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Safety and efficacy of the feed additive consisting of protease produced by *Bacillus licheniformis* DSM 33099 (ProAct 360) for use in poultry species for fattening or reared for laying/breeding (DSM Nutritional Products Ltd)

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Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of a protease (ProAct 360) produced by a genetically modified strain of *Bacillus licheniformis* (DSM 33099) as a zootechnical feed additive for poultry species for fattening or reared for laying/breeding. The production strain and its recombinant DNA were not detected in an intermediate concentrated product representative of the final formulation. The final product did not trigger a safety concern with regard to the genetic modification. The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) concluded that ProAct 360 is considered safe for all growing poultry species at the recommended inclusion level of 30,000 NFP/kg complete feed. The use ProAct 360 as a feed additive did not give rise to concerns for the consumers or the environment. The additive is not an eye or a dermal irritant but should be considered a respiratory sensitiser. In the absence of data, no conclusions could be reached on the skin sensitisation potential of the additive. The FEEDAP Panel concluded that the additive has the potential to be efficacious at 30,000 NFP/kg complete feed for all poultry species for fattening or reared for laying/breeding.

Keywords: zootechnical additive, digestibility enhancer, protease, ProAct 360, all poultry species for fattening or reared for laying/breeding, safety, efficacy

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1. Introduction

1.1. Background and terms of reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from DSM Nutritional Products Ltd[2] for the authorisation of the additive consisting of protease produced by *Bacillus licheniformis* DSM 33099 (ProAct 360), when used as a feed additive for all poultry species for fattening or reared for laying/reproduction[3] (category: zootechnical additives; functional group: digestibility enhancers).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 18 March 2022.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the feed additive consisting of protease (EC 3.4.21.62) produced by *Bacillus licheniformis* DSM 33099 (ProAct 360), when used under the proposed conditions of use (see **Section 3.1.5**).

1.2. Additional information

The subject of the assessment is the feed additive consisting of a protease produced by *Bacillus licheniformis* DSM 33099 (ProAct 360). It has not been previously 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[4] in support of the authorisation request for the use of the product consisting of a protease produced by *Bacillus licheniformis* DSM 33099 (ProAct 360) as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed.[5]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of active substance (trade name of the product) is in line with the principles laid down in Regulation (EC) No 429/2008[6] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c),

[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 Ltd, represented in the EU by Nutritional Products Sp. z.o.o., Wurmisweg 576, 4303, Kaiseraugst, Switzerland.
[3] Technical dossier/Annex I and Supplementary information June 2023.
[4] FEED dossier reference: FAD-2021-0025.
[5] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en
[6] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

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

This opinion assesses the safety and efficacy of the product that contains a protease (IUBMB EC 3.4.21.62) produced by *Bacillus licheniformis* DSM 33099 as a zootechnical additive (functional group: digestibility enhancers) for all poultry species for fattening or reared for laying/reproduction. It will be hereafter referred to as ProAct 360.

3.1. Characterisation

3.1.1. Characterisation of the production organism

The active substance is a protease produced by a genetically modified strain of *Bacillus licheniformis* which is deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) with accession number DSM 33099.[7]

[redacted] confirmed its identity as *B. licheniformis*. [redacted][8]

The susceptibility of the production strain was tested against the list of antibiotics recommended for *Bacillus* by the FEEDAP Panel (EFSA FEEDAP Panel, 2018b).[9] [redacted] Therefore, *B. licheniformis* DSM 33099 is considered susceptible to all the tested antibiotics, but resistant to clindamycin.

The WGS-based data of the production strain were interrogated for the presence of antimicrobial resistance (AMR) genes [redacted][10] No hits of concern were found [redacted] Although the strain was resistant to clindamycin, this resistance raises no safety concerns since no acquired AMR genes were found in the WGS.

