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Safety and efficacy of a feed additive consisting of 25-hydroxycholecalciferol (produced by *Pseudonocardia autotrophica* DSM 32858) for all pigs, all poultry for fattening and ornamental birds and other poultry species (Huvepharma NV)

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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 feed additive consisting of 25-hydroxycholecalciferol (produced by *Pseudonocardia autotrophica* DSM 32858) for all pigs, all poultry for fattening and ornamental birds and other poultry species. The production strain *P. autotrophica* DSM 32858 is not genetically modified however, uncertainties remain on the possible presence of its viable cells in the final product. Due to the lack of adequate safety data and uncertainty on the presence of nano particles, the FEEDAP Panel cannot conclude on the safety of the additive for the target species and the consumer. The additive was shown not to be irritant to skin or eyes and it is not a skin sensitiser. Considering the low dusting potential of the additive, the FEEDAP Panel concluded that the exposure through inhalation is unlikely. However, the FEEDAP Panel considered that uncertainties remain on genotoxicity and on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product which might have an impact on the safety for the users. The use of the feed additive is considered safe for the environment. The Panel concluded that the additive has a potential to be efficacious under the proposed conditions of use.

Keywords: nutritional additives, vitamins, *Pseudonocardia autotrophica*, safety, efficacy, vitamin D_3, 25-hydroxycholecalciferol

Requestor: European Commission

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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 NV[2] for the authorisation of the additive consisting of 25-hydroxycholecalciferol produced by *Pseudonocardia autotrophica* DSM 32858, when used as a feed additive for all pigs, all poultry for fattening and ornamental birds and other poultry species (category: nutritional additives; functional group: vitamins, pro-vitamins and chemically well-defined substances having similar effect).

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 25 November 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 25-hydroxycholecalciferol when used under the proposed conditions of use (see Section 3.1.7).

1.2. Additional information

The feed additive consisting of 25-hydroxycholecalciferol (25-OH-D_3) produced by *Pseudonocardia autotrophica* DSM 32858 is not currently authorised for use in animal feed.

The FEEDAP Panel issued two opinions on the use of 25-OH-D_3 as a feed additive: one for chickens for fattening, turkeys and laying hens (EFSA, 2005) and the other one for poultry and pigs (EFSA, 2009).

Another opinion was issued on the safety of calcidiol monohydrate (25-hydroxycholecalciferol monohydrate) produced by chemical synthesis as a novel food (EFSA NDA Panel, 2021).

25-OH-D_3 is currently authorised for use in feed for chickens for fattening, turkeys for fattening, other poultry and pigs under the identification number 3a670a.[3]

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 25-OH-D_3 produced by *P. autotrophica* DSM 32858 as a feed additive.

The dossier was received on 1/10/2021 and the general information and supporting documentation is available at https://open.efsa.europa.eu/questions/EFSA-Q-2021-00641.

The confidential version of the technical dossier was subject to a target consultation of the interested Member States from 9 March 2022 to 9 June 2022 for which the received comments were considered for the assessment.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' (elicitation) knowledge, to deliver the present output.

[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 NV, Uitbreidingstraat 80, Berchem (Belgium).
[3] Commission Regulation (EC) No 887/2009 of 25 September 2009 concerning the authorisation of a stabilised form of 25-hydroxycholecalciferol as a feed additive for chickens for fattening, turkeys for fattening, other poultry and pigs. OJ 254/68, p. 3.
[4] FEED dossier reference: FAD-2021-0057.

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 25-OH-D_3 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), 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

25-OH-D_3 produced with *P. autotrophica* DSM 32858 is intended to be used as a nutritional additive (functional group: vitamins, pro-vitamins and chemically well-defined substances having similar effect) in feed and water for all pigs, all poultry for fattening and ornamental birds and other poultry species as a source of Vitamin D_3.

3.1. Characterisation

3.1.1. Characterisation of *Pseudonocardia autotrophica* DSM 32858

The active substance is produced using a non-genetically modified strain of *P. autotrophica* which is deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under the accession number DSM 32858.[7]

[redacted][8]

The minimum inhibitory concentration (MIC) of *P. autotrophica* DSM 32858 to the list of antibiotics for [redacted] Consequently, the production strain is considered to be resistant to these antibiotics.[9]

The WGS data of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes against [redacted][10] No genes of concern were identified. [redacted]

[5] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2021-0057_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.
[7] Technical dossier/Section II/Annex_II.2.2.
[8] Technical dossier/Supplementary Information (June 2022)/Section II/Appendix 1.
[9] Technical dossier/Section II/Annex_II.2.18.
[10] Technical dossier/Supplementary Information (June 2022)/Section II/Appendix 2.

[redacted] these resistances raise no safety concerns since no acquired AMR genes were found in the WGS.

The WGS data of *P. autotrophica* DSM 32858 was also examined for virulence factors [redacted] No genes of concern were identified.[10,11]

[redacted] No antimicrobial activity was detected.[12]

3.1.2. Manufacturing process

[redacted]

3.1.3. Characterisation of the active substance

The active substance is 25-OH-D_3.

The Chemical Abstracts Service (CAS) number is 19356–17-3, the European Inventory of Existing Commercial Chemical Substances (EINECS) number is 242–990-9, molecular formula $C_{27}H_{44}O_2$ and the molecular weight 400.64 g/mol. It has a melting point of 107–109°C and a boiling point of 592.25°C. It is insoluble in water.[13]

The structural formula of the active substance is presented in Figure 1.

No purity data on the active substance were provided by the applicant.

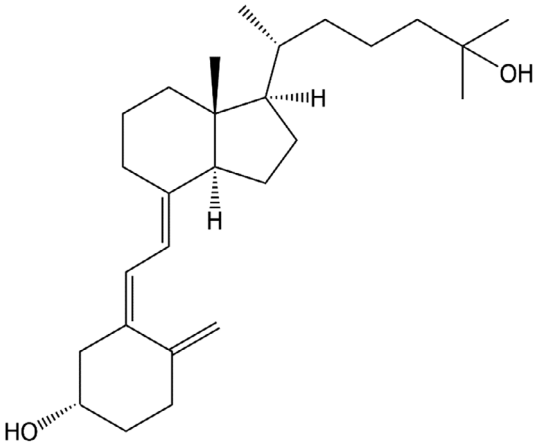

Figure 1: Structural formula of 25-hydroxycholecalciferol (25- OH-D3)

3.1.4. Characterisation of the additive

The final formulation of the additive contains a stabilised form of the active substance (25-OH-D_3) at a minimum of 1.25%. Maltodextrin is used as a carrier. In addition to 25-OH-D_3, the final formulation of the additive also contains other sterol derivatives, including residues of cholecalciferol (the starting substrate) and other sterols produced in minor amounts during the manufacturing process.

