050 to 3,280 XGU/g) for Huvezym® neXo 100 G and 20,258 EPU (ranging from 19,800 to 20,750 EPU/g), 3,202 CU (ranging from 3,080 to 3,260 CU/g), 3,152 XGU (ranging from 3,100 to 3,190 XGU/g) for Huvezym® neXo 100 L. The results support the compliance of the final additive with the minimum specifications in terms of enzyme activities. The batches analysed showed ratios of xylanase:glucanase, xylanase:xyloglucanase and glucanase: xyloglucanase similar to 6, 6 and 1. Those ratios are lower than the ones specified for the final additive, but in line with the ones for the fermentation product. The FEEDAP Panel notes that the specifications for the fermentation-derived multi-enzyme product with the specified enzymes ratios of [redacted] (see Section 3.1.2) would not allow to obtain [redacted] final formulations of the additive having the specified enzymes ratios of 15, 15 and 1. The applicant, upon request, explained that the

[11] Technical dossier/Section II/Annexes_Sect.II/Annex_II_59 and Supplementary information May 2022/Annexes/RTQ_04.
[12] One EPU is the amount of enzyme which releases 0.0083 µmol of reducing sugars (xylose equivalent) per minute from oat spelt xylan at pH 4,7 and 50°C.
[13] One CU is the amount of enzyme that liberates 0,128 µmol of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4,5 and 30°C.
[14] One XGU is the amount of enzyme that releases low-molecular fragments from dyed xyloglucan in amount equal to the amount of sugars liberated from 1 unit enzyme standard at pH 4.5 and 50°C.
[15] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_26.
[16] Technical dossier/Supplementary information May 2022/FAD 2021_0036 Huvezym neXo poultry piglets RTQ.
[17] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_05 and 06 and Supplementary information May 2022/FAD 2021_0036 Huvezym neXo poultry piglets RTQ.

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products are formulated to comply with the minimum specifications in terms of enzyme activities. Considering the inconsistencies between the specifications of the additive and the analysed values, the FEEDAP Panel is not in the position to characterise the additive under assessment. The FEEDAP Panel considers that the batches of the additive analysed are compliant with the specifications in terms of enzyme activities but are not compliant in the ratios between the enzymes.

Huvezym® neXo 100 G contains the fermentation-derived multi-enzyme product (1.75% w/w), pregelatinised starch (1% w/w) and wheat meal (up to 100% w/w).

Huvezym® neXo 100 L contains the fermentation-derived multi-enzyme product (1.75% w/w), sodium benzoate (0.25% w/v), glycerol (50% v/v) and water (up to 100% v/v).

The applicant set specifications for chemical and microbiological contamination which include arsenic (< 4 mg/kg), lead (< 10 mg/kg), cadmium (< 0.5 mg/kg), mercury (< 0.2 mg/kg), total aerobic microbial count (TAMC) (< 10^4 colony forming units (CFU)/g), total coliforms (< 30 CFU/g), *Escherichia coli* (not detected in 25 g) and *Salmonella* (not detected in 25 g). Three batches of each formulation were tested for the presence of arsenic (< limit of detection (LOD)), lead (< LOD), cadmium (0.033–0.037 mg/kg for the solid form and < LOD for the liquid form) and mercury (< LOD) and showed compliance with the specifications set. The same three batches were also analysed for aflatoxins (B1, B2, G1 and G2) which were in all cases < LOD.[18] Analytical data to confirm the specifications set for the microbiological contamination were provided for each form of the additive: TAMC ranged between 3.3×10^2 and 6.0×10^3 CFU/g and between $<1.0\times10^2$ and 3.1×10^2 CFU/g for the solid and liquid form (nine batches tested), respectively; total coliforms were < 30 CFU/g (six batches tested) and *E. coli* and *Salmonella* were not detected in 25 g (six and nine batches tested, respectively).[19] Three additional batches of Huvezym® neXo 100 G and of Huvezym® neXo 100 L were tested for the presence of Enterobacteriaceae. A pre-enrichment step was included in the method and the results showed no detection in 10 g samples.[20]

Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and coplanar dioxin-like polychlorinated biphenyls (coplanar PCBs) were analysed in three batches of each form and found below the corresponding limit of quantification (LOQ).[21] The calculated (upper bound) levels of dioxins and the sum of dioxins and dioxin-like-PCBs were 0.137 ng WHO-PCDD/F-TEQ/kg and 0.269 ng WHO-PCDD/F-PCB-TEQ/kg in all batches.

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

The presence of viable cells of the production strain was investigated [redacted][22]. Therefore, no viable cells of the production strain were found.

3.1.4. Physical properties of the additive

Huvezym® neXo 100 G appears as beige to brownish granules with poured and tapped densities ranging from 420 to 470 kg/m^3 and from 490 to 540 kg/m^3, respectively.[23] The dusting potential of three batches of the additive was determined using the Stauber–Heubach method and showed values on average of 68.3 mg/m^3 (range 55–80 mg/m^3).[24] The particle size distribution was analysed in other three batches of Huvezym® neXo 100 G by sieve method; the results showed that on average 88.9% of particles had a size between 100 μm and 800 μm and 11.1% a diameter below 100 μm.[23]

[18] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_10. Limit of detection (LOD) = Limit of quantification (LOQ) in mg/kg were: 0.04 for arsenic, 0.01 for cadmium, 0.005 for mercury and 0.05 for lead; in μg/kg was 1 for aflatoxins (B1, B2, G1 and G2).
[19] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_11 and Supplementary information May 2022/Annexes/RTQ_03.
[20] Technical dossier/Supplementary information May 2022/Annexes/RTQ_03.
[21] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_10.
[22] Technical dossier/Supplementary information May 2022/Annexes/RTQ_05 and RTQ_06 and Supplementary information September 2022/RTQII FAD 2021-0036/FAD 2021_0036 Huvezym neXo poultry piglets RTQ II.
[23] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_12.
[24] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_13.

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Huvezym® neXo 100 L is a yellowish to brown liquid with a density of 1,100–1,300 kg/m^3 (analysis in six batches showed values ranging from 1,150 to 1,200 kg/m^3).[25] Three batches of Huvezym® neXo 100 L were analysed for viscosity and the average value was 12.1 cP (range 12.0–12.2 cP).[26]

3.1.5. Stability and homogeneity

The studies were conducted with the batches analysed for the batch-to-batch variation.