[redacted] No lysis of Vero cells was detected, so *B. licheniformis* DSM 33099 is considered to be not toxigenic.[11] [redacted]

3.1.1.1. Information related to the genetically modified microorganism

Characterisation of the parental microorganism

[redacted][12]

[7] Technical dossier/Section II/Annex 2–7.
[8] Technical dossier/Section II/Annex 2–9, Annex 2–10 and Annex 2–11.
[9] Technical dossier/Section II/Annex 2–9 and Annex 2–12 and Supplementary information January 2023/Annex 2-12.
[10] Technical dossier/Section II/Annex 2–9 and Supplementary information January 2023/Annex 2–36, Annex 2–37 and Annex 2–38.
[11] Technical dossier/Section II/Annex 3–4.
[12] Technical dossier/Section II/Annex 2–9.

Characterisation of the donor sequence

[redacted][13]

Description of the genetic modification

[redacted][13]

3.1.2. Manufacturing process

[redacted][14]

3.1.3. Characterisation of the additive

ProAct 360 is a coated granulated product containing: the protease (12% w/w estimated total organic solids), sodium sulfate (45%), dextrin (7%) and cellulose (6%) as carriers and calcium carbonate (17%), hydrogenated palm oil (8%) and magnesium sulfate (4%) as coating agents. Water represents ≤ 1%.[15] The minimum guaranteed enzyme activity is 600,000 NFP[16]/g.

The batch-to-batch variation was studied in eight independent batches showing a mean enzyme activity of 730,000 NFP/g additive (range: 700,000–804,000 NFP/g).[17]

The applicant set specifications for chemical and microbiological contamination which include arsenic (≤ 3 mg/kg), lead (≤ 5 mg/kg), cadmium (≤ 0.5 mg/kg), mercury (≤ 0.5 mg/kg), total viable counts ($< 5 \times 10^4$ colony forming units (CFU)/g), total coliforms (< 30 CFU/g), *Escherichia coli* (no detection in 25 g) and *Salmonella* (no detection in 25 g). Three batches of the additive were analysed for chemical contaminants[17] and all values for arsenic, lead, mercury and cadmium fell below the limit of quantification of the analytical methods, except for two batches that showed an average cadmium concentration of 0.21 mg/kg (0.20–0.22 mg/kg).[18] Eight batches were analysed for microbial contamination and showed total viable counts of < 100 CFU/g in four batches and an average of 150 CFU/g in the remaining batches, < 10 CFU/g for coliforms and no *Escherichia coli* or *Salmonella* spp. detection in 25 g of additive. All the results showed compliance with the specifications set. Additional five batches were analysed for Enterobacteriaceae and total yeasts and filamentous fungi which showed levels < 10 CFU/g.[19]

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

Although the strain belongs to a species not expected to produce antimicrobial substances, the applicant submitted an experiment to test the antimicrobial activity of one batch of the final additive

[13] Technical dossier/Section II/Annex 2–9, Annex 2–11, Annex 2–13, and Annex 2–15 and Supplementary information January 2023/Annex 2–11_New version.
[14] Technical dosser/Section II/Annex 2–5 and Annex 2–6.
[15] Technical dossier/Section II.
[16] One NFP unit is defined as the amount of enzyme that releases ~ 1 μmol of p-nitroaniline from 1 mM substrate (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide) per minute at pH 9.0 and 37°C.
[17] Technical dossier/Section II/Annex 2–2.
[18] Limit of quantification (LOQ) for arsenic: 0.3 mg/kg, cadmium and mercury: 0.05 mg/kg, lead: 0.5 mg/kg.
[19] Technical dossier/Supplementary information January 2023/Annex 2–39.

(enzyme activity 814,500 NFP/g) according to the FEEDAP Guidance (EFSA FEEDAP Panel, 2018b).[20] No antimicrobial activity was detected.

The presence of viable cells of the production strain was analysed [redacted][21] No colonies were detected.

The presence of DNA of the production strain was analysed [redacted][22] No DNA was detected in the samples.