The applicant proposed specifications of the additive as follows: content of 25-OH-D_3 not less than (NLT) 1.25%, 1α,25-dihydroxycholecalciferol (1α,25-$(OH)_2$-D_3) not more than (NMT) 0.03%, cholecalciferol NMT 0.5%, loss of drying NMT 7%. Other sterol derivatives might be present and should be NMT 40% (of 1.25% of 25-OH-D_3) expressed as the area per cent of the corresponding

[11] Technical dossier/Section II/Annex_II.2.30.
[12] Technical dossier/Section II/Annex_II.2.28.
[13] https://pubchem.ncbi.nlm.nih.gov/compound/Calcifediol#section=Computed-Properties

chromatographic peak (% HPLC area), assuming the sum of chromatographic areas of all detected sterols as 100%, any individual sterol derivative NMT 10% expressed as % HPLC area.

Analytical data to confirm the specifications were provided for five batches of the additive, showing the following average values: 25-hydroxycholecalciferol mean 1.3% (range 1.28–1.32%), 1α,25-dihydroxycholecalciferol mean 0.015% (range 0.013–0.017%), cholecalciferol mean 0.34% (range 0.3–0.4%), total related sterols (expressed as % LC area) mean 18.6% (range 16–21%), any individual sterol (expressed as % HPLC area) mean 5% (range 4–6%).[14]

In addition, the applicant sent certificates of analysis for five pilot scale batches to confirm compliance with specifications.[15]

Three pilot scale batches of the additive were analysed for the content of crude protein (mean: 7.34%; range: 6.52–8.33%), total of fat and crude fibre (mean: < 10%), total ash (mean: 6.62%; range: 3.11–9.97%) and moisture (mean: 4.02%; range: 3.78–4.23%).[16]

Three batches of the additive were analysed for cadmium < 0.01 mg/kg, lead < 0.05 mg/kg, mercury < 0.005 mg/kg and arsenic < 0.04 mg/kg. Individual aflatoxin (B1, B2, G1, G2) levels were < 1 μg/kg and the total aflatoxin level was < 1.5 μg/kg. Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/PCDF) were 0.129 (0.128–0.131) ng TEQ-WHO/kg and dioxin-like polychlorinated biphenyls (PCBs) were 0.123 ng TEQ-WHO/kg resulting in a total of 0.252 (0.251–0.254) ng TEQ-WHO/kg PCDD/F/PCB (expressed as 88% dry matter) in all batches.[17]

Microbiological contamination was analysed in six batches of the additive by determination of total aerobic counts (1.06×10^3 CFU/g; range 3.5×10^2–2.4×10^3 CFU/g), coliforms (< 30 CFU/g) and yeasts and mould counts (< 10^2 CFU/g). *Escherichia coli* and *Salmonella* spp. were not detected in 25 g.[18]

[redacted][19]

The Panel considered that the microbial contamination and the amounts of the detected impurities do not raise safety concerns.

The presence of viable cells of *P. autotrophica* DSM 32858 was investigated in three batches of the product tested in triplicate with a total of nine samples.[20,21] For each sample, 10 g of the product was suspended in sterile water to reach a final volume of 100 mL. Then 10 mL (corresponding to 1 g of the product) were centrifuged, the pellet suspended in 0.2 mL, and used to inoculate nutrient agar plates. Incubation was done at 28°C for 8 days. A proper positive control was included in the analysis. Colonies were found in the non-spiked samples analysed and, according to the applicant, they were identified and excluded to belong to *P. autotrophica* species. However, the FEEDAP Panel notes that the identification of the colonies was not performed using molecular methods, but by morphology, microscopic analysis and using the semi-automatic identification system BD BBL Crystal Identification system. Those methods are considered not conclusive and would not allow to unequivocally exclude the presence of viable cells of the production strain in the additive. Therefore, uncertainty remains on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product.

The applicant also investigated the presence of DNA of *P. autotrophica* DSM 32858 in three batches of the product tested in triplicate. The extraction procedure included a lysis step using glass beads. The primers amplified a specific segment of the *Pseudonocardia* spp. 16S rRNA gene of 645 bp with a limit of detection of 10 ng of genomic DNA/mL. Relevant controls were included in the analysis. DNA of *P. autotrophica* DSM 32858 was not detected.[22,23]

3.1.5. Physical properties of the additive

The bulk and tapped density were measured on five pilot scale batches of the additive and showed on average 351 kg/m^3 (332–386 kg/m^3) and 403 kg/m^3 (377–438 kg/m^3), respectively.

[14] Technical dossier/Section II/Annex_II.1.1.
[15] Technical dossier/Section II/Annex_II.1.2.
[16] Technical dossier/Section II/Annex_II.1.4.
[17] Technical dossier/Supplementary information (June 2022)/Appendices/Appendix 5.6 and 7.
[18] Technical dossier/Supplementary information (June 2022)/Appendices/Appendix 8.
[19] Technical dossier/Section II/Annex_II.1.12.
[20] Technical dossier/Section II/Annex_II.2.29.
[21] Technical dossier/Supplementary Information (June 2022)/Section II/Appendix 4.
[22] Technical dossier/Section II/Annex_II.2.19.
[23] Technical dossier/Supplementary Information (June 2022)/Section II/Appendix 3.

