SCIENTIFIC OPINION

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Safety and efficacy of a feed additive consisting of endo-1,3(4)-beta-glucanase produced by *Aspergillus fijiensis* CBS 589.94 (RONOZYME® VP (CT/L)) for chickens for fattening and weaned piglets (DSM Nutritional Products AG)

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of endo-1,3(4)-beta-glucanase produced by *Aspergillus fijiensis* CBS 589.94 (RONOZYME® VP (CT/L)) as a zootechnical feed additive for chickens for fattening and weaned piglets. Based on the no observed adverse effect level identified in a subchronic oral toxicity study in rats and the tolerance trials provided, the additive was considered safe for chickens for fattening and weaned piglets at the proposed conditions of use. The Panel also concluded that the use of the product as a feed additive does not rise concerns for consumers and the environment. Owing to the lack of data obtained with the final formulations, the Panel could not conclude on the potential of the additive to be irritant to skin and eyes or on its potential as a dermal sensitiser. Due to the proteinaceous nature of the active substance, the additive is considered a respiratory sensitiser. The Panel concluded that the additive is efficacious as a zootechnical additive in chickens for fattening and weaned piglets at the minimum recommended level of 10 FBG/kg feed.

Keywords: zootechnical additives, digestibility enhancers, safety, efficacy, endo-1,3(4)-beta-glucanase, chickens for fattening, weaned piglets

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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 10(2) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of seven years after the entry into force of this Regulation.

The European Commission received a request from DSM Nutritional Products AG[2] for the re-evaluation of the additive consisting of endo-1,3(4)-beta-glucanase produced by *Aspergillus fijiensis*[3] CBS 589.94 (RONOZYME® VP (CT/L)), when used as a feed additive for chickens for fattening and weaned piglets (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 10(2) (re-evaluation of an authorised 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 25 September 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 feed additive consisting of endo-1,3(4)-beta-glucanase produced by *A. aculeatus* CBS 589.94 (RONOZYME® VP (CT/L)), when used under the proposed conditions of use (see **Section 3.1.5**).

1.2. Additional information

The additive is currently authorised for use in chickens for fattening[4] and weaned piglets.[5]

2. Data and Methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[6] in support of the authorisation request for the use of endo-1,3(4)-beta-glucanase produced by *A. fijiensis* CBS 589.94 (RONOZYME® VP (CT/L)) as a feed additive.

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

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.[7]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of endo-1,3(4)-beta-glucanase produced by *A. fijiensis* CBS 589.94 (RONOZYME® VP (CT/L)) is in line with the principles laid down in Regulation (EC) No 429/2008[8] and the relevant guidance documents: Guidance

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] DSM Nutritional Products AG, represented in the EU by DSM Nutritional Products Sp. Z o.o., Poland, Tarczynska 113, 96–320, Mszczonow, Poland.
[3] Formerly identified as *Aspergillus aculeatus*.
[4] Commission Regulation (EC) No 1259/2004 of 8 July 2004 concerning the permanent authorisation of certain additives already authorised in feedingstuffs. OJ L 239, 9.7.2004, p. 8.
[5] COMMISSION REGULATION (EC) No 1811/2005 of 4 November 2005 concerning the provisional and permanent authorisations of certain additives in feedingstuffs and the provisional authorisation of a new use of an additive already authorised in feedingstuffs. OJ L 291, 5.11.2005, p. 12.
[6] Dossier reference: FAD-2010-0194.
[7] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2010-0194_en
[8] 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.

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 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) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The endo-1,3(4)-beta-glucanase (EC 3.2.1.6; glucanase) produced with *A. fijiensis* (CBS 589.94; formerly identified as *Aspergillus aculeatus*), herein and after named as Ronozyme® VP, is subject to its re-evaluation as a zootechnical additive in feed for chickens for fattening and weaned piglets (functional group: digestibility enhancers).

3.1. Characterisation

3.1.1. Characterisation of the additive

The glucanase present in the additive is produced with a non-genetically modified strain of the fungal species *A.fijiensis* (declared and deposited as *Aspergillus aculeatus*). The strain was deposited at the Centraalbureau voor Schimmelcultures with the accession number CBS 589.94.[9]

The taxonomic identification of the strain was done by phylogenetic analysis [redacted].[10] The data allowed to identify the production strain CBS 589.94 as *A. fijiensis*, a species within the *Aspergillus aculeatus* clade (*Nigri* section).

The species to which the production strain belongs is known to produce secalonic acid and the production strain has been shown to be capable to produce it.[11] The applicant provided analysis of the content of secalonic acid in the final additive (see Section 3.1.3).