3.1.5.1. Huvezym® neXo 100 G

The shelf life of Huvezym® neXo 100 G was studied in three batches when stored in paper bags with internal polyethylene layer at 25 and 40°C for up to 24 and 6 months, respectively.[27] Mean loss of xylanase/glucanase/xyloglucanase activity after 24 months at 25°C was of 9.3/9.6/9.5%. Mean loss of xylanase/glucanase/xyloglucanase activity after 6 months at 40°C was 19.4/26.2/25.4%.

Three batches of the additive were added to a vitamin-mineral complete premixture for poultry (including choline chloride) and in a vitamin-mineral premixture for pigs.[28] Samples were stored in polyethylene bags and placed into double paper bags at 25°C for 6 months and at 40°C for 3 weeks. Mean loss of xylanase/glucanase/xyloglucanase activity after 6 months at 25°C was 12.1/12.9/15.8% in the premixture for poultry and 13.2/14/13.4% in the premixture for pigs. Mean loss of xylanase/glucanase/xyloglucanase activity after 3 weeks at 40°C was 16.5/18.8/20.6% in the premixture for poultry and 16.8/18.6/17.1% in the premixture for pigs.

Three batches of the additive were mixed in a complete feed (mash form) for poultry based on maize and wheat.[29] Samples were kept in polyethylene bags and placed into double paper bags at 25 and 40°C for 3 months and 3 weeks, respectively. Mean enzyme activity loss of xylanase/glucanase/xyloglucanase after 3 months at 25°C was 5.6/5.3/5.6% and after 3 weeks at 40°C was 11.2/10.8/10.8%. The mash feed was pelleted at 85°C. Mean enzyme activity loss of xylanase/glucanase/xyloglucanase after pelleting was 3.2/5.3/4.8%. Samples of the pelleted feed were stored in polyethylene bags and placed into double paper bags at 25 and 40°C for 3 months and 3 weeks, respectively. Mean enzyme activity loss of the activities present after pelleting for xylanase/glucanase/xyloglucanase after 3 months storage at 25°C was 6.4/7/7.1% and after 3 weeks at 40°C was 11.6/11.9/12.4%.

Three batches of the additive were also mixed in a complete feed (mash form) for pigs based on maize and wheat.[30] Samples were kept in polyethylene bags and placed into double paper bags at 25 and 40°C for 3 months and 3 weeks, respectively. Mean enzyme activity loss of xylanase/glucanase/xyloglucanase after 3 months at 25°C was 4.8/5.6/6% and after 3 weeks at 40°C was 9.7/10.6/10.4%. The mash feed was pelleted at 85°C. Mean enzyme activity loss of xylanase/glucanase/xyloglucanase after pelleting was 3.4/6.3/4.8%. Samples of the pelleted feed were stored in polyethylene bags and placed into double paper bags at 25 and 40°C for 3 months and 3 weeks, respectively. Mean enzyme activity loss of the activities present after pelleting for xylanase/glucanase/xyloglucanase after 3 months storage at 25°C was 6.4/7.6/7.8% and after 3 weeks at 40°C was 10.6/11.9/12.6%.

The capacity of Huvezym® neXo 100 G to homogeneously distribute was studied in mash feed for poultry and for pigs by analysing 10 subsamples from three batches, respectively.[31] The coefficient of variation in mash feed for poultry ranged between 10.4 and 12% for xylanase, 9.6 and 10% for glucanase, and 8.9 and 10.7% for xyloglucanase. The coefficient of variation in mash feed for pigs ranged between 9 and 10.3% for xylanase, 9.2 and 10.1% for glucanase, and 9 11.4% for xyloglucanase.

3.1.5.2. Huvezym® neXo 100 L

The shelf life of Huvezym® neXo 100 L was studied in three batches stored in small size plastic bottles at 25 and 40°C for up to 12 and 6 months, respectively.[32] Mean loss of xylanase/glucanase/xyloglucanase activity after 12 months at 25°C was 10/11/10.1%. Mean loss of xylanase/glucanase/xyloglucanase activity after 6 months at 40°C was 21.4/26.8/26.1%.

[25] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_06.
[26] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_14.
[27] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_29.
[28] Technical dossier/Section II/Annexes_Sect.II/Annex_II_31 and 32.
[29] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_33.
[30] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_35.
[31] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_33 and 35.
[32] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_30.

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Three batches of the additive were sprayed onto pelleted feed for poultry based on wheat and maize.[33] Samples were kept in polyethylene bags and placed into double paper bags at 25 and 40°C for 3 months and 3 weeks, respectively. Mean enzyme activity loss of xylanase/glucanase/xyloglucanase after 3 months at 25°C was 7.7/8.1/8.4% and after 3 weeks at 40°C was 13.8/13.5/14.3%.

Three batches of the additive were also sprayed onto pelleted feed for pigs based on wheat and maize.[34] Samples were kept in polyethylene bags and placed into double paper bags at 25 and 40°C for 3 months and 3 weeks, respectively. Mean enzyme activity loss of xylanase/glucanase/xyloglucanase after 3 months at 25°C was 7.7/7.6/8.4% and after 3 weeks at 40°C was 13.2/13.8/14%.

The capacity of Huvezym® neXo 100 L to homogeneously distribute was studied in pelleted feed for poultry and for pigs by analysing 10 subsamples from three batches, respectively.[35] The coefficient of variation in pelleted feed for poultry ranged between 6.7 and 7.4% for xylanase, 7.2 and 8.1% for glucanase, and 6.8 and 7.4% for xyloglucanase. The coefficient of variation in pelleted feed for pigs ranged between 6.9 and 7.1% for xylanase, 7.3 and 8% for glucanase, and 7 and 7.7% for xyloglucanase.

3.1.6. Conditions of use

The additive is intended for use in feed for all poultry species, ornamental birds and piglets (weaned and suckling) at a proposed minimum level of 1,500 EPU, 100 CU and 100 XGU/kg complete feed.