The dusting potential of three pilot scale batches (four replicates per batch) of the additive was determined using the Stauber-Heubach method and showed values on average of 42 mg/m^3 (range 30–60 mg/m^3) (mg airborne dust per m^3 of air).[24] The dust of the same three batches (four replicates per batch) of the additive was tested to evaluate the concentration of 25-OH-D_3 and showed values on average of 2.62 mg 25-OH-D_3/g (range 1.85–3.26 mg/g).[25]

The particle size of the additive was analysed by laser-diffraction method: 10% of the particles are smaller than 13.21 μm, 50% of the particles are smaller than 58.72 μm, 90% of the particles are smaller than 214 μm; the average particle size was 92.72 μm.[26]

The applicant analysed the final formulation of the additive (containing 25-OH-D_3 at a minimum of 1.25% and maltodextrin) by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)[27] The analysis showed that the sample contained predominantly amorphous material as well as a low level of small particles which are classified as nanoparticles as they have one or more external dimension in the nano size range. However, it was not possible to differentiate the 25-OH-D_3 particles (insoluble in water) from the particles of the maltodextrin (very soluble in water) used as carrier. Therefore, data does not allow to conclude whether 25-OH-D_3 contains a fraction of small particles including nanoparticles.

3.1.6. Stability and homogeneity

3.1.6.1. Shelf life of the additive

The shelf life of the additive was studied on three batches when stored at 25°C (60% relative humidity - RH) for 24 months and at 40°C (75% RH) for 6 months.[28] The stability in terms of loss of drying (< 7%, ranging 3.6–5.5%), content of 25-OH-D_3 (> 1.25%, ranging 1.32–1.43%), cholecalciferol (< 0.5, ranging 0.13–0.16%), related sterols (< 40%, ranging 19–22%) and content of 1α,25-dihydroxycholecalciferol (< 0.03, ranging 0.017–0,021%) was demonstrated under the two conditions studied.

3.1.6.2. Stability of the additive in premixtures and feedingstuffs

Stability in premixtures

The stability of the additive in premixtures for pigs and poultry was studied in three batches when supplemented at 627.3 mg 25-OH-D_3/kg and stored at 25°C (60% RH) for 24 months and at 40°C (75% RH) for 6 months in standard packaging.[29] Losses in terms of content of 25-OH-D_3 ranged 3.2–11.3% after 24 months storage at 25°C (60% RH) and 11.9–13.9% after 6 months storage at 40°C (75% RH).

Stability in feeds

The stability of three batches of the additive in mash and pelleted feed for poultry was investigated at two different inclusion levels of 50 or 100 μg 25-OH-D_3/kg and when stored at 25°C (60% RH) for 3 months and at 40°C (75% RH) for 4 weeks.[30] At the lowest inclusion level (50 μg/kg) the losses of 25-OH-D_3 ranged from 0% to 8.4% after 3 months at ambient temperature, while when stored at 40°C, after 4 weeks the loss ranged from 7.7% to 23.2%, for mash feed. For pelleted feed, the loss after 3 months at ambient temperature ranged from 11.6% to 14.7% and after 4 weeks at 40°C ranged from 15.8% to 22.9%. At the highest inclusion level (100 μg/kg) the loss of 25-OH-D_3 ranged from 3.6% to 4.4% after 3 months at ambient temperature, while when stored at 40°C, after 4 weeks the loss ranged from 17.2% to 18.1%, for mash feed. For pelleted feed, the loss after 3 months at ambient temperature ranged from 12.2% to 21.1% and after 4 weeks at 40°C ranged from 15.4% to 18.4%.

The same conditions as described above were also applied to mash and pelleted feed for pigs but with different inclusion levels (25 or 50 μg 25-OH-D_3/kg). At the lowest inclusion level (25 μg/kg) the losses of 25-OH-D_3 ranged from 2.4% to 18% after 3 months at ambient temperature, while when

[24] Technical dossier/Section II/Annex_II.1.9.
[25] Technical dossier/Section II/Annex_II.1.10.
[26] Technical dossier/Section II/Annex_II.1.11.
[27] Technical dossier/Supplementary Information (January 2023)/Section II/Appendix 1.
[28] Technical dossier/Section II/Annex_II.4.1.
[29] Technical dossier/Section II/Annex_II.4.2.
[30] Technical dossier/Section II/Annex_II.4.3.

stored at 40°C, after 4 weeks the loss ranged from 7.3% to 20%, for mash feed. For pelleted feed, the loss after 3 months at ambient temperature ranged from 0% to 2.5% and after 4 weeks at 40°C ranged from 2.7% to 12.8%. At the highest inclusion level (50 μg/kg) the losses in terms of content of 25-OH-D_3 ranged from 11.6 to 14.4% after 3 months at ambient temperature, while when stored at 40°C, after 4 weeks the loss ranged from 16.8% to 20.4%, for mash feed. For pelleted feed, the loss after 3 months at ambient temperature ranged from 0% to 4.9% and after 4 weeks at 40°C ranged from 7.4% to 12.5%.

The applicant has also provided data on the effects on stability of the pelleting process (at 80°C) in feed for poultry and pigs. The loss of 25-OH-D_3 ranged between 0% and 14.7% and from 0% to 11.5% for poultry and pigs feeds, respectively.

3.1.6.3. Stability of the additive in water for drinking

The stability of the additive in water for drinking was studied in two types of water: soft water (pH between 5–7 and ≤ 60 mg/mL of calcium carbonate) and hard water (pH 8–9 and 180–350 mg/L of calcium carbonate) supplemented with 25-OH-D_3 at 0.01%. Samples were stored at ambient temperature and refrigerated (between 3 and 8°C) for 48 h. The losses in terms of content of 25-OH-D_3 in the soft water was 9.7% at ambient temperature after 48 h and 10.7% and 21% in the refrigerated sample after 24 and 48 h, respectively. In the hard water samples, there were no losses at ambient temperature or after 24 h refrigerated but after 48 h refrigerated the loss was 16.5%.