3.1.2. Manufacturing process[12]

The enzyme present in the additive is produced by fermentation with the production strain. [redacted]

The applicant states that no antimicrobial substances are used in the manufacturing process.

3.1.3. Characterisation of the additive

The solid formulation, RONOZYME® VP (CT), contains enzyme concentrate (7% as dry matter), dextrin (4%), kaolin (8%), cellulose (7%), palm oil (7%), calcium carbonate (9%), sodium sulphate (57.7%) and water (0.3%). This formulation ensures a minimum enzyme activity of 50 FBG[13]/g of product. The batch-to-batch variation was studied in five batches and the mean value was 58.7 FBG/g product, ranging from 57.4 to 60.4 FBG/g.[14] The mean particle size measured in three batches by laser diffraction was 540 μm, with less than 0.5% of particles below 250 μm.[15] The dusting potential

[9] Technical dossier/Section II/Annex 2.22 and supplementary information September 2021/Annex 1c.
[10] Technical dossier/Supplementary information February 2022 and related references.
[11] Technical dossier/Section II/Annex 2.23 and supplementary information July 2021/Appendix 1d.
[12] Technical dossier/Section II/Supplementary information July 2021 – February 2022 and May 2022.
[13] 1 fungal beta-glucanase unit (FBG) is the amount of enzyme, which under standard conditions (pH 5.0 and 30 °C) liberates glucose or other reducing carbohydrates at a rate correponding to 1 micromol glucose per minute.
[14] Technical dossier/Section II/Annex 2.2.
[15] Technical dossier/Section II/Annex 2.21.

measured in three batches by Heubach method I was negligible (< 1 mg dust in 60 g product), the applicant provided further data for three more batches which showed a dusting potential ranging from 8 to 24 mg/m^3.[16] The bulk density of the product is of 1,100 kg/m^3.[15]

The liquid formulation, RONOZYME® VP (L), is based on the enzyme concentrate (11% as dry matter), sucrose (23%), potassium sorbate (0.1%), sodium chloride (10%) and water (55.9%). This formulation ensures a minimum enzyme activity of 120 FBG/mL of product. The batch to batch variation of this formulation was studied in five batches and the mean value was 136.5 FBG/mL, ranging from 135.2 to 138.7 FBG/mL.[14] This form of the additive has a viscosity of approximately 7 mPa·s at 20°C and a surface tension of 44 dyn/cm^2.[17]

Three batches of each of the two formulations were analysed for chemical and microbiological impurities.[18] The analysis of the chemical contamination included total heavy metals (< 15 mg/kg and <5 mg/L), lead (< 2 mg/kg and < 0.5 mg/L), and arsenic (< 2 mg/kg and < 0.1 μg/L). Microbial analysis included total coliform bacteria (< 10 Colony Forming Units (CFU)/g), total viable counts (up to 18,000 CFU/g in the solid < 100 CFU/mL in the liquid), *Escherichia coli* (not detected in 25 g) and *Salmonella* spp. (not detected in 25 g). The presence of secalonic acid is checked in all production batches and the applicant provided data on a total of 13 batches for each formulation in which secalonic acid was not detected.[19]

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

No antimicrobial activity was detected in three batches of each formulation.[20]

Three batches of an intermediate product, representative of the final formulations,[21] were analysed in triplicate for the presence of viable cells of the production strain. For each sample, 10 mL were diluted with 90 ml of saline water and 10 mL were passed through a 0.45-μm filter. The filter was then cultured onto potato dextrose agar plates. A positive control with the production strain was also included. The plates were incubated at 26°C for 4 days (5 days used for the optimisation). No growth was observed in the samples tested and the positive controls performed as expected. Therefore, viable forms of the production strain were not detected.

3.1.4. Stability and homogeneity

The shelf-life of Ronozyme® VP (CT and L) was assessed (in three batches for each formulation) when stored in closed glass vials at 10 or 25°C for up to 104 weeks or at 40°C for up to 13 weeks.[22] For the solid formulation, the enzyme activity loss was below 10% of the initial ones when stored for 104 weeks at 10 and 25°C or after 13 weeks stored at 40°C. For the liquid formulation, the enzyme activity loss was below 10% of the initial ones when stored at 10 and 25°C for 52 weeks but decreased to about 80% after 104 weeks. The samples stored at 40°C showed losses of 20% after 13-week storage.

Three batches of the solid formulation were mixed with two different premixtures (one with choline chloride and the other one without) at 1,250 FBG/kg and samples were stored for 6 months at 25°C.[23] The enzyme activity loss after 6 months were 2% and 27% for the premixture with choline chloride and the one without choline chloride, respectively.