3.2. Safety

3.2.1. Toxicological studies

3.2.1.1. Test item used

The applicant submitted genotoxicity studies and a subchronic oral toxicity study performed with batches of the intermediate concentrate multi-enzyme product used in the formulation of Huvezym® neXo 100 G/L produced in 2013 (enzymes activity per gram of product of 1,181,000 EPU, 88,900 CU and 82,300 XGU, resulting in enzymes ratios of 13, 14 and 1 (*in vitro* mammalian cell micronucleus test and sub-chronic oral toxicity study) and of 1,243,800 EPU, 102,000 CU and 95,300 XGU, resulting in enzymes ratios of 12, 13 and 1 (bacterial reverse mutation test)).[36]

The glucanase and the xyloglucanase activities in the test items were lower than the specifications set by the applicant for the intermediate dry concentrate ([redacted]). Moreover, the xylanase:glucanase or xylanase:xyloglucanase ratios analysed in the test items were higher than the ones expected according the specifications set for the fermentation-derived dry concentrate multi-enzyme product ([redacted]).

According to the applicant, the batches tested in the toxicological studies were one of the first that were produced and had a lower enzyme activity yield for glucanase and xyloglucanase.[37] The data made available for the test items used and the batches of the fermentation product used for the formulation of the additive (see Sections 3.1.2 and 3.1.3) allowed comparison of the enzyme activities but no other relevant comparison was possible. The test items used in the toxicological studies may not reflect the intermediate product currently used in the formulation of the additive.

However, the test items would reflect the xylanase:glucanase, xylanase:xyloglucanase and glucanase:xyloglucanase ratios (15, 15 and 1, respectively) as per specifications of the final forms of the additive.

The genotoxicity studies and the subchronic oral toxicity study submitted are presented under this section.

[33] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_34.
[34] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_36.
[35] Technical dossier/Section II/ Annexes_Sect.II/Annex_II_34 and 36.
[36] Technical dossier/Supplementary information May 2022/FAD 2021_0036 Huvezym neXo poultry piglets RTQ and clarifications received by email on 24th June 2022.
[37] Technical dossier/Supplementary information September 2022/RTQII FAD 2021-0036/FAD 2021_0036 Huvezym neXo poultry piglets RTQ II.

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3.2.1.2. Bacterial reverse mutation test

In order to investigate the potential of the test item to induce gene mutations in bacteria, an Ames test was performed according to OECD Test Guideline (TG) 471 (OECD, 1997) and following Good Laboratory Practice (GLP) in four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535, TA1537) and *Escherichia coli* WP2 uvrA, in the presence and absence of metabolic activation.[38] Two independent experiments were performed applying the plate-incorporation and pre-incubation methods. Based on the results of a dose-finding test, the test item was tested at six concentrations ranging from 17 to 5,000 μg/plate. Appropriate positive and negative control chemicals were used and the results obtained confirmed that the experimental system was sensitive and valid. No precipitate and cytotoxicity 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 metabolic activation.

The FEEDAP Panel concluded that the test item did not induce gene mutations in bacteria under the experimental conditions applied in this study.

3.2.1.3. *In vitro* mammalian cell micronucleus test

To evaluate the potential of the test item to induce chromosomal damage, an *in vitro* micronucleus test was performed in human peripheral blood lymphocytes according with OECD TG 487 (2010) and following GLP.[39] Three concentrations were selected for the analysis of micronuclei (i.e., 0.05, 0.2 and 1 mg/ml) based on a preliminary experiment showing precipitation at 1, 3 and 5 mg/ml and interference with the scoring at 3 mg/ml and above. At the highest concentration tested, the limited solubility did not interfere with the conduct of the assay. A short treatment (4 + 26 h of recovery) in the absence and presence of metabolic activation and a continuous treatment (26 + 0 h of recovery) without metabolic activation were applied. Appropriate positive and negative control chemicals were used and the results obtained confirmed that the experimental system was sensitive and valid. No cytotoxicity was observed. No significant increase in the frequency of binucleated cells with micronuclei was induced by the test item at any tested concentration.

Based on these results, the FEEDAP Panel concluded that the test item did not induce structural and numerical aberrations under the experimental conditions applied in this study.

3.2.1.4. 90-day toxicity study

In a GLP study performed following the OECD TG 408 (1998), 10 Wistar rats/sex/group were administered by gavage the test item as water solution (volume of 1 ml/100 g of body weight (bw)) at a dose of 100, 300 and 1,000 mg/kg bw per day for 90 days.[40] An additional group of 10 animals/sex served as control. In the control and in the high-dose groups, there were another five males and five females which were allowed to survive for the subsequent 28-day period after the end of administration. Results of clinical signs of toxicity observations, and sensory activity, grip strength and motor activity assessments were not reported in tabular form. One female at 1,000 mg/kg bw per day died during the study. Marked purulent bronchopneumonia, pleuritis, and pericarditis were found as a consequence of previous incorrect administration of the test item. There were no treatment related clinical signs of toxicity or body weight effects. A statistically significant food consumption decrease was observed in females at 300 mg/kg bw per day at week 7 and 12, and at 1,000 mg/kg bw per day at weeks 1, 2, 6 and 7. On the contrary a significant increase of food consumption was observed during the recovery period in these animals. None of these changes affected body weight. No treatment-related findings were observed during ophthalmoscopic examination. A statistically significant higher erythroblast count and partial thromboplastin time was reported in females at 1,000 mg/kg bw per day. Given that almost all individual values fell within the concurrent control range, the absence of significant changes in related haematological or clotting parameters, or changes in the bone marrow differential counts, these findings were considered likely to be incidental and of no toxicological concern. No treatment related changes were observed for the clinical chemistry or urine parameters. A significantly higher absolute weigh of epididymides (+12%) and decreased relative thymus weight (−25%) in males at 1,000 mg/kg bw per day of the recovery phase were reported. As these changes were limited to the absolute or relative weight only and there was no treatment-related

[38] Technical dossier/Section III/Annexes_Sect_III/Annex_III_04.
[39] Technical dossier/Section III/Annexes_Sect_III/Annex_III_05.
[40] Technical dossier/Section III/ Annexes_Sect_III/Annex_III_06.

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histopathology finding for these organs, these changes were considered of no toxicological significance. There were no histopathology treatment-related effects.

Therefore, under the conditions of the study, the no observed adverse effect level (NOAEL) is considered to be the highest dose tested, 1,000 mg/kg bw per day.

3.2.1.5. Conclusion on the toxicological studies

The product tested did not show genotoxicity potential and the results obtained in a subchronic oral toxicity study raised no concerns regarding the product.