3.1.6.4. Homogeneity

The capacity for homogeneous distribution of the additive, was evaluated in feeds for poultry and pig in two presentations each (mashed and pelleted) and supplemented with two levels of inclusion. Poultry feed was supplemented with 74–97 μg/kg or 161–205 μg/kg and pigs feed with 45–52 μg/kg or 83–92 μg/kg. The coefficient of variation (CV) was calculated in 10 subsamples of each feed. At the lowest inclusion level (74–97 μg/kg), the CV ranged from 3 to 5.9 and from 1.8 to 4.3 for mashed and pellet poultry feed respectively and from 3.5 to 7.4 and from 3.4 to 4.8 for mashed and pellet pigs feed respectively. At the highest inclusion level (161–205 μg/kg), the CV ranged from 5 to 6.1 and from 2 to 6.2 for mashed and pellet poultry feed, respectively and from 3.5 to 4.5 and from 2.3 to 3.7 for mashed and pellet pigs feed, respectively.[30]

3.1.7. Conditions of use

The additive is intended for use in feed for the following animal species/categories:

- Poultry for fattening and ornamental birds: the additive is intended to be used to provide a typical content of 34.85 μg of 25-OH-D_3/kg feed and a maximum content of 100 μg of 25-OH-D_3/kg feed. Considering that water intake would be 2–3 times higher than feed intake (DM), the typical inclusion level in water for drinking would correspond to 11.6–17.5 μg of 25-OH-D_3/L and the maximum content to 33.3–50 μg of 25-OH-D_3/L in water for drinking.
- Poultry for laying and breeding: the additive is intended to be used to provide a typical content of 34.85 μg of 25-OH-D_3/kg feed and a maximum content of 80 μg of 25-OH-D_3/kg feed. Considering that water intake would be 2–3 times higher than feed intake (DM), the typical inclusion level in water for drinking would correspond to 11.6–17.5 μg of 25-OH-D_3/L and the maximum content to 26.6–40 μg of 25-OH-D_3/L in water for drinking.
- Pigs: the additive is intended to be used to provide a typical content of 25 μg of 25-OH-D_3/kg feed and a maximum content of 50 μg of 25-OH-D_3/kg feed. Considering that water intake would be 2–3 times higher than feed intake (DM), the typical inclusion level in water for drinking would correspond to 8.4–12.5 μg of 25-OH-D_3/L and the maximum content to 16.6–25 μg of 25-OH-D_3/L in water for drinking.

The maximum amount of the combination of 25-OH-D_3 with vitamin D_3[31] should be:

1) ≤ 125 μg (equivalent to 5,000 IU of vitamin D_3) for chickens for fattening and turkeys for fattening.
2) ≤ 80 μg (equivalent to 3,200 IU of vitamin D_3) for poultry for laying and breeding.
3) ≤ 50 μg (equivalent to 2,000 IU of vitamin D_3) for pigs.

[31] 40 IU 25-OH-D_3 = 1 μg 25-OH-D_3.

3.2. Safety

3.2.1. Toxicological studies

Genotoxicity studies

The applicant has provided two Ames tests[32,33] and an *in vitro* micronucleus test.[32] One of the two Ames tests was performed with the active substance produced by the strain under assessment (*P. authotrophica* DSM 32858). The test item used in the other studies was the final formulation of the additive containing 25-OH-D_3, produced enzymatically during fermentation of [redacted].

Due to the lack of confirmation of the toxicological equivalence between the [redacted], used in some of the genotoxicity studies, and the strain under assessment, *P. autotrophica* DSM 32858, the FEEDAP Panel considered that only the Ames test conducted with 25-OH-D_3 produced by the strain under assessment can be further considered for the risk assessment.

In addition, the *in vitro* micronucleus test provided by the applicant, beside the limitation of the test item used, showed: [redacted] The Panel noted that these results did not fulfil the criteria of OECD TG 487 for a clearly positive or a clearly negative result, and therefore judged the study as equivocal.

Bacterial reverse mutation assay

25-OH-D_3, produced by fermentation of *P. autotrophica* DSM 32858, was tested for the induction of reverse mutations [redacted]

Repeated dose toxicity studies

The applicant submitted a 28-day[34] and a 90-day[35] toxicity studies to support the safety of the additive for the consumers.

The FEEDAP Panel noted that both studies were performed with [redacted] The FEEDAP Panel considered the lack of confirmation of the toxicological equivalence between the [redacted] and the strain under assessment, *P. autotrophica* DSM 32858. Moreover, this test item is not representative of the additive under assessment and does not reflect the realistic manufacturing conditions. Therefore, the studies submitted cannot be further considered for risk assessment.

[32] Technical dossier/Section III/Annex_III_2_9.
[33] Technical dossier/Supplementary information (June 2022)/Appendices/Appendix 9.
[34] Technical dossier/Section III/Annex_III.2.10.
[35] Technical dossier/Section III/AnnexIII.2.11.

3.2.1.1. Conclusion on Toxicology

Based on the data available, the FEEDAP Panel concluded that 25-OH-D_3 did not induce gene mutations, provided that it is not in the nano form accordingly to the criteria described in the relevant EFSA Guidance (see Section 4.2.1 of the Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles; EFSA Scientific Committee, 2021). Due to the lack of adequate data, the FEEDAP Panel cannot conclude on the potential of 25-OH-D_3 to induce chromosomal damage. The toxicological dataset provided does not allow to conclude on possible adverse effects.

3.2.2. Safety for the target species

In support of the safety for the target species, the applicant provided a combined tolerance/efficacy study[36,37] conducted in chickens for fattening and results of a literature search. The test item used in the study was the final formulation of the additive containing 25-OH-D_3, [redacted]

Due to the lack of confirmation of the toxicological equivalence between the [redacted] and the strain under assessment, *P. autotrophica* DSM 32858, the FEEDAP Panel did not further consider this study for the risk assessment.

The results of the literature search performed did not show any negative effect of 25-OH-D_3 when given to chickens for fattening up to 5,520 IU/kg feed (138 μg/kg),[31] to laying hens up to 9,000 IU/kg (225 μg/kg) and to piglets up to 40,000 IU/kg feed (1,000 μg/kg). However, the studies provided present some limitations (e.g. limited number of replicates, the source of 25-OH-D_3 used in the studies is not clearly reported or described, not clear study design), which prevents them to be used to support the safety of the additive under assessment for the target species.

3.2.2.1. Conclusions on safety for the target species

Due to the lack of adequate data, the FEEDAP Panel cannot conclude on the safety of the additive for the target species. Moreover, the FEEDAP Panel considered that uncertainties remain on genotoxicity and on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product.

3.2.3. Safety for the consumer

3.2.3.1. Absorption, distribution, metabolism and excretion

No ADME studies performed with the additive under assessment were carried out. The applicant submitted reviews and experimental studies retrieved from the literature that are herein described.