The stability of Ronozyme® VP (CT) to pelleting was studied in three batches by adding the additive at 20 FBU/kg feed to a feed for poultry.[24] Pelleting temperature was 75°C, no activity losses were observed. The stability of the enzyme in mash/pelleted feed was studied in the same feeds (mash or pelleted) when stored in closed vials at 25 or 35°C for up to 3 months. Losses of the initial enzyme activity in samples stored at 25°C were 15% for pellets and 4% for mash, the corresponding values for samples stored at 35°C were 30 and 19%.

The stability of Ronozyme® VP (L) in pelleted feed was studied (three batches) by adding the additive at 20 FBU/kg feed to a pelleted feed for poultry which was stored in closed vials at 25 or 35°C

[16] Technical dossier/Section II/Annex 2.2. and Supplementary information July 2021/Annex 3 and February 2022/Annex 3.1.
[17] Technical dossier/Section II/Annex II.1.5.4.
[18] Technical dossier/Section II/Annex 2.5.
[19] Technical dossier/Section II/Annex 2.5 and 2.2 and supplementary information July 2021/Annex 5 [redacted]
[20] Technical dossier/Section II/Annex 2.12.
[21] Technical dossier/Supplementary information July 2021/Annex 4b and Supplementary information February 2022 and May 2022.
[22] Technical Dossier/Section II/Appendix 2.34 and 2.35.
[23] Technical dossier/Supplementary information July 2021/Annex 3.
[24] Technical dossier/Section II/Annex 2.36 and 2.37.

for up to 13 weeks.[25] Losses of the initial enzyme activity in samples stored at 25°C was 19% and at 35°C was 25%.

The capacity to homogeneously distribute of the enzyme was studied for the CT formulation when added to feed, mash and pelleted feeds described above. The analysis of 8 subsamples of each feed showed coefficients of variation up to 13% in the mash feed and up to 19% in the pelleted feed.

3.1.5. Conditions of use

The additive is proposed to be used in feed for chickens for fattening and weaned piglets at a level ranging from 10 to 20 FBG/kg feed.

3.2. Safety

3.2.1. Toxicological studies

In the bacterial reverse mutation assay, *in vivo* chromosome aberration test and subchronic oral toxicity study the test product was in solid form [redacted], while in the *in vitro* micronucleus test the test product was liquid and showed [redacted]. Therefore, the Panel concludes that all the batches tested in the toxicological studies can be considered representative of the fermentation product used in the final formulations of the additive.

3.2.1.1. Bacterial reverse mutation assay

In order to investigate the potential of the fermentation product to induce gene mutations in bacteria, an Ames test was performed according to OECD Test Guideline (TG) 471 (1983) and following Good Laboratory Practice (GLP) in four strains of *Salmonella* Typhimurium (TA98, TA100, TA1535, TA1537) and *Escherichia coli* WP2*uvr*A, in the presence or absence of metabolic activation applying the plate incorporation method.[26] Five enzyme concentrations were tested from 0.1 to 10 mg/mL; positive and negative controls were included. 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 increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains with or without S9-mix. The test item did not induce gene mutations in the bacterial reverse mutation assay under the test conditions employed for this study.

3.2.1.2. *In vivo* chromosome aberration test

In order to investigate the potential of the test item to induce chromosome aberrations *in vivo*, a bone marrow chromosomal aberration test was conducted in CD rats.[27] A preliminary toxicity test was performed, and no clinical signs of toxicity or animal deaths were observed at 5,000 mg/kg b.w. On this basis, the animals (5 males and 5 females per dose and sampling time) were treated orally with 0, 500, 1,600 and 5,000 mg/kg per day and bone marrow was sampled at 6, 24 and 48 h after treatment. Fifty metaphase cells per animal were scored for the analysis of chromosome aberrations. Cyclophosphamide (40 mg/kg body weight (bw)) was given as the positive control and induced a statistically significant increase of chromosome aberrations. No significant increase of chromosome aberrations was observed in the groups treated with the test item compared to the negative controls at any harvest time and with any dose level used. However, the Panel notes that a low number of cells was analysed per animal and no evidence of exposure of the bone marrow was provided and, therefore, considered the results obtained of limited validity.