Toxicological tests for fermentation products should be conducted with a fermentation product identical to that to be used in the commercial product. The test items used in the toxicological studies may not reflect the intermediate product used in the formulation of the additive as presented in the characterisation (see Section 3.1), and therefore, do not allow conclusions for the batches used in the characterisation. However, the test item would reflect a manufacturing resulting in xylanase:glucanase, xylanase:xyloglucanase and glucanase:xyloglucanase ratios (15, 15 and 1, respectively) as per specifications of the final forms of the additive.

3.2.2. Safety for the target species

The applicant provided three studies to support the safety for the target animals: one in chickens for fattening, one in laying hens and one in weaned piglets. However, in the trial in weaned piglets,[41] the high rate of morbidity (> 27%) during the trial suggested a poor health status of the animals. Therefore, this study could not be considered further as supporting evidence of the additive's safety for weaned piglets.

The studies were conducted in 2019–2020 using test items that would reflect the xylanase: glucanase, xylanase:xyloglucanase and glucanase:xyloglucanase ratios (15, 15 and 1, respectively) as per specifications of the final forms of the additive.

3.2.2.1. Safety for chickens for fattening

A total of 1,170 one-day-old male chickens for fattening (Ross 308) were distributed in 78 pens and randomly allocated to 3 dietary treatments (26 replicates per treatment).[42] Three (starter – days 1–10; grower – days 11–24; and finisher – days 25–35) basal diets based on wheat, soya bean meal and maize were either not supplemented (control) or supplemented with the test item to provide 1,500/100/100 (1× minimum recommended level) or 150,000/10,000/10,000 (100×) EPU/CU/XGU per kg feed. The enzyme activities were confirmed analytically.[43] The experimental feeds were offered *ad libitum* in mash form for 35 days.

General health and mortality (including culling) were recorded daily, including probable causes of death/culling. The birds were weighed at the beginning of the experiment (day 1). Thereafter, the pen body weight and feed intake were measured at every diet change (days 10, 24 and 35). The average daily feed intake (ADFI), average daily gain (ADF) and feed to gain ratio were calculated and corrected for mortality for the 1–10, 11–24, 25–35 and overall periods.

The safety of the additive was evaluated with a non-inferiority test, considering ADG (non-inferiority margin (Δ) = 2.49 g/bird) and ADFI (Δ = 4.67 g/bird) as reference parameters. The experimental data were analysed with an analysis of variance (ANOVA), including the diet as a fixed effect and the replicate as a random effect. The Wilcoxon test was used when normality or homogeneity of variances were not ensured. Mean groups were compared with Tukey's test. The significance level applied was 0.05.

Mortality including culling was 2.3, 0.8 and 1.3% for the control, 1× and 100× diets, respectively, and no differences were found between treatments.

Based on the results of the non-inferiority test, the supplementation of the diet with the additive at 1× or 100× minimum recommended level proposed did not negatively affect the ADG and ADFI in comparison with the control diet. The birds receiving the diet supplemented with 100× use level showed higher final body weight (C = 2,032 vs 1 × MRD = 2,011 vs 100 × MRD = 2,151 g) and ADG

[41] Technical dossier/Section III/Annex III_03.
[42] Technical dossier/Section III/Annex III_01.
[43] Starter/Grower/Finisher (EPU-CU-XGU/kg complete feed): 280–90-100/300–80-70/310–100-120, 2,130–240-210/1,930–220-230/1,940–230-240, and 153,400-10,900-10,500/155,100-11,300-10,900/152,000-10,500–11,300 for the control, 1×, and 100× groups, respectively.

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(56.6 vs 56.2 vs 60.1 g), a reduced feed to gain ratio (1.483 vs 1.477 vs 1.438), and no differences in the ADFI (average = 83.5 g) in comparison with the control and the 1× diets.

The FEEDAP Panel concludes that the additive is safe for chickens for fattening at 1,500 EPU-100 CU-100 XGU/kg complete feed with a margin of safety of 100.

3.2.2.2. Safety for laying hens

A total of 384 25-week-old laying hens (Novogen brown) were distributed in 96 cages (4 hens per cage) and allocated to three dietary treatments (32 replicates per treatment).[44] A basal diet based on wheat, barley and sunflower meal was either not supplemented (control) or supplemented with the test item to provide 1,500/100/100 (1× minimum recommended level) or 150,000/10,000/10,000 (100×) EPU/CU/XGU per kg feed. The enzymatic activity of the feeds was analytically confirmed.[45] The experimental diets were offered *ad libitum* in mash form for 84 days.

General health was monitored daily and mortality (including culls) together with the most likely cause of death were recorded as it occurred. Body weight was recorded per replicate at the beginning and at the end of the trial, and the average daily weight gain (ADG) calculated. Laying rate, egg weight and feed intake were recorded for each cage on a 4-week basis, and the egg mass, average daily feed intake (ADFI) and feed to egg mass ratio calculated for the corresponding periods and overall.

The data on laying rate (Δ = 3.39%) and ADFI (Δ = 5.51 g/bird) were subjected to a non-inferiority test comparing the control and supplemented diets (1× and 100×). The significance level applied was 0.10. The performance data were analysed with a generalised linear model including the diet as a fixed effect and the replicate (cage) as a random effect. A nonparametric test (Welch's) was used when normality or homogeneity of variances were not achieved. Group means were compared with Tukey's test. The significance level applied was 0.05.

Mortality including culling was 1.56% for all treatments. The supplementation of the diet with either 1× or 100× maximum recommended dose of the additive showed not to be inferior ($p < 0.10$) to the control diet in terms of laying rate and ADFI. No differences were observed between groups in the laying rate (C = 94.9%), egg weight (62.0 g), daily egg mass (234.7 g/cage), final body weight (1,927 g), ADFI (125.1 g/hen) and egg mass to feed ratio (0.473) in the overall experimental period.

The FEEDAP Panel concludes that the additive is safe for laying hens at 1,500 EPU-100 CU-100 XGU/kg complete feed with a margin of safety of 100.