Humans

In humans and other species, the intestinal absorption of 25-OH-D_3 is consistently higher than that of vitamin D_3. 25-OH-D_3 is absorbed via the vena porta and vitamin D_3 via the lymph pathway and this can in part explain the greater bioavailability of 25-OH-D_3. The mean increase of 25-OH-D_3 in human serum (considering nine randomised control studies reviewed) after intake of 1 μg vitamin D_3 per day was 1.53 ± 0.89 nmol/L, whereas the mean increase after oral ingestion of 1 μg 25-OH-D_3 per day was 4.76 ± 1.17 nmol/L. Based on this calculation, the overall relative potency of oral 25-OH-D_3 was 3.1 fold greater when compared with oral vitamin D_3 (Quesada-Gomez and Bouillon, 2018).

25-OH-D_3 is the major circulating form of vitamin D_3, formed mainly in the liver from vitamin D_3 by hydroxylation catalysed by the cytochrome P450 enzyme, the vitamin D_3 25-hydroxylase (CYP2R1). This reaction can also occur in intestine and kidney at a lower extension. From the liver, 25-OH-D_3 enters the circulatory system transported by the vitamin D-binding protein to the kidney where a second hydroxylation at the C-1 position occurs by the 25-OH-D_3-1-α-hydroxylase (CYP27B1), originating 1α,25-$(OH)_2$-D_3. In kidney, another hydroxylated vitamin D metabolite is produced, 24R,25-$(OH)_2D_3$, catalysed by the 25-hydroxyvitamin D_3-24R-hydroxylase. CYP24A1 can also metabolise 25-OH-D_3 and 1α,25-(OH)2-D_3 into 24-hydroxylated metabolites. In vertebrates, 1α,25-

[36] Technical dossier/Section III/Annex III_1_1.
[37] Technical dossier/Section IV/Annex_IV_1_3.

(OH)2-D_3 and 24R,25-dihydroxyvitamin D_3 (24R,25-(OH)2D_3) are secreted from kidney into blood. Many other metabolites are formed, being the structure of 37 compounds already elucidated (Norman and Henry, 2007). Later, more than 50 metabolites were referred to be already identified, being calcitroic acid and lactones the final metabolites of this complex biotransformation pathways (Quesada-Gomez and Bouillon, 2018).

Vitamin D and its metabolites are excreted mainly in the faeces with the aid of bile salts, only a small fraction being excreted in the urine.

In humans, the blood half-life of 25-OH-D_3 is of 15 days while it is of 4–6 h for vitamin D (Jones, 2008).

Besides the reviews above referred, the applicant also submitted some experimental studies, mainly comparing the absorption of vitamin D_3 with 25-OH-D_3.

Rats

In the rat, Maislos et al. (1981) studied the absorption of vitamin D_3 and of two hydroxylated metabolites (25-OH-D_3, and 1,25-$(OH)_2$-D_3) from intestine into the mesenteric lymphatic and portal venous systems. With this aim, rats with mesenteric lymph fistula and portal vein cannulation were intraduodenally given the same dose of the labelled compounds. Mesenteric lymph was collected 30 min before the administration of the compounds, every 15 min in the first hour and every 30 min thereafter up to 4 h after administration. Also, blood samples were taken from both the portal and iliac veins at 0, 15, 30, 45, 60 min and every 30 min thereafter. After 4 h, the rats were killed and organs exsanguinated and removed (heart, lung, liver, spleen, kidney, duodenum, jejunum, ileum, epididymal fat and skeletal muscle) for analysis. Radioactivity was measured in lymph, serum, and dried chloroform extract of tissue homogenates. In the lymph, the peak concentration of compounds was 66, 110, and 190 pmol/h, attained at 15–30, 45–60, and 150–160 min for 1,25-OH-D_3, 25-OH-D_3, and vitamin D_3, respectively. The data show that vitamin D_3 was slowly absorbed as compared with its hydroxy metabolites. In tissues, the largest amount of vitamin D and 25-OH-D_3 was present in the intestinal wall, mainly in jejunum. Both hydroxy compounds were distributed in liver and kidney, being the levels very low in the other tissues. The kidney extract showed an additional, more polar metabolite of 25-OH-D_3 that was not identified. The sum of the concentrations of 25-OH-D_3 measured in blood, liver, kidney and heart 4 h after intraduodenal administration was approximately the double of vitamin D_3 (10.4% and 5.4% of the administered dose, respectively). Concluding, 25-OH-D_3 and 1,25-$(OH)_2$-D_3, are mainly absorbed directly into portal blood and in a higher extension as compared with vitamin D_3.

Target species

Studies in target species were also submitted, two in pigs with performance evaluation purposes (Lauridsen et al., 2010; Witschi et al., 2014) and one in birds (Bar et al., 1980).

Lauridsen et al. (2010) carried out a study in gilts from the first oestrus until day 28 of gestation and in pregnant multiparous sows from the first day of mating until weaning by giving in the diet several levels of vitamin D_3 or 25-OH-D_3 (200, 800, 1,400, 2,000 IU each). Blood samples and uterine fluids were collected from gilts at day 28 and blood at several days from sows, and also from piglets at days 4, 16 and 28 of age. Both vitamins were analysed in plasma and uterine fluids of gilts by an isotope dilution assay with HPLC-MS (LOQ: 5 ng/mL). The levels of 25-OH-D_3 increased linearly in plasma of gilts with increasing dietary levels of the two compounds, although in gilts supplemented with 25-OH-D_3, the plasma level of 25-OH-D3 was significantly higher than in gilts supplemented with vitamin D_3. No detectable 25-OH-D_3 was found in uterine fluid samples.

25-OH-D_3 in plasma of sows and piglets was measured by HPLC after saponification and extraction into heptane. In plasma of sows the levels of 25-OH-D_3 were significantly higher in animals given 25-OH-D_3 than in those given vitamin D_3 in diet at 800 IU and higher. In plasma of piglets, 25-OH-D_3 was only detected in 154 of 576 samples analysed, being the concentrations very low, although higher in piglets born of sows fed with 25-OH-D_3 (mean 4.3 ng/mL). These data show that very low levels of the compounds were transferred to the progeny.