3.2.1.3. *In vitro* micronucleus test

To evaluate the potential to induce chromosomal damage of the test item, an *in vitro* micronucleus test was carried out in whole blood human lymphocytes according to OECD Test Guideline 487 (2010) and following GLP. In the report, test item concentrations were expressed in terms of total organic solids ([redacted]). Based on a preliminary cytotoxicity test, the concentrations selected for the analysis of micronuclei ranged from 500 to 5,000 μg TOS/mL for the

[25] Technical dossier/Section II/Annex 2.38.
[26] Technical dossier/Section III/Annex 3.3.
[27] Technical dossier/Section III/Annex 3.4.

short treatment (3 + 21 h of recovery) in the presence and absence of metabolic activation and 200 to 700 μg TOS/mL for the continuous treatment (24 + 0 h of recovery) in the absence of metabolic activation. Cytochalasin B was used to obtain binucleated cells. A concentration-related increase in cytotoxicity was observed in all the experimental conditions, up to 67% after short treatment in the presence of metabolic activation. No significant increase in the frequency of micronuclei was induced by treatment with the test item. Exception was observed at the intermediate concentration (3,000 μg TOS/mL) after short treatment in the presence of metabolic activation where a statistically significant increase in the frequency of micronuclei was recorded. The increase was not concentration-related and the value was within the historical negative control range; therefore, the FEEDAP Panel considered that it was not biologically relevant. The test item did not induce structural and numerical chromosome aberrations in human lymphocytes both in the presence and absence of metabolic activation.

3.2.1.4. Subchronic oral toxicity study

In a study conducted in accordance with OECD TG 408, groups of 20 Wistar rats of each sex, caged in groups of two and given dietary concentrations of 0, 5,000, 15,000 or 50,000 mg/kg (representing 0, 4,750, 14,250 or 47,500 FBG/kg feed). Enzyme activities were confirmed by analysis to be within the expected range. Throughout the experimental period, the animals were fed *ad libitum* and observed at least once daily. The body weight of the rats was recorded weekly throughout the study and food intake was measured continuously. Water consumption was measured over three 5-day periods, during weeks 1, 6 and 11 of the study. During the last week of the study, urinalysis was conducted and blood samples were taken from 10 animals of each sex from each treatment group for haematological and biochemical analyses. All rats were necropsied and samples were taken and organ weights recorded. Microscopic examination was confined to the control and high-dose groups, apart from the kidneys, which were examined for all groups of females. Among treated females a dose-related increase in nephrocalcinosis accompanied by an increase in inflammatory foci was observed. The test item contained 3.6% phosphorus and 0.05% calcium. Increased amounts of the test item resulted in lower Ca:P ratios in the diet. Notwithstanding the different Ca:P ratios in the different batches of the basal diet, the relative decrease of the Ca:P ratio, within a group level and compared to control, was similar at all different time intervals. The addition of the test item resulted in a dose-related increase in the incidence of renal calculi in female rats which is considered to be related to a dietary imbalance (Ca/P) resulting from the addition of the test item. Since no other adverse effects have been observed, the highest dose tested was considered as a no observed adverse effect level. The NOAEL is determined to be 50,000 mg test item/kg feed, resulting in 4,500 mg/kg bw per day and day or 4,275 FBG/kg bw per day.

3.2.1.4.1. Conclusion on toxicology

The FEEDAP Panel concludes that the fermentation product showed no genotoxicity potential in tests addressing gene mutations, numerical and structural chromosome aberrations. Moreover, the results obtained in a subchronic oral toxicity study raised no concerns regarding the product and allowed to derive a NOAEL of 4,275 FBG/kg bw per day, the highest dose tested.

3.2.2. Safety for the target species

The applicant referred to the results in the subchronic oral toxicity study and submitted two tolerance trials.

3.2.2.1. Calculation of the maximum safe level in feed

The subchronic oral toxicity study in rats has been described in Section 3.2.1.4. The NOAEL identified (4,275 FBG/kg bw and day) was used to calculate the maximum safe level in chickens for fattening and piglets in accordance with the procedure described in the Guidance on the safety for the target species (EFSA FEEDAP Panel, 2017b). The calculated maximum safe level for chickens for fattening is 476 FBG/kg feed and for weaned piglets is 855 FBG/kg complete feed.