ProAct 360 is an off-white to light brown colour granulate with a bulk density of 1,000 kg/m^3 and a tapped density of 1,100 kg/m^3. The preparation tested by the Heubach method showed a dusting potential with a mean value (three batches) of 25 mg/m^3 (range: 21–30 mg/m^3) and ~ 80% of particles with a diameter in the range of 150–850 μm and no particles with a size < 150 μm, tested by laser diffraction.[23]

3.1.4. Stability and homogeneity

The shelf-life of ProAct 360 was evaluated in three batches when stored in glass vials sealed with metal caps for up to 19.5 months at 10, 25 and 30°C and up to 24 months at 40°C[24] and in glass vials with seals simulating the original packaging for up to 24 months at 30°C/65% relative humidity (%RH). Recoveries were expressed as % of the activity of the sample at time zero (696,204; 749,027; and 814,797 NFP/g). No or negligible losses were observed at any time or conditions, except for one batch stored at 30°C/65% RH that showed losses of 11% after 24 months.

The stability of ProAct 360 (three batches) in two vitamin–mineral premixtures (containing choline chloride) for chickens was studied when added at 3,000,000 NFP/kg and stored in plastic bags at 25°C for up to 6 months.[25] No or negligible losses were observed after 3 months while after 6 months the losses were up to 14%.

The stability of ProAct 360 (three batches) in mash feed for chickens (based on wheat and maize) was studied when added at 30,000 NFP/kg feed and stored in plastic bags at 4°C for up to 3 months.[25] Losses up to 3% were observed after 3 months. Subsamples of the same feed were subjected to pelleting at 83°C and stored at the same conditions. Losses after 3 months reached 16%.

To test the stability of ProAct 360 (four batches) to pelleting process, a mash feed for chickens (based on wheat and maize) added with the additive at 30,000 NFP/kg was subjected to pelleting at 80°C and 90°C.[25] Losses after the pelleting process were 2% in both cases.

The capacity for homogeneous distribution of the additive in a mash feed for chickens for fattening was studied in 10 subsamples. The coefficient of variation was 13%.[25]

3.1.5. Conditions of use

ProAct 360 is intended to be used in feed for all poultry species for fattening or reared for laying/reproduction at the recommended level of 30,000 NFP/kg complete feed.[3]

[20] Technical dossier/Section II/Annex 2–21 and Annex 2–22.
[21] Technical dossier/Section II/Annex 2–16, Annex 2–18 and Annex 2–20.
[22] Technical dossier/ Section II/Annex 2–16 and Annex 2–17 and Supplementary information June 2020/Annex_1.
[23] Technical dossier/Section II/Annex 2–4 and Supplementary information January 2023/DSM ProAct 360 SIn reply - Main Annex.pdf.
[24] Technical dossier/Section II/Annex 2–23.
[25] Technical dossier/Section II/Annex 2–4.

3.2. Safety

3.2.1. Safety of the production organism

The production strain *B. licheniformis* DSM 33099 [redacted] The production strain belongs to a species, *Bacillus licheniformis*, that is suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2023). [redacted] None of the introduced modifications raise a safety concern. The identity of the strain has been unambiguously established. Evidence was provided on the lack of toxigenic potential of the strain and on the absence of acquired antimicrobial resistance genes. The production strain and its DNA were not detected in [redacted] the final additive. Therefore, the final product does not give raise to any safety concern with regard to the genetically modified production strain.

3.2.2. Toxicological studies

Toxicological studies are not required for fermentation products produced by a genetically modified microorganism for which the recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment and for which the genetic modification raises no concerns. The production strain *B. licheniformis* DSM 33099 qualifies for the QPS approach and the genetic modifications performed to obtain it are not expected to have an impact on the toxicological profile of the product.