Concluding, at concentrations higher than 200 IU, 25-OH-D_3 showed to be more bioavailable than vitamin D_3 both in gilts and in sows, being the plasma levels of 25-OH-D_3 increased by a factor of 2 to 3 when the 25-OH-D_3 was included in the diet.

Witschi et al. (2014) carried out a study in piglets by comparing the effects of vitamin D_3 with 25-OH-D_3 on growth, blood status and various bone traits of pigs from birth to 77 days of age. With this aim, primiparous and multiparous sows (13 in each treatment) were given diets supplemented with

either 5 or 50 μg of vitamin D_3 or 50 μg of 25-OH-D_3 per kg feed. By week 3 of lactation, piglets had access to a creep diet containing the same levels of the two vitamins as the corresponding dams and after weaning up to reaching a BW of approximately 20 kg. Blood was collected from randomly selected piglets of each group treatment on days 21, 33 (just before weaning) and 77 of age. 25-OH-D_3 was measured in serum by an isotope dilution assay using a reversed-phase HPLC-mass spectrometry. Serum levels of 25-OH-D_3 of piglets from sows given 25-OH-D_3 were numerically higher as early as day 21, and significantly higher in the following days 33 and 77, as compared with the two groups given vitamin D_3. At day 77, serum levels of piglets given diet supplemented with 25-OH-D_3 or vitamin D_3 were ~ 50 ng/mL and 15 ng/mL, respectively. Serum concentrations of 25-OH-D_3 decreased numerically between day 21 and day 33 in both groups given diets with vitamin D_3 but not in the group given 25-OH-D_3.

The absorption and excretion of vitamin D_3 and 25-OH-D_3 was studied in chicks and turkeys and their metabolism was tentatively evaluated (Bar et al., 1980). Animals were fed diets deficient in vitamin D_3 from day 1 to day 14. In the following 6 days, they received the same diet supplemented with labelled vitamin D_3 at 15 or 50 μg/kg feed or labelled 25-OH-D_3 at 50 μg/kg feed. At the end of day 6, animals were killed, intestine removed and divided in five segments, the contents collected for radioactivity measurement.

The absorption of 25-OH-D_3 was significantly higher than vitamin D_3 in chicks (84% vs. 67%) and (84% vs. 75%) in turkeys. The absorption of both compounds was almost complete in the upper jejunum in both species. 25-OH-D_3, polar metabolites and non-polar metabolites and esters were secreted in duodenum, in significantly greater levels in turkeys than in chicks. Some of these metabolites were reabsorbed, mainly in the upper jejunum. These metabolites were analysed by thin layer chromatography (TLC), thus their identity was not clarified. The overall daily excretion of vitamin D_3 metabolites was significantly higher (20% and 14% of the daily intake in turkeys and chicks, respectively) as compared with 7% excretion of 25-OH-D_3 metabolites.

Conclusions on ADME

Overall, the limited data available on ADME of 25-OH-D_3 in animals show that it is more rapidly and extensively absorbed than vitamin D_3, is metabolised to several hydroxylated metabolites, has a longer blood half-life, being mainly excreted in faeces as calcitroic acid and lactones. The same pattern of disposition seems to happen in humans.

If nanoparticles of 25-OH-D_3 are present, they are expected to partition and quickly solubilise into the lipophilic cell compartments, suggesting that systemic distribution of particles is unlikely to occur.

3.2.3.2. Residue studies

The applicant has provided information on residues of 25-OH-D_3 derived from a combined tolerance/efficacy study conducted in chickens for fattening. Publications were retrieved through a literature search reporting data on residues in tissues and organs of pigs, birds and ruminants, as well as residues of 25-OH-D_3 in milk. Reference was made to the previous EFSA FEEDAP Panel Opinions (EFSA, 2005, 2009) where, residue data for eggs and tissue and organs of pigs could be identified.

When evaluating the studies, the FEEDAP Panel considered that (i) 25-OH-D_3 contributes to the exposure of the consumer to vitamin D_3, for which a UL is available, (ii) other forms of vitamin D_3 are available on the market and (iii) residues of vitamin D_3 in tissues and products from target species other than poultry and pigs contribute to the exposure of the consumer; therefore, studies reporting residue data in target species different from pigs and poultry were also considered. Among the studies submitted, the FEEDAP Panel selected the studies with the more relevant design (e.g. supplementation level) and among those, the studies which showed the highest values for the residues of 25-OH-D_3 in tissues, organs and products for each class (mammals, birds).

The studies considered by the FEEDAP Panel as relevant for the estimate of the consumer exposure are herein described.

Regarding poultry, recent residue data were available from the combined tolerance-efficacy study performed with the additive containing 25-OH-D_3 produced by [redacted] (Schothorst, 2019;[38] Section 3.2.2). [redacted]

[38] Technical dossier/Section III/Annex_III_1_1_Schothorst_2019_CONF.pdf

Table 1:

Celi et al. (2018)[39] fed growing cattle (106 kg bw, 10 animals/group) for 90 days with increasing doses of 25-OH-D_3: 1.7 μg, 5.1 μg and 8.5 μg/kg bw (corresponding to 56.4, 168.7 and 249.1 μg/kg feed, respectively). The control group received 0.75 μg vitamin D_3/kg bw. Serum 25-OH-D_3 increased from 46 (control group without calcidiol) to 107, 188 and 217 ng/mL for the 25-OH-D_3 supplemented groups, respectively. All calves in the four groups gained weight continually: no growth depression was observed. No adverse effects of 25-OH-D_3 were observed for any of the haematology[40] and serum chemistry parameters[41] measured monthly or during the routine clinical examinations. In the post-mortem evaluation, no adverse effects of the different 25-OH-D_3 doses were observed, neither during the gross pathology nor in the histopathological examination. The data allow the conclusion that about 10,000 IU vitamin D from 25-OH-D_3 was well tolerated by growing cattle. Serum, fat, muscle, kidney and liver samples (10 per treatment group) were collected to evaluate the concentration of 25-OH-D_3. The concentrations of 25-OH-D_3 at day 90 of treatment in liver, muscle, kidney and fat were significantly increased in comparison to the control group (Table 2).