3.2.2.2. Chickens for fattening

A total of 1,600 one-day-old male chickens for fattening (Ross 308) were distributed in 32 pens in groups of 50 animals and allocated to four dietary treatments (8 replicates per treatment).[28] Two basal diets (starter, from day 1 to 21; and grower, from day 22 to 35) based on barley and soya bean meal were either not supplemented (control) or supplemented with Ronozyme® VP (CT) to provide 10 (0.5× maximum recommended level), 20 (1×) or 200 (10×) FBG per kg feed. The enzyme activity in feed was analytically confirmed. The experimental diets were offered *ad libitum* in pelleted form for 35 days. Mortality and health status were checked daily, and the most probable cause of death was recorded. The birds were weighed at the start of the trial (day 1). Thereafter, body weight and feed intake were recorded at days 22 and 35 and average daily gain, average daily feed intake and feed to gain ratio were calculated and corrected for mortality. On day 35, blood samples were obtained from two birds per pen for haematology[29] and blood biochemistry analysis.[30] The data were analysed with analysis of variance (ANOVA), considering the treatment and the block (location in the house) as the main effects, and the group means were compared with Duncan's multiple range test. Significance level was set at 0.05.

Mortality registered during the study was on average 6.5% and no differences were observed between the treatments. The inclusion of the additive in the diet of chickens for fattening from 0.5× maximum recommended level reduced the feed to gain ratio in comparison with the control diet (1.73, 1.65, 1.67 and 1.62 for the control, 0.5×, 1× and 10×, respectively). No other differences between treatments were observed in any other zootechnical parameter (results for control group: mean daily feed intake 105 g; mean final body weight 2,174 g; and average daily gain 61.0 g). No differences were observed in any of the blood haematological parameters analysed. Regarding the blood biochemistry, protein and albumin were lower in the 1× and 10× groups compared to control (protein: 3.64 and 3.76 vs 4.14 g/dL; albumin 1.27 and 1.31 vs 1.41 g/dL) and uric acid in the 1× was lower compared to the control (3.96 vs 5.12 mg/dL). These differences in the biochemical parameters were considered not biologically relevant. Therefore, feeding the birds with 10× the maximum recommended level did not show adverse effects on the zootechnical performance and the blood parameters measured.

3.2.2.3. Safety for weaned piglets

A total of 180 crossbred[31] weaned piglets (females and castrated males; ca. 27 days of age; initial body weight = 7.7 kg) were distributed in 30 pens in groups of 6 animals and allocated to three dietary treatments (10 replicates per treatment).[32] A basal diet based on wheat, barley and soya bean meal was either not supplemented (control) or supplemented with Ronozyme® VP (CT) to provide 10 (0.5× maximum recommended dose) or 2,500 (125×) FBG per kg feed. The enzyme activity in feed was confirmed by analysis. The experimental diets were offered *ad libitum* in pelleted form for 42 days. Mortality and health status were checked daily, and the most probable cause of death was recorded. The animals were weighed at the start of the trial (day 1). Thereafter, body weight and feed intake were recorded on days 14 and 42 and the average daily feed intake, average daily gain and feed to gain ratio calculated. The faecal consistency was scored (1 = liquid stools; 10 = hard and dry stools) twice a week on a pen basis. The data were analysed with an ANOVA, considering the treatment and the block (location in the house) as the main effects, and the group means were compared with Duncan's multiple range test.[33] Significance level was set at 0.05.

No mortality occurred throughout the experiment. The inclusion of up to 125× maximum recommended level of Ronozyme® VP (CT) showed improvements in the final body weight, average daily weight gain and feed to gain ratio compared to control and 0.5×. Final body weights were 26.3, 26.4 and 27.3 kg for control, 0.5× and 125×. The corresponding values for average daily gain were 441, 443 and 465 g and for feed to gain 1.65, 1.66 and 1.63.

[28] Technical dossier/Section III/Appendix_3.2 and Supplementary information July 2021/appendix 7. Study designed in compliance with the guidance in force at the time of submission of the application.
[29] Haematocrit, haemoglobin, mean corpuscular volume, mean corpuscular haemogloin, mean corpuscular haemoglobin concentration, erythrocytes.
[30] Serum aspartate amino transferase, alanine aminotransferase, gamma glutamin transpeptidase, uric acid, albumin and total protein.
[31] Taht × (GYz × Finnish Landrace).
[32] Technical dossier/Section III/Appendix_3.1.
[33] The statistical output was not provided by the applicant.

Therefore, feeding the weaned piglets with 125× the maximum recommended dose did not show adverse effects on the zootechnical parameters measured.

3.2.2.4. Conclusions on safety for the target species

Based on the results of the sub-chronic toxicity study, the calculated maximum safe concentration of the additive in feed would correspond to 476 FBG/kg feed for chickens for fattening and to 855 FBG/kg feed for weaned piglets. The results of the two tolerance studies showed that 200 FBG per kg feed and 2,500 FBG per kg feed were tolerated by chickens for fattening and weaned piglets, respectively. Therefore, the FEEDAP Panel concludes that the additive is safe at the highest level recommended of 20 FBG/kg feed.