Despite the above, the applicant submitted a battery of toxicological studies consisting of a bacterial reverse mutation test, an *in vitro* mammalian cell micronucleus test and a 90-day oral toxicity study. The FEEDAP Panel noted that all these studies were performed using a protease batch ([redacted]) which was concentrated by evaporation instead of by ultrafiltration, as described in the manufacturing process (Section 3.1.2). The evaporation step used was not thoroughly described in terms of temperature and duration applied. Depending on the conditions applied, the evaporation could inactivate possible toxins produced during the fermentation by the microorganism as well as the enzyme. However, as the production strain qualifies for the QPS approach, the risk of toxins production is excluded. The possible inactivation of the enzyme caused by the evaporation step was also ruled out, since the enzymatic activity in the test item was confirmed by certificates of analysis. Therefore, the FEEDAP Panel considered the test item used in the toxicological studies to be representative of the additive under assessment.

3.2.2.1. Bacterial reverse mutation assay

The test item was tested for the induction of reverse mutations in Salmonella Typhimurium tester strains (TA1535, TA1537, TA98 and TA100) and in *Escherichia coli* strain [WP2uvrA (pKM101)].[26] The experimental protocol was in line with the Organization for Economic Cooperation and Development (OECD) testing guideline (TG) 471 and claimed to be conducted according to the good laboratory practice (GLP). The test item was dissolved in water and tested both in the presence and absence of metabolic activation. For testing with S9 mix, heat-treated (90°C for 30 min) test item was used as the intrinsic properties of the active enzyme in the present test item cause reduction in the performance of S9 due to degradation of active components in the S9. All concentrations of protease were expressed in terms TOS. Seven concentrations up to 5,000 μg/mL were tested in two independent studies, with triplicate plating at each concentration. Positive and negative controls were included. All plates were incubated at 34–39°C for 69 h. No toxicity or precipitate was seen in the first experiment at concentrations up to 5,000 μg/mL. No indication of mutagenic activity was observed in any experimental condition, while a significantly increased number of revertant colonies was observed in the positive controls.

3.2.2.2. *In vitro* mammalian cell micronucleus test

The test item was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy.[27] The experimental protocol was stated to be line with OECD TG 487, but some deviations were noted. The maximum tested

[26] Technical dossier/Section III/Annex 3–2.
[27] Technical dossier/Section III/Annex 3–3.

concentrations were established with a preliminary cytotoxicity experiment. Tests were conducted both in the presence and absence of a post-mitochondrial supernatant fraction (S9) obtained from the livers of rats treated with Aroclor 1254. Cells were human lymphocytes stimulated for 48 h with phytohaemagglutinin (PHA) and then treated for 3 h (followed by a 21-h recovery period) with the protease dissolved in water, both in the absence and in the presence of S9-mix. For the S9 condition, the test article was heat treated prior to dilution and treatment, at 90°C for a 30-min period. An extended exposure study was also performed by exposing cells for 24 h and allowing 24 h of recovery before addition of cytochalasin B (CytB). The Panel noted that the timing for CytB addition in the extended treatment is a limitation of the experimental design, possibly affecting the sensitivity of the assay. During the extended treatment, cells should be treated for 1.5–2 normal cell cycle lengths in the presence of CytB. In the study, CytB was added only 24 h after the exposure and after removal of test item. The cytotoxicity was studied after 3 h, at 100; 250; 500; 1,000; 2,000; 3,000; 4,000 and 5,000 μg TOS/mL and at the two highest concentrations (4,000 and 5,000 μg TOS/mL) the levels of cytotoxicity were 50% and 45% without S9 and 20% and 11% with S9. In a parallel assay, cells were treated for 24 h with 0 or 13 different concentrations of the test item from 1 to 100 μg of TOS/mL in the absence of S9-mix with 24 h of recovery period. The highest concentration induced 90% cytotoxicity. There were two replicate cultures per treatment and 1,000 cells per replicate (i.e. 2,000 cells per dose) were scored for cytotoxicity, except for the for 3 + 21 h treatments -S9, where only 500 cells were scored per replicate, meaning 1,000 cells per dose. Marked increases in osmolality were observed at concentrations between 4,000 and 5,000 μg TOS/mL. There was no evidence that the test item induced chromosomal damage or aneuploidy in either the presence or absence of S9 mix, up to 5,000 μg TOS/mL (3-h exposure) or up to the cytotoxic dose of 40 μg TOS/mL (24 + 24 h treatment). The micronucleus frequency of all the protease treated cultures (all concentrations, all treatments) fell within the 95th percentile of the current observed historical vehicle control ranges. The positive controls performed as expected.