3.2.2.3. Toxicological data

The results of the subchronic oral toxicity study described above (see Section 3.2.1.4) were used to support the safety for the target species. The NOAEL identified (1,000 mg/kg bw per day representing 1,181,000 EPU, 88,900 CU and 82,300 XGU/kg bw per day) was used to calculate the maximum safe level in feed for the different target species in accordance with the procedure described in the Guidance on the safety for the target species (EFSA FEEDAP Panel, 2017b). The calculated maximum safe concentrations in feed are presented in Table 1.

Table 1: Maximum safe concentration of the additive in feed

	Body weight (kg)	Feed intake (Kg DM/day)	Daily feed intake (g DM/kg bw)	Maximum safe concentration (EPU-CU-XGU/kg feed)(1)
Chicken for fattening	2	0.158	79	131,554-9,903-9,167
Turkey for fattening	3	0.176	59	177,150-13,335-12,345
Laying hen	2	0.106	53	196,090-14,760-13,665
Piglet	20	0.88	44	236,200-17,780-16,460

DM; dry matter; bw: body weight.
(1): Complete feed containing 88% dry matter, milk replacer 94.5% dry matter.

The maximum safe levels obtained are higher than the minimum recommended use level of 1,500 EPU, 100 CU and 100 XGU/kg complete feed for chickens and turkeys for fattening, laying hens and

[44] Technical dossier/Section III/Annex 2.
[45] 450–40-30, 2,370–185-170, and 179,000-12,100–11,300 EPU-CU-XGU/kg complete feed for the control, 1×, and 100× groups, respectively.

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piglets. Therefore, the Panel concludes that the additive is safe for all poultry species and piglets (weaned or suckling).

3.2.2.4. Conclusions on safety for the target species

Based on the data available, the FEEDAP Panel concludes that the product tested is safe for poultry species, ornamental birds and weaned and suckling piglets at 1,500 EPU, 100 CU and 100 XGU/kg complete feed.

The test items used in the tolerance trials and in the sub-chronic oral toxicity study showed ratios of enzymes that would allow conclusions on the additive as per specifications. However, those would not allow conclusions for the batches used in the characterisation.

3.2.3. Safety for the consumer

The results obtained in the genotoxicity and subchronic oral toxicity studies performed with the test item did not indicate safety concerns. The test items used showed ratios of enzymes that would allow conclusions on the additive as per specifications. However, those would not allow conclusions for the batches used in the characterisation.

3.2.4. Safety for the user

3.2.4.1. Effect on respiratory system

The dusting potential of the solid formulation is up to 80 mg/m^3. Based on the proteinaceous nature of the active substances, the additive is considered a respiratory sensitiser.

3.2.4.2. Effect on skin and eyes

The studies were conducted in 2022 using final formulations of the additive which were compliant with the specifications in terms of enzyme activities. The batches showed xylanase:glucanase, xylanase:xyloglucanase and glucanase:xyloglucanase ratios (similar to 13, 13 and 1, respectively) compliant with the specifications of the final forms of the additive.

3.2.4.2.1. Huvezym® neXo 100 G

The skin corrosion potential of Huvezym® neXo 100 G was investigated using the 'In Vitro Skin Corrosion Reconstructed Human Epidermis (RhE) test method' (2019) according to OECD TG 431 and following the principles of GLP.[46] The results of the study indicated that the additive can be categorised as "non-corrosive" according to the UN GHS classification system. No studies investigating the skin irritation potential of the additive were submitted.

The skin sensitisation potential of Huvezym® neXo 100 G was evaluated using 'the Local Lymph Node Assay in Mice' (2010) according to OECD TG 429 and following the principles of GLP.[47] The results of the study indicated that the additive is a potential skin sensitiser.

The eye irritation potential of Huvezym® neXo 100 G was investigated using 'Acute Eye Irritation/ Corrosion' using New Zealand White Rabbits according to OECD TG 405 and following the principles of GLP.[48] According to the GHS Classification Criteria, the additive can be classified as 'No Category' (not an eye irritant).

3.2.4.2.2. Huvezym® neXo 100 L

The skin corrosion potential of Huvezym® neXo 100 L was investigated using the 'In Vitro Skin Corrosion Reconstructed Human Epidermis (RhE) test method' (2019) according to OECD TG 431 and following the principles of GLP.[49] The results of this study indicate that the additive can be categorised as 'non-corrosive' according to the UN GHS classification system. No studies investigating the skin irritation potential of the additive were submitted.

The skin sensitisation potential of Huvezym® neXo 100 L was evaluated using 'the Local Lymph Node Assay in Mice' (2010) according to OECD TG 429 and following the principles of GLP.[50] The results of this study indicated that the additive has no skin sensitisation potential.

[46] Technical dossier/Supplementary information May 2022/RTQ_12.
[47] Technical dossier/Supplementary information May 2022/RTQ_13.
[48] Technical dossier/Supplementary information May 2022/RTQ_14.
[49] Technical dossier/Supplementary information May 2022/RTQ_15.
[50] Technical dossier/Supplementary information May 2022/RTQ_16.

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The eye irritation potential of Huvezym® neXo 100 L was investigated using 'Acute Eye Irritation/ Corrosion' using New Zealand White Rabbits according to OECD TG 405 and following the principles of GLP.[51] According to the GHS Classification Criteria, the additive can be classified as 'No Category' (not an eye irritant).

3.2.4.3. Conclusions on safety for the user

Huvezym® neXo 100 G is neither skin corrosive nor eye irritant but should be considered a potential skin sensitiser. Huvezym® neXo 100 L is neither skin corrosive nor sensitising and it is not an eye irritant. Due to lack of data, no conclusions can be drawn on the skin irritation of the final formulations of the additive. Due to the proteinaceous nature of the active substances, the additive is considered a respiratory sensitiser.

The test items used showed ratios of enzymes that would allow conclusions on the additive as per specifications. However, those would not allow conclusions for the batches used in the characterisation.

3.2.5. Safety for the environment

The active substances of the additive are proteins, 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 studies were conducted in 2018–2019 using test items declared to be the final formulation 100 G and diluted 5 times with wheat flour. The ratios of the enzymes in the products tested would reflect the xylanase:glucanase, xylanase:xyloglucanase and glucanase:xyloglucanase ratios (15, 15 and 1, respectively) as per specifications of the final forms of the additive.