3.2.3. Safety for the consumer

The results obtained with the fermentation product, considered representative of the product used to formulate the additive, in the genotoxicity studies and the subchronic oral toxicity study, do not indicate any reason for concern for consumer safety arising from the use of the product as a feed additive.

3.2.4. Safety for the user

3.2.4.1. Effects on respiratory system

No specific studies were provided by the applicant regarding the toxicity of the additive on the respiratory system. The solid formulation has a low dusting potential (highest value was 24 mg/m^3) and therefore the exposure is expected to be low. However, the additive is assumed to be a respiratory sensitiser.

3.2.4.2. Effects on skin and eyes

Two *in vivo* studies were conducted to test the potential of the fermentation product used for the formulation of the additive to be irritant to skin and eyes.[34] Each study was conducted with six rabbits which showed that the test item tested was not irritant to skin or eyes. The applicant requested also to consider the CLP classification of the ingredients; the enzyme protein should be classified as a respiratory sensitiser and potassium sorbate as an eye irritant.[35] No data was submitted regarding the skin sensitisation potential.

Owing to the lack of data with the final formulations the FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin or eyes or on its potential to be a dermal sensitiser.

3.2.4.3. Conclusions on safety for the user

The FEEDAP Panel cannot conclude on the potential of the additive to be irritant to skin/eyes or its potential to sensitise the skin. Owing to the nature of the active substance the additive is considered a respiratory sensitiser.

3.2.5. Safety for the environment

The active substance is a protein; thus, it will be degraded/inactivated during the passage through the digestive tract of animals or in the environment. Therefore, no risks to the environment are expected when the additive is used in chickens for fattening and weaned piglets.

3.3. Efficacy

3.3.1. Efficacy for chickens for fattening

Three trials, including the tolerance-efficacy trial described above in Section 3.2.2.2, were submitted to support the efficacy of the additive in chickens for fattening. The three studies shared a common experimental design; the details on the study design are provided in Table 1 and the main results in Table 2. In all three trials, the basal diets (starter, from day 1 to 21; grower, from day 22 to 35) were either non-supplemented (control) or supplemented with Ronozyme VP (CT) to provide 10 or

[34] Technical dossier/Section II/Annexes 3.9 and 3.10.
[35] Technical dossier/Supplementary information February 2022.

20 FBG/kg complete feed. The enzyme activity of the diets was confirmed by analysis. The experimental diets were offered *ad libitum* for 35 days.

Mortality and health status were monitored daily, and the most probable cause of death was recorded. The birds were weighed at the start of the trial (day 1). Thereafter, body weight and feed intake were recorded at days 21 and 35 and average daily gain, average daily feed intake and feed to gain ratio were calculated and corrected for mortality. The experimental data were analysed with ANOVA, considering the treatment and the block (location in the house) as the main effects, and the group means were compared with Tukey's (trial 1) or Duncan's multiple range test (trials 2 and 3). Significance level was set at 0.05.

Table 1: Trial design and analysed enzyme activities of the diets of the efficacy trials performed in chickens for fattening

Trial	Total No of animals (animals × replicate) replicates × treatment	Breed sex (duration - days)	Composition feed (Form)	Groups (FBG/kg feed)	
				Intended	Analysed
1[36]	2,160 (60) 12	Ross 308 50%♀:50%♂ 35	Maize, lupins and soya bean meal (mash first week then pellet)	0 10 20	4/2 10/12 17/18
2[37]	1,200 (50) 8	Ross 308 Males 35	Barley and soya bean meal (pelleted)	0 10 20	Below LOQ 14.1/8.9 22.3/20.4
3[38]	600 (25) 8	Ross 308 Males 35	Barley and soya bean meal (pelleted)	0 10 20	Below LOQ 10.5/17.0 22.6/23.4

Mortality was within the normal ranges in all trials with no differences between the treatments. In all trials, the inclusion of the additive in the birds' diet from the minimum use level (10 FBG/kg feed) showed lower feed to gain ratio in comparison with the control. No significant differences were observed in any other zootechnical parameter measured with the exception of a lower feed intake in trial 1 for the group receiving 20 FBG/kg feed.