3.2.2.3. 90-day study

Han Wistar rats (four groups of 10 male and 10 female) were administered by gavage the protease at 0 (control), 300,000; 990,000 or 3,020,000 NFP/kg bw per day for 13 weeks.[28] The study was conducted following the OECD TG 408 and as claimed by the applicant, following the principles of GLP. There was no evidence of any adverse effect at any of the administered doses. Consequently, the FEEDAP Panel identified a no observed adverse effect level (NOAEL) of 3,020,000 NFP/kg bw per day, the highest dose tested.

3.2.2.4. Conclusion on the toxicological studies

Based on the results obtained, the FEEDAP Panel concluded that the test item did not induce gene mutations nor chromosomal damage. These results were used as supporting evidence for the safety of the additive.

No adverse effects were identified in a 90-day oral toxicity study. The FEEDAP Panel identified a NOAEL of 3,020,000 NFP/kg bw per day, the highest dose tested.

3.2.3. Safety for the target species

No tolerance studies in relevant target species were submitted. In order to support the safety of the additive for the target species, the applicant referred to the 90-day toxicity study that is described above (see Section 3.2.2.3). The NOAEL identified (3,020,000 NFP/kg bw per day) was used to calculate the maximum safe level in feed for chickens and turkeys for fattening in accordance with the procedure described in the Guidance on the safety for the target species (EFSA FEEDAP Panel, 2017b). The calculated maximum safe concentration in feed was 336,405 NFP/kg complete feed for chickens for fattening and 453,000 NFP/kg complete feed for turkeys for fattening. These values are higher than the recommended use level of 30,000 NFP/kg feed for other growing poultry species. Therefore, the Panel concludes that the additive is safe for all poultry species for fattening or reared for laying/breeding.

[28] Technical dossier/Section III/Annex 3–5.

3.2.4. Safety for the consumers

The enzyme is produced by a genetically modified strain of *B. licheniformis*; this species is considered to qualify for the QPS approach to safety assessment. The identity of the strain was established, the qualifications were met and the genetic modification of the production strain raises no concerns as regards to the toxicological profile of the production strain. Therefore, the production strain is presumed safe for production purposes and no safety concerns would raise for the consumer from the fermentation product obtained from this strain. The results obtained in the genotoxicity studies and the 90-day study support this conclusion. The FEEDAP Panel concludes that the use of ProAct 360 in animal nutrition under the proposed conditions of use is safe for the consumer.

3.2.5. Safety for the user

The dusting potential of the additive is up to 30 mg/m^3. The FEEDAP Panel considered that exposure via inhalation is unlikely. However, owing to the nature of the active substance, the additive should be considered a respiratory sensitiser.

The skin irritation potential of the additive (enzyme activity: 785,470 NFP/g)[3] was studied in an *in vitro* assay according to OECD TG 439.[29] Based upon the results, it is concluded that the test item is non-irritant to the skin.

The eye irritation potential of the additive was investigated *in vitro* according to OECD TG 438.[30] Based upon the results, the product is non-irritant to the eye.

No study on skin sensitisation was provided. In the absence of data, no conclusions on the skin sensitisation potential can be reached.

3.2.5.1. Conclusions on safety for the user

The additive is not irritant to skin and eyes. The FEEDAP Panel cannot conclude on the potential of the additive to be a skin sensitiser. Owing to the proteinaceous nature of the active substance, the additive should be considered a respiratory sensitiser.

3.2.6. Safety for the environment

Viable cells of the production strain and its DNA were not detected in an intermediate concentrate representative of the final formulation. The additive does not raise safety concerns for the environment with regard to the genetic modification of the production strain *B. licheniformis* DSM 33099. The active substance of the additive is a protein, and as such 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 and no further environmental risk assessment is required.