3.3.1. Efficacy for chickens for fattening

The applicant submitted three long-term trials to evaluate the effect of the product on the productive performance of chickens for fattening. Trials 2 and 3 included a measurement of the apparent metabolisable energy (AME) of the diets. In all trials, the birds were distributed in two experimental groups: the basal diet either not supplemented (control) or supplemented with the test item to provide 1,500 EPU, 100 CU and 100 XGU per kg feed. The experimental diets were offered *ad libitum*, and the enzyme activity was confirmed analytically for the starter, grower and finisher diets in each trial. The finisher diets in trials 2 and 3 included an external marker for the digestibility analysis. The details of the experimental design of the trials are included in Table 2, and the results of the productive performance and energy utilisation parameters are in Table 3.

Table 2: Trial design and analysed enzyme activities of the diets of the efficacy trials performed in chickens for fattening

Trial	Total N (bird/rep.) Rep/treat.	Breed Sex	Duration (Starter/ Grower/ Finisher)	Composition feed (form)	Groups (EPU-CU-XGU/kg feed)	
					Intended	Analysed
1[52]	660 (22) 15	Ross 308 50% ♂:♀	42 days (1–14/ 15–28/29–42)	Wheat, soya bean meal, barley (mash)	0-0-0 1,500-100-100	210-310/21-31/23-39 1,620-2,170/140-170/160-185
2[53]	480 (20) 12	Ross 308 Male	35 days (1–14/ 15–28/29–35)	Wheat, soya bean meal, barley, maize (mash)	0–0-0 1,500-100-100	180-280/210-310/90-120 1,660-2,170/350-420/210-270

[51] Technical dossier/Supplementary information May 2022/RTQ_17.
[52] Technical dossier/Section IV/Annex IV_1.
[53] Technical dossier/Section IV/Annex IV_2.

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Trial	Total N (bird/rep.) Rep/treat.	Breed Sex	Duration (Starter/ Grower/ Finisher)	Composition feed (form)	Groups (EPU-CU-XGU/kg feed)	
					Intended	Analysed
3[54]	374 (17) 11	Ross 308 Male	35 days (1–10/ 11–21/22–35)	Maize, wheat, soya bean meal (pellets)	0-0-0 1,500-100-100	210-280/30-50/25-30 1,870-1,970/150-180/145-150

In all trials, the general health status of the birds was monitored daily, and the mortality (including culls) was recorded as it occurred, including the most likely cause of death. The weight of the animals was recorded on a pen basis at the start of the trial. Thereafter, body weight and feed intake were measured at every diet change and at the end of the trial. The ADG, ADFI and feed-to-gain ratio were calculated and corrected for mortality for the starter, grower and finisher phases, and the overall period. In trial 2, spot samples of the excreta were collected during the last 4 days of the study (days 32–35) and pooled per pen. The feed and excreta samples were analysed for the content of the external marker, gross energy and nitrogen, and the AME of the diets calculated. In trial 3, at day 25, two birds per pen were moved to metabolic cages, and after an adaptation period of 7 days, excreta samples were collected daily from each cage during the last 4 days of the trial (days 32–35) by the total collection sampling method. The feed and excreta samples were analysed for the content of gross energy, nitrogen and the external marker to calculate the nitrogen-corrected apparent metabolisable energy (AMEn). The experimental data were statistically analysed with ANOVA (trials 1 and 3) or t-test (trial 2) considering the diet as a fixed effect. The significance level applied was 0.05. The supplementation of the diets with the test item to provide 1,500 EPU, 100 CU and 100 XGU per kg feed showed an improved growing performance of the birds in trials 1 (higher final body weight and ADG, and lower feed to gain ratio) and 3 (lower ADFI and feed to gain ratio), and higher dietary AME in trial 2 in comparison with the control diets.

Table 3: Effects of test item on the zootechnical performance and energy utilisation of chickens for fattening

Trial	Groups (EPU-CU-XGU/kg feed)	Daily feed intake (g)	Final body weight (g)	Average daily weight gain (g)	Feed to gain ratio	AME (n)* (MJ/ kg)	Mortality and culling (%)
1	0-0-0	89.0	2,232^{b}	52.1^{b}	1.71^{a}	–	2.4
	1,500-100-100	89.6	2,328^{a}	54.4^{a}	1.65^{b}	–	3.3
2	0-0-0	98.0	2,001	55.8	1.76	10.5^{b}	2.2
	1,500-100-100	101	2,048	57.2	1.74	11.1^{a}	1.3
3	0-0-0	121,7^{a}	2,466	69.3	1.53^{a}	12.0	1.1
	1,500-100-100	119,9^{b}	2,442	68.6	1.51^{b}	11.9	1.1

^{a,b}: Mean values within a trial and within a column with a different superscript are significantly different $p < 0.05$.
*: AME in trial 2/AMEn in trial 3.

3.3.2. Efficacy for laying hens

The applicant submitted three trials to support the efficacy of additive in laying hens: one short-term digestibility trial assessing the energy utilisation of the diets (trial 1), one long-term trial assessing the zootechnical performance parameters, including a digestibility trial to evaluate the effect of the additive on the diet energy utilisation (trial 2), and one long-term trial exclusively assessing the zootechnical performance (trial 3). However, the calcium level of the experimental diets in trial 3[55] was much lower (< 25 g/kg) than the recommended ones, which may imply a detrimental effect on the mineral balance of the birds. Therefore, this trial was not considered further as supporting evidence of the efficacy.

[54] Technical dossier/Section IV/Annex IV_3.
[55] Technical dossier/Section IV/Annex IV_6.

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In the short-term trial,[56] two-hundred and eighty-eight 50-week-old laying hens (ISA Brown) were distributed in 36 enriched cages (8 hens per cage) and randomly allocated to two dietary treatments (18 replicates per treatment). The basal diet based on wheat, soya bean meal and maize was either not supplemented (control) or supplemented with the test item to provide 1,500 EPU, 100 CU and 100 XGU/kg feed. The enzyme activities of the diets were analytically confirmed (Control = 60-110-75/ Treatment = 2,230-260-210 EPU-CU-XGU/kg complete feed). The experimental diets were offered *ad libitum* for 28 days and contained an external marker for the digestibility analysis. At the beginning and end of the experimental period, animals were individually weighed, and the cage feed intake measured. Egg quality and laying performance parameters were daily recorded. From days 22 to 25, excreta samples were collected daily by the total collection method and pooled per cage. The diet and excreta samples were analysed for the content of dry matter, external marker, gross energy and nitrogen, and the dietary AMEn was calculated. The experimental data were analysed with ANOVA with the treatment as fixed effect. The significance level applied was of 0.05. No mortality was recorded during the whole experimental period. The supplementation of the diets with the test item to provide 1,500 EPU, 100 CU and 100 XGU per kg feed showed higher ($p < 0.05$) dietary AMEn in comparison with the control diet (C = 10.9 vs T = 11.3 MJ/kg feed).