Table 2: Effects of Ronozyme® VP on the zootechnical performance of chickens for fattening

Trial	Groups (FBG/kg feed)	Daily feed intake (g)	Final body weight (g)	Average daily weight gain (g)	Feed to gain ratio	Mortality (%)
1	Control	85.2^{a}	1,893	53.0	1.61^{a}	2.98
	10	83.7^{a,b}	1,878	52.5	1.60^{b}	3.51
	20	83.5^{b}	1,889	52.8	1.58^{c}	2.48
2	Control	105.4	2,174	61.0	1.73^{a}	5.42
	10	104.0	2,245	63.0	1.65^{b}	5.41
	20	105.3	2,250	63.2	1.67^{b}	6.41
3	Control	105.3	2,156	60.3	1.75^{a}	2.59
	10	104.8	2,201	61.6	1.70^{b}	0.50
	20	104.8	2,212	61.9	1.70^{b}	4.06

a,b,c: Mean values within a trial and within a column with a different superscript are significantly different $P < 0.05$.

3.3.2. Efficacy for weaned piglets

A total of five long-term trials were submitted by the applicant to support the efficacy of the additive on weaned piglets. All trials addressed the effect of the dietary supplementation of the additive in the zootechnical performance of the piglets. Out of the five, two studies were not further considered due to the short duration[39] or the high mortality during the study (overall 9%).[40]

[36] Technical dossier/Section IV/Annex 4.1 and supplementary information July 2021/appendixes 10, 12a and 12a1.
[37] Technical dossier/Section IV/Annex 4.2 and supplementary information July 2021/Appendix 12b.
[38] Technical dossier/Section IV/Annex 4.3 and supplementary information July 2021/Appendix 11 and 12c.
[39] Technical dossier/Section IV/Annex 4.4.
[40] Technical dossier/Section IV/Annex 4.5.

The other three trials followed a similar design. The details are provided in Table 3 and the main results in Table 4. In all cases, the basal feeds (pre-starter from 1 to 14 days; starter from 15 to 42 days) were either not supplemented (control) or supplemented with the additive to provide 10 FBG/kg complete feed. The experimental feeds were offered *ad libitum* for 42 days and the enzyme activities were confirmed by analysis.

Mortality and health status were checked daily throughout all trials, and the most probable cause of death was recorded. The piglets were weighed at the start of the trial (day 1). Thereafter, body weight and feed intake were recorded every 2 weeks (trials 1 and 3) until the end of the experiment (day 42), or at days 14 and 42 (trial 2). The average daily gain, average daily feed intake and feed to gain ratio were calculated and corrected for mortality. The experimental data were analysed with ANOVA, considering the treatment, sex (trials 2 and 3), block (trials 2 and 3) and run (trial 3) as fixed effects. The group means were compared with Duncan's multiple range test (trial 1) or Tukey's (trial 3). Significance level was set at 0.05.

No mortality (including culling) was reported in any trial, except for the control group in trial 1, which showed 3.2%. In all cases, the inclusion of the additive in the diet of weaned piglets at the minimum recommended use level (10 FBG/kg complete feed) showed lower feed to gain ratio in comparison with the control. In trial 1, also higher final body weight and average daily gain were observed, while trials 2 and 3 showed no other significant difference in any performance parameters.

Table 3: Trial design and analysed enzyme activities of the diets of the efficacy trials performed in weaned piglets

Trial	Total No of animals (animals × replicate) replicates × treatment	Breed Sex (duration)	Composition feed (form)	Groups (FBG/kg feed)	
				Intended	Analysed
1[41]	274 (10–15) 18/22	(Duroc × Piétrain) × (Landrace × Large White) 51%♀:49%♂ 42 days	Barley, wheat and soya bean meal (mash)	0 10	< 5/0.005 9.5
2[42]	144 (4) 18	(Duroc × LW) × Piétrain 45%♀:55%♂ 42 days	Barley, soya bean meal and rapeseed meal (pelleted)	0 10/10	LOD 7.9/13.1
3[43]	120 (1) 60	Large White 50%♀:50%♂ 42 days	Barley, soya bean meal and rapeseed meal (pelleted)	Control – 0 Pre-st / St – 10/10	LOQ 9.8/10.6

Table 4: Effects of Ronozyme® VP on the zootechnical performance of weaned piglets

Trial	Groups (FBG/kg feed)	Daily feed intake (g)	Initial Body weight (kg)	Final body weight (kg)	Average daily weight gain (g)	Feed to gain ratio	Mortality and culling (%)
1	Control	731	7.9	23.1[b]	360[b]	2.04[a]	3.5
	10	741	8.0	25.5[a]	416[a]	1.78[b]	0
2	Control	569	7.9	22.8	355	1.61[a]	0
	10	537	7.9	22.9	357	1.51[b]	0
3	Control	1,009	8.0	34.5	632	1.60[a]	0
	10	1,011	8.0	35.3	650	1.56[b]	0

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

[41] Technical dossier/Section IV/Annex 4.6 and suplementary information July 2021/Appendixes 13 and 13b.
[42] Technical dossier/Section IV/Supplementary information July 2021/Annex Pa.
[43] Technical dossier/Section IV/Supplementary information July 2021/Annex Pb.