3.2.7. Extensive literature search on the safety of the product

The applicant also performed a literature search to retrieve any published evidence on the safety of the active substance and of the species to which the ProAct 360 production strain belongs, for the target species, consumers, users and the environment.[31] The search involved a total of three databases (Web of Science Core Collection, Biosis Citation Index and Medline), covered the period 2000 to January 2021 and the terms and strategy used were provided. Proximity operators and truncation were applied to optimise the search. The search identified 12 relevant hits. However, nine of these were not considered further since they referred to the use of *B. licheniformis* as a human probiotic (one hit), to food-borne outbreaks related to *Bacillus* spp. (six hits), to the allergenicity properties of food proteases or protease-containing laundry and cleaning products in humans (two hits) that do not bring new relevant information, or to the use of a *B. licheniformis* metabolite as a bioactive molecule (one hit). The remaining two papers addressed the production of lichenysin in different *B. licheniformis* genotypes. However, this last concern can be excluded by the negative results of the cytotoxicity test.

[29] Technical dossier/Section III/Annex 3–6.
[30] Technical dossier/Section III/Annex 3–7.
[31] Technical dossier/Section III/Annex 3–27 and Annexes 3–9 to 3–20.

3.3. Efficacy

ProAct 360 is intended to be used as a zootechnical additive (digestibility enhancer) in feed for all poultry species for fattening or reared for laying/reproduction at the recommended level of 30,000 NFP/kg complete feed.

3.3.1. Efficacy for chickens for fattening

A total of four trials with chickens for fattening sharing a similar design were submitted. The details of the study design are provided in Table 1 and the main results in Table 2.

Table 1: Trial design and use level of the efficacy trials performed in chickens for fattening

Trial	Total N (animals/ replicate) Reps./ treatment	Breed sex	Duration (Starter/Grower/ Finisher)	Composition feed (form)	Groups (NFP/kg feed)	
					Intended	Analysed*
1[32]	870 (15) 29	Ross 308 Male	35 days (1–14/15–28/29–35)	Maize, wheat, soya bean meal (mash)	0 30,000	– 33,897
2[33]	1,056 (22) 24	Ross 308 Male	35 days (1–14/15–28/29–35)	Wheat, soya bean meal, rapeseed meal (mash)	0 30,000	– 30,863
3[34]	576 (12) 24	Ross 308 Male	35 days (1–14/15–28/29–35)	Wheat, soya bean meal (crumble/ pellet)	0 30,000	– 29,240
4[35]	450 (10) 15	Ross 308 Male	35 days (1–11/12–21/22–35)	Wheat, maize, soya bean meal (crumble/pellet)	0 30,000 PC	– 23,625 –

PC: positive control.
*: Average value of the starter/grower/finisher feeds.

In all trials, 1-day-old male Ross 308 chicks were distributed in pens and randomly allocated to two dietary groups. Three basal diets (starter; grower; finisher) were either not supplemented (control) or supplemented with the test item to provide 30,000 NFP/kg complete feed. The experimental diets were offered ad libitum during a 35-day period. The enzyme activity in the feeds was confirmed analytically (see Table 1). Trial 4 included an additional group (considered by the applicant as a positive control) in which the chickens were fed the control diet with 3–4% higher crude protein and amino acid content.

Mortality and health status of the animals were daily monitored, and the most likely reason for culling/death recorded. The birds were individually weighed at the start of the trial (day 1). Thereafter, the feed intake and body weight of each pen were recorded at every diet change and at the end of the trial. The average daily feed intake, average daily gain feed and feed to gain ratio were calculated and corrected for mortality for every diet period and the whole production period.

The productive performance data were analysed with Student's t-test (trial 1) or one-way analysis of variance (ANOVA) (trials 2–4) with the diet as fixed effect. In trial 4, means were compared with Duncan's test. The experimental unit used was the pen in all cases. The significance level applied was 0.05.