In the long-term trial 2,[57] one-hundred 23-week-old laying hens (Hy-Lyne Brown) were distributed in 50 cages and allocated to two dietary treatments (25 replicates per treatment). The basal diet based on wheat, triticale and sunflower meal, was either not supplemented (control) or supplemented with the test item to provide 1,500 EPU, 100 CU and 100 XGU/kg feed. The enzyme activity of the feed was analytically confirmed (Control = 230-40-30/Treatment = 1,940-210-170 EPU-CU-XGU/kg complete feed). The experimental diets were offered *ad libitum* in mash form for 84 days and included an external marker for the digestibility analysis. At the beginning and end of the experimental period, the cage weight was measured, while the cage feed intake was recorded every 4 weeks. Eggs were sampled 3 days per week, egg weight recorded, and the egg mass, laying rate and feed to egg mass ratio were calculated for every four-weeks and the overall 84-days experimental period. From days 42 to 45, excreta samples were collected and pooled twice per day from 10 replicates per treatment (2 cages of 2 hens each were considered one replicate; the selection of cages was done according to the distribution of the cages within the experimental room). The diet and excreta samples were analysed for the content of dry matter, external marker and gross energy. The AME of the diet was calculated. The experimental data were analysed with t-test. The significance level applied was 0.05. No mortality was recorded during the whole experimental period. No significant differences between treatments were observed in any of the productive performance parameters measured (average final body weight = 2,050 g/hen; daily feed intake = 121 g; laying rage = 96.3%; egg weight = 60.6 g; feed to egg mass ratio = 2.06). The diet supplemented with the test item to provide 1,500 EPU, 100 CU and 100 XGU per kg feed showed higher ($p < 0.05$) AME in comparison with the control diet (C = 10.75 vs T = 10.88 MJ/kg feed).

3.3.3. Efficacy for weaned piglets

The applicant submitted three long-term trials to assess the effect of the additive on the productive performance of weaned piglets. However, trial 2[58] was not considered further for the assessment due to the high number of medical treatments applied during the experiment (> 20% of animals) and the high mortality rate (control = 6.3%).

Trials 1 and 3 followed a similar experimental design in which the piglets were distributed in two experimental groups: the basal diets either not supplemented (control) or supplemented with the test item to provide 1,500 EPU, 100 CU and 100 XGU per kg feed. The experimental diets were offered *ad libitum*, and the enzyme activity confirmed analytically for the pre-starter and starter diets in each trial. The details of the design are included in Table 4, and the results of the productive performance parameters in Table 5.

[56] Technical dossier/Section IV/Annex IV_4.
[57] Technical dossier/Section IV/Annex IV_5.
[58] Technical dossier/Section IV/Annex IV_8.

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Table 4: Trial design and analysed enzyme activities of the diets of the efficacy trials performed in weaned piglets

Trial	Total N (aals/ rep.) Rep/ treat	Breed Sex	Duration (pre-/ starter)	Composition feed (form)	Groups (EPU-CU-XGU/kg feed)	
					Intended	Analysed
1[59]	192 (8) 12	(Lan × LW) × Pietrain 50% ♂:♀	42 days (1–14/15–42)	Wheat, barley and soybean meal (pellets)	0-0-0 1,500-100-100	270–370/170–210/110–130 1,850–1,960/340–350/ 230–250
3[60]	168 (4) 21	LW x Duroc 50% ♂:♀	45 days (1–14/15–45)	Wheat, corn, soybean meal, barley (mash)	0-0-0 1,500-100-100	125–240/60–70/55 1,250–1,805/205–230/ 180–210

The general health was daily monitored, and mortality (including culls) was recorded as it occurred including the most likely cause of death. The piglets were weighed at the start of the trial. Thereafter, the bodyweight and the feed intake were recorded at the diet change and at the end of the trial. The average daily weight gain, feed intake and feed to gain ratio were calculated and corrected for mortality for pre-starter, starter and the overall experimental period. The experimental data of each trial were analysed with a generalised linear model including the treatment and the block (weaning batch) in trial 1 as fixed effects. The initial body weight was included as a covariate in both trials. In trial 3, data were analysed with Wilcoxon rank test when normality or homogeneity of variances were not achieved. The significance level applied was 0.05. Mortality including culling for the whole period was on average 2.7 and 1.2% for trials 1 and 3, respectively, and no differences were found between treatments. In both trials, the animals fed with the diets supplemented with the test item to provide 1,500 EPU, 100 CU and 100 XGU per kg feed showed lower feed to gain ratio in comparison with the control. No effect on any other performance parameter was observed.

Table 5: Effects of test item on the zootechnical performance of weaned piglets

Trial	Groups (EPU-CU-XGU/kg feed)	Daily feed intake (g/day)	Initial body weight (kg)	Final body weight (kg)	Average daily weight gain (g)	Feed to gain ratio	Mortality and culling (%)
1	0-0-0	514	7.1	21.0	331	1.57[a]	1.0
	1,500-100-100	517	7.1	22.3	357	1.46[b]	4.2
3	0-0-0	648	8.2	25.0	373	1.74[a]	1.2
	1,500-100-100	663	8.0	26.1	400	1.67[b]	1.2

[a,b]: Mean values within a trial and within a column with a different superscript are significantly different $p < 0.05$.

3.3.3.1. Conclusions on efficacy

The Panel concludes that the product tested has the potential to be efficacious in chickens for fattening when added to feed at 1,500 EPU, 100 CU and 100 XGU per kg feed. This conclusion can be extended for chickens reared for laying and breeding and extrapolated to all growing poultry and ornamental birds. Due to the lack of sufficient data, the Panel is not in a position to conclude on the efficacy in laying hens and weaned piglets.

The test items used showed ratios of enzymes that would allow conclusions on the additive as per specifications. However, those would not allow conclusions for the batches used in the characterisation.