Table 2: Tissue content of 25-OH-D_3 (μg/kg), 10 samples per treatment

Tissue	Control	56.4 μg 25-OH-D_3/kg feed	168.7 μg 25-OH-D_3/kg feed	249.1 μg 25-OH-D_3/kg feed
Liver	4.5^c	14.5^b	27.4^a	31.2^a
Kidney	7.2^c	23.1^b	44.0^a	39.7^a
Muscle	1.8^c	5.7^b	10.8^a	12.3^a
Fat	4.1^c	13.2^b	20.7^a	26.4^a

Different letters within a row indicate statistical significance ($p < 0.05$).

For calculating consumer exposure, an average between the groups with 56.4 and 168.7 μg 25-OH-D3/kg feed was used (liver: 21.0, kidney: 33.6, muscle 8.3, fat 17.0 μg/kg) as representative of the value expected from the use of 25-OH-D_3 at the concentration of 100 μg/kg complete feed which correspond to the maximum authorised concentration for vitamin D_3 of 4,000 IU/kg complete feed.[42] Since these values were higher than those reported in studies available with pigs (Jacobsen et al.,

[39] Technical dossier/Section III/Spontaneous submission (February 2023)/Celi et al.pdf
[40] WBC, RBC, Haemoglobin, HTC, MCV, MCH, MCHC, platelets, neutrophils, Neu % of WBC, Lymphocytes, Lym % of WBC, monocytes, Mon % of WBC, eosinophils, Eos % of WBC, basophils, Bas % of WBC, large unstained cells, Luc % of WBC, protrombine time, activated partial thromboplastine time.
[41] Urea, creatinine, urea/creatinine, glucose, sodium, potassium, chloride, ALP, ALT, AST, LDH, CPK, CGT, total protein, albumin, globulin, A/G, calcium, phosphate, magnesium, cholesterol, amylase, total bile acids.
[42] Commission Implementing Regulation (EU) 2017/1492 of 21 August 2017 concerning the authorisation of cholecalciferol as a feed additive for all animal species. OJ L 216, 22.8.2017, p. 19–22.

2007; Höller et al., 2010; Burild et al., 2016; von Rosenberg et al., 2016; Duffy et al., 2018a,b), values from cattle study were taken for the consumer exposure assessment.

In the study by Rodney et al. (2018),[43] a total of 25 mid-lactation Holstein dairy cows (blocked by age and milk production) were randomly distributed to five treatment groups. The diet of the control group was left unsupplemented while the diet of the other four was supplemented with 0.5, 1, 2 or 4 mg 25-OH-D_3/cow per day for 30 days. Data on feed intake were not provided. Corresponding levels in a complete feed could be calculated based on the default values for daily feed intake of dairy cows of DM content of 88% and 20 kg DM/day (EFSA FEEDAP Panel, 2017a,b,c) and were 22, 44, 88, and 176 μg/kg complete feed. Milk volume was measured, and milk samples taken every 2 weeks to analyse milk levels of protein and fat, as well as protein and fat yield, somatic cell count and 25-OH-D_3 concentrations. The 25-OH-D_3 concentration in milk of the group fed with 88 μg 25-OH-D_3/kg complete feed was taken as representative of the value expected from the use of 25-OH-D_3 at the concentration of 100 μg/kg complete feed which correspond to the maximum authorised concentration for vitamin D_3 of 4,000 IU/kg complete feed. Following the guidance, two times the SD was added to the mean resulting in 0.465 (0.4145 + 2 × 0.0254) μg 25-OH-D_3/kg milk.

3.2.3.3. Assessment of consumer exposure

In its first opinion (EFSA, 2005), the FEEDAP Panel proposed a provisional upper tolerable limit (UL) for 25-OH-D_3 of 10 μg/day in adults and adolescents (11–17 years) and 5 μg/day in children (0–10 years). This was based on the UL for vitamin D_3 (50 μg/day in adults and 25 μg/day in children up to 11 years) and a relative biological activity factor of 5.

In the opinion of 2009, the FEEDAP Panel conducted a 'worst-case scenario' exposure assessment for the consumer, based on the consumption model described in Regulation (EC) No 429/2008 and on data from studies done with the additive at the maximum use levels for pigs and poultry species. The results indicated that exposure of adults was below the provisional UL for 25-OH-D_3 set for adults (69%) but that of children would be exceeded (138%). A refined calculation of exposure based on more realistic data (SCOOP) indicated that exposure for both adults and children would be below the provisional UL (24% and 49%, respectively). Based on that, the Panel concluded that the total exposure resulting from the use of 25-OH-D_3 in all poultry and pig categories at the maximum levels would not represent a risk for the consumer.

The FEEDAP Panel notes that the NDA Panel of EFSA has revised the UL levels for vitamin D_3 for all age groups (EFSA NDA Panel, 2012, 2018): 25 μg/day for children up to 6 months, 35 μg/day for children 6–12 months, 50 μg/day for children 1–10 years and 100 μg/day for adolescents (11–17 years) and adults, including pregnant women.

The FEEDAP Panel would like to withdraw the provisional UL for 25-OH-D_3, since also cholecalciferol from other food and body's own synthesis would enter the body's store of 25-OH-D_3. It is therefore considered reasonable to apply the UL for vitamin D_3 and to introduce the 25-OH-D_3 intake multiplied with a biopotency factor of 5.

In reassessing consumer exposure, the FEEDAP Panel is aware of the ongoing evaluation by the NDA Panel of setting a conversion factor for 25-OH-D_3 into vitamin D_3. At the time of the adoption of the current FEEDAP Panel's scientific opinion, the work of the NDA Panel has not been completed. Therefore, as a pragmatic approach, the Panel considered that the residues of 25-OH-D_3 deposited in edible tissues and products should be expressed in terms of vitamin D_3 activity, and therefore, multiplied by 5 to consider the relative biological activity of the different compounds (Table 1). This was then compared with the UL established by the NDA Panel for vitamin D_3.

New data, from literature search and a tolerance trial performed in chickens, have been made available with regard to the deposition of 25-OH-D_3 in tissues or products of pigs, poultry and ruminants not considered in the past (see 3.2.3.2). Therefore, the Panel used the new residue data to perform an exposure assessment following the methodology described in the Guidance on consumer safety (EFSA FEEDAP Panel, 2017a). The input data are reported in Table 3.