3.3.2.1. Conclusions on efficacy

Based on the results obtained in the efficacy trials in chickens for fattening and weaned piglets the FEEDAP Panel concludes that the additive is efficacious as a zootechnical additive at the level of 10 FBG/kg feed.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[44] and Good Manufacturing Practice.

4. Conclusions

The additive is safe for chickens for fattening and weaned piglets at the maximum recommended level of 20 FBG/kg feed.

The use of Ronozyme® VP in feed for chickens for fattening and weaned piglets is of no concern for consumer safety.

The use of the additive as a feed additive in chickens for fattening and weaned piglets is considered safe for the environment.

The Panel cannot conclude on the potential of the additive to be an irritant to skin or eyes or on the dermal sensitisation potential. Owing to the proteinaceous nature of the active substance, the additive is considered a respiratory sensitiser.

The Panel concludes that the additive is efficacious as a zootechnical additive for chickens for fattening and weaned piglets at 10 FBG/kg feed.

5. Documentation provided to EFSA/Chronology

Date	Event
05/11/2010	Dossier received by EFSA. Ronozyme VP for chickens for fattening and piglets. Submitted by DSM Nutritional Products Sp. z o.o.
19/08/2019	Reception mandate from the European Commission
25/09/2019	Application validated by EFSA – Start of the scientific assessment
29/11/2019	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 of analysis, characterisation of the additive, safety and efficacy*
29/12/2019	Comments received from Member States
11/11/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
22/11/2021	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
18/11/2021	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, safety and efficacy*
21/02/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
06/04/2022	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: safety*
10/06/2022	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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[44] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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Abbreviations

ANOVA	analysis of variance
bw	body weight
CFU	colony forming unit
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
NOAEL	no observed adverse effect level
OECE	Organisation for Economic Co-operation and Development
TG	Test Guideline
TOS	total organic solids

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 Ronozyme® VP (CT/L)

Ronozyme® VP is currently authorised as feed additive for chickens for fattening and piglets (weaned) by Commission Regulation (EC) No 1259/2004 and Commission Regulation (EC) No 1811/2005 respectively. In the current application authorisation is sought under Article 10 (2) for Ronozyme® VP under the category/functional "zootechnical additives"/"digestibility enhancers". The authorisation is sought for the use of the feed additive for chickens for fattening and piglets (weaned).

According to the Applicant, the active agent of Ronozyme® VP is endo-1,3(4)-beta-glucanase (glucanase) produced by Aspergillus aculeatus. The Applicant expressed the glucanase enzymatic activity in fungal beta-glucanase units (FBG), where one FBG is defined as "the amount of enzyme which under standard conditions (pH 5.0 and 30°C) liberates glucose or other reducing carbohydrates at a rate corresponding to 1 μmol glucose per minute".

The product is intended to be marketed as solid (CT) and liquid (L) formulations having a guaranteed minimum glucanase activity of 50 FBG/g and 120 FBG/mL respectively. The feed additive formulations are intended to be included through premixtures (solid) or directly in feedingstuffs (solid and liquid) to obtain a minimum activity of 10 FBG/kg feedingstuffs.

For the quantification of the glucanase activity in the feed additive the Applicant provided a single-laboratory validated and further verified colorimetric method. Glucanase cleaves non-starch polysaccharides (NSP) releasing glycosylic moieties with reducing ends from beta-glucan. The reducing moieties are oxidized in an alkaline milieu by forming orange-yellow compounds with the 2-hydroxy-3,5-dinitrobenzoic acid. These orange-yellow compounds are measured at a wavelength of 530 nm and quantified against a validated Ronozyme® VP standard available from the Applicant upon request.

For the quantification of the glucanase activity in premixtures and feedingstuffs the Applicant proposes a single-laboratory validated and further verified colorimetric method based on the quantification of the water soluble dyed fragments produced by the action of endo-1,3(4)-beta-glucanase on azo-barley beta-glucan substrate. The quantification of the glucanase activity is determined by using a standard curve of a certified Ronozyme® VP glucanase standard available from the Applicant upon request.

Based on the satisfactory performance characteristics the EURL recommends for official control the colorimetric methods mentioned above for the quantification of the glucanase 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.