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[61] and Good Manufacturing Practice.

[59] Technical dossier/Section IV/Annex IV_7.
[60] Technical dossier/Section IV/Annex IV_9.
[61] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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4. Conclusions

The information regarding the production strain does not allow to confirm its taxonomic identification as *Trichoderma citrinoviride*.

The Panel concludes that the batches used for the characterisation of the final formulations showed compliance with the minimum specifications of the additive in terms of enzyme activities but showed ratios between the enzymes lower than the ones specified for the additive.

The FEEDAP Panel considers that the below conclusions apply to the final formulations containing > 15,000 EPU, > 1,000 CU and > 1,000 XGU per gram and with a xylanase:glucanase, xylanase: xyloglucanase and glucanase:xyloglucanase ratios of 15, 15 and 1, respectively:

- The additive is safe for poultry species, ornamental birds and piglets (weaned and suckling) at 1,500 EPU, 100 CU and 100 XGU/kg complete feed,
- The additive is safe for the consumers and the environment,
- Huvezym® neXo 100 G is not skin corrosive nor eye irritant but should be considered a potential skin sensitiser. Huvezym® neXo 100 L is not skin corrosive nor sensitiser and it is not an eye irritant. Due to lack of data, no conclusions can be drawn on the skin irritation of the final formulations of the additive. Due to the proteinaceous nature of the active substances, the additive is considered a respiratory sensitiser,
- The additive has the potential to be efficacious in chickens for fattening, chickens reared for laying and breeding, and all growing poultry and ornamental birds at the minimum intended level of 1,500 EPU, 100 CU and 100 XGU/kg complete feed. Owing to the lack of sufficient data, the Panel cannot conclude on the efficacy of the additive for laying hens and weaned piglets.

5. Documentation provided to EFSA/Chronology

Date	Event
17/03/2021	Dossier received by EFSA. Huvezym neXo (endo-1,4-beta-xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-beta-1,4-glucanase) for poultry and pigs. Submitted by Huvepharma EOOD.
19/04/2021	Reception mandate from the European Commission
29/07/2021	Application validated by EFSA – Start of the scientific assessment
03/11/2021	Comments received from Member States
24/11/2021	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, targets species safety, consumer safety, user safety, efficacy*
29/11/2021	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
24/06/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
25/07/2022	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, safety for the target species, efficacy*
25/09/2022	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*
03/10/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
23/11/2022	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ADFI	average daily feed intake
ADG	average daily gain
AME	apparent metabolisable energy
AMEn	nitrogen-corrected apparent metabolisable energy
ANOVA	analysis of variance
bw	body weight
CFU	colony forming unit
DM	dry matter
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
LOD	limit of detection
LOQ	limit of quantification
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin
PCDF	polychlorinated dibenzofuran
TAMC	total aerobic microbial count
TEQ	toxic equivalents
TG	Test Guideline
WHO	World Health Organization

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Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for the preparation of endo-1,4-beta-xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-1,4-beta-1,4-glucanase (Huvezym® neXo 100 G/L)

In the current application an authorisation of a *preparation of endo-1,4-beta-xylanase, endo-1,4-beta-glucanase and xyloglucan-specific-endo-1,4-beta-1,4-glucanase* is sought under Article 4 under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for poultry species, ornamental birds and piglets (weaned and suckling).

According to the Applicant, the *feed additive* contains three active substances: (i) *endo-1,4-beta-xylanase* (EC 3.2.1.8), (ii) *endo-1,4-beta-glucanase* (EC 3.2.1.4) and (iii) *xyloglucan-specific-endo-beta-1,4-glucanase* (EC 3.2.1.151), which are produced by *Trichoderma citrinoviride B-125 (DSM 33578)*.

The activity of: (i) *endo-1,4-beta-xylanase* is expressed in endo-pentosanase units (EPU), where one EPU unit is the amount of enzyme, which liberates 0.0083 micromoles of reducing sugars (xylose equivalents) from oat spelt xylan per minute at pH 4.7 and 50°C; (ii) *endo-1,4-beta-glucanase* is expressed in cellulase units (CU), where one CU unit is the amount of enzyme that liberates 0.128 micromoles of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4.5 and 30°C; and (iii) *xyloglucan-specific-endo-beta-1,4-glucanase* is expressed in xyloglucanase unit (XGU), where one XGU unit is the amount of enzyme that releases low-molecular fragments from dyed xyloglucan in amount equal to the amount of such fragments liberated from 1 unit enzyme standard under the conditions of the assay (50°C and pH 4.5). The *feed additive* is intended to be marketed as solid and liquid formulations having the following guaranteed minimum activities: 15000 EPU/g for *endo-1,4-beta-xylanase*, 1000 CU/g for *endo-1,4-beta-glucanase* and 1000 XGU/g for *xyloglucan-specific-endo-beta-1,4-glucanase*. The solid product is intended to be incorporated through *premixtures* or directly into *feedingstuffs* while the liquid formulation should be applied after the pelleting process by spraying it on the pellets to obtain a minimum content for *endo-1,4-beta-xylanase*, *endo-1,4-beta-glucanase* and *xyloglucan-specific-endo-beta-1,4-glucanase* respectively of 1500 EPU, 100 CU and 100 XGU/kg *feedingstuffs*.

For the quantification of the *endo-1,4-beta-xylanase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted single-laboratory validated and further verified colorimetric methods based on the quantification of water soluble dyed fragments produced at pH 4.7 and 50°C, by the action of *endo-1,4-beta-xylanase* on commercially available azurine cross-linked wheat arabinoxylan substrates from Megazyme.

For the quantification of the *endo-1,4-beta-glucanase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-beta-glucanase* on commercially available azurine cross-linked cellulose substrate from Megazyme.

For the quantification of *xyloglucan-specific-endo-beta-1,4-glucanase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method based on the quantification of soluble dyed labelled fragments produced at pH 4.5 and 50°C by the action of *xyloglucan-specific-endo-beta-1,4-glucanase* on commercially available xyloglucan substrate from Megazyme.

Based on the acceptable performance characteristics presented the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods submitted by the Applicant for the determination of the activity of *endo-1,4-beta-xylanase, endo-1,4-beta-glucanase* and *xyloglucan-specific-endo-beta-1,4-glucanase* 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.

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