[32] Technical dossier/Section IV/Annex 4–1.
[33] Technical dossier/Section IV/Annex 4–2.
[34] Technical dossier/Section IV/Annex 4–3.
[35] Technical dossier/Supplementary information January 2023/Annex 4add_Trial PL2022.

Table 2: Effects of ProAct 360 on the performance of chickens for fattening

Trial	Groups (NFP/kg feed)	Average daily feed intake (g)	Initial body weight (g)	Final body weight (g)	Average daily weight gain (g)	Feed to gain ratio	Mortality and culling (%)
1	0	90.0	44.0	2,182	61.1	1.48[a]	2.8
	30,000	89.0	44.0	2,191	61.3	1.45[b]	1.8
2	0	104	47.7	2,114[b]	58.7[b]	1.78[a]	2.8
	30,000	103	47.7	2,201[a]	61.2[a]	1.68[b]	2.7
3	0	92.0[b]	43.0	2,260[b]	63.0[b]	1.48[a]	1.04[b]
	30,000	95.0[a]	43.0	2,530[a]	67.0[a]	1.40[b]	5.56[a]
4	0	90.4	39.4	1,948[b]	54.5[b]	1,66[a]	0
	30,000	88.6	39.8	2,028[b]	56.8[b]	1,56[b]	0.67
	PC	92.2	39.9	2,133[a]	59.8[a]	1,54[b]	0.67

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

In four trials, chickens for fattening fed with the additive at 30,000 NFP/kg complete feed showed improvements of the feed-to-gain ratio in comparison with the control group. In two trials (trial 2 and 3), higher final body weight and average daily gain were seen in the supplemented group compared to the control, as well as higher average daily feed intake in trial 3. Hoverer, in trial 3, the overall mortality and culling were higher in the supplemented group compared to the control, which prevented the use of the results of this study to support the efficacy of the additive. Overall, positive effects on the zootechnical performance (namely in feed to gain ratio) were observed in three trials when chickens were fed the additive at 30,000 NFP/kg complete feed.

3.3.1.1. Conclusions on efficacy

The Panel concludes that additive has the potential to be efficacious as a zootechnical additive in chickens for fattening at 30,000 NFP/kg complete feed. This conclusion can be extrapolated to all poultry species for fattening or reared for laying/breeding.

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[36] and Good Manufacturing Practice.

4. Conclusions

ProAct 360, manufactured with the production strain *Bacillus licheniformis* DSM 33099, does not give rise to safety concerns with regard to the genetic modification of the production strain. No viable cells or DNA of the production strain were detected in an intermediate concentrate representative of the final formulation.

ProAct 360 is considered safe for all poultry species for fattening or reared for laying/breeding at the recommended inclusion level of 30,000 NFP/kg complete feed.

The use of ProAct 360 in animal nutrition under the proposed conditions of use is of no concern for consumers safety.

ProAct 360 is not irritant to skin and eyes. The FEEDAP Panel cannot conclude on the potential of the additive to be a skin sensitiser. Owing to the proteinaceous nature of the active substance, the additive is considered a respiratory sensitiser.

The use of ProAct 360 as a feed additive is considered safe for the environment.

The additive is considered to be efficacious in feedingstuffs for all poultry species for fattening or reared for laying/breeding at the recommended use level of 30,000 NFP/kg complete feed.

[36] 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

AMR	antimicrobial resistance
ANI	average nucleotide identity
ANOVA	one-way analysis of variance
BIOHAZ	EFSA Panel on Biological Hazards
BW	body weight
CFU	colony-forming unit
CV	coefficient of variation
CytoB	cytochalasin B
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLP	good laboratory practice
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
NOAEL	no observed adverse effect level
OECD	Organization for Economic Cooperation and Development
PHA	phytohaemagglutinin
QPS	qualified presumption of safety
RH	relative humidity
TG	testing guideline
TOS	total organic solid
WGS	whole genome sequence