[43] Technical dossier/Section III/Spontaneous submission (February 2023)/Rodney et al., 2018.pdf

Table 3: Input data on 25-OH-D_3 and vitamin D_3 content in food of animal origin used for the consumer exposure assessment

Tissue/product	µg 25-OH-D_3/kg wet tissue/product	µg Vitamin D_3 equivalent/ kg wet tissue/product*	Reference
Birds fat tissue	24.12§	120.6	Schothorst (2019)[38]
Birds liver	29.36§	146.8	Schothorst (2019)[38]
Birds meat**	9.2§	46	Schothorst (2019)[38]
Birds offals and slaughtering products (other than liver)	25.42§	127.1	Schothorst (2019)[38]
Mammals fat tissue	17	85	Celi et al. (2018)
Mammals liver	21	105	Celi et al. (2018)
Mammals meat†	10	50	Celi et al. (2018)
Mammals offals and slaughtering products (other than liver)	33.6	168	Celi et al. (2018)
Whole eggs	13.3	66.5	EFSA (2005)
Milk	0.47	2.35	Rodney et al. (2018)

*: Calculated from the residues of 25-OH-D_3 by multiplying with the relative biological activity factor of 5.
**: 90% breast muscle + 10% skin/fat.
†: 80% muscle +20% fat.
§: Data are the mean + 2 SD.

The results of the dietary exposure to residues of 25-OH-D_3 calculated as vitamin D_3 equivalents for the different population categories are reported in Table 4. The detailed results are given in Appendix A.

Table 4: Chronic human dietary exposure to 25-OH-D_3 expressed as vitamin D_3 equivalents

Population class	Maximum HRP* (µg/kg body weight per day)	Default body weight† (kg)	Exposure (µg/day)	Upper tolerable level (µg/day)	% UL
Infants	0.7450	5	2.8	25	11.2
Toddlers	0.8759	12	10.51	50	21.05
Other children	0.8390	23	19.29	50	38.58
Adolescents	0.5617	52.4**	29.43	100	29.43
Adults	0.4243	70	29.7	100	29.7
Elderly	0.3217	70	22.4	100	22.4
Very elderly	0.3335	70	23.34	100	23.34

*: HRP: highest reliable percentile
**: Average of 43.4 and 61.3 kg.
†: EFSA Scientific committee (2012).

The FEEDAP Panel noted that some uncertainties exist in relation to the exposure calculation (e.g. the standard deviations and the levels of 1,25-$(OH)_2$-D_3, an active metabolite of 25-OH-D_3, were not available). However, the FEEDAP Panel considered these limitations not having a significant impact on the exposure calculation.

To compare the exposure to residues of 25-OH-D_3 transformed to vitamin D_3 to the UL of vitamin D_3, the FEEDAP Panel used the highest reliable percentile (HRP) for the different population categories and converted it from µg/kg body weight (bw) to µg/person and day using the default values for body weight (EFSA Scientific Committee, 2012). For the population group infants, the lowest UL of 5 µg/day has been used. The contribution to consumer exposure to Vitamin D_3 from products of animals fed with the additive ranged from 11.2% to 38.58% of the UL (Table 4).[38]

The FEEDAP Panel concludes that there is no safety concern for the consumer resulting from the intake of food from pigs, poultry and ruminants fed with 25-OH-D_3.

3.2.3.4. Conclusions on safety for the consumer

The FEEDAP Panel concluded that the exposure calculation does not indicate any risk for the consumer resulting from the use of food originating from animals fed with 25-OH-D_3. However,

considering the lack of adequate toxicological data and the uncertainties on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product, the FEEDAP Panel cannot conclude on the safety for the consumers.

3.2.4. Safety for the user

No data on respiratory toxicity was provided. Considering the low dusting potential of the additive (up to 60 mg/m^3), the FEEDAP Panel considered that exposure through inhalation is unlikely.

The skin irritation potential of the additive was tested *in vitro* according to OECD TG 439.[44] The results of the study indicated that the formulated additive is not irritant to the skin and is, therefore, classified according to the UN GHS as 'No Category'.

The eye irritation potential of the additive was investigated *in vitro* according to OECD TG 438.[45] The results of the study showed that the formulated additive is not irritant to eyes and is, therefore, classified according to the UN GHS as 'No Category'.

The skin sensitisation potential of the additive was investigated in a study conducted in compliance with the OECD TG 429.[30] The results of the study indicated that the formulated additive is not a skin sensitiser.

3.2.4.1. Conclusions on safety for the user

Based on the studies submitted, the additive was shown not be irritant to skin or eyes. It is not a skin sensitiser. Considering the low dusting potential of the additive, the FEEDAP Panel considered that the exposure through inhalation is unlikely.

However, the FEEDAP Panel considered that uncertainties remain on genotoxicity and on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product.

3.2.5. Safety for the environment

The active substance present in the additive occurs in nature, and its use in animal nutrition is not expected to substantially increase the concentration in the environment. The strain *P. autotrophica* DSM 32858 is non-genetically modified, and no DNA of the production strain was detected in the additive using a test with a sensitivity of 10 ng of genomic DNA/mL. Although, uncertainty remains on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product, a risk for the environment resulting from the use of the additive under assessment in animal nutrition is not foreseen.

3.3. Efficacy

According to Regulation (EC) No 429/2008, efficacy studies are not required for vitamins, pro-vitamins and chemically defined substances having similar effects that are already authorised as feed additives under Directive 70/524/EEC.

Since 25-OH-D_3 is already authorised for chickens for fattening, turkeys for fattening, other poultry and pigs,[46] and EFSA concluded that its use is efficacious as a substitute for vitamin D_3 (EFSA, 2005, 2009), no new demonstration of efficacy would be necessary.

The applicant has provided three studies in chickens for fattening to support the efficacy of 25-OH-D_3. Two of them are available in publications (Goodgame et al., 2011; Leyva-Jimenez et al., 2019), and the third one is the combined tolerance-efficacy study[37] already mentioned under Section 3.2.2. The test article used in the three studies in chickens for fattening was a fermentation product containing the active substance 25-OH-D_3 produced by [redacted] (the production strain of the additive under assessment). The Panel considers that these studies could be used to support the efficacy of the additive, as the effects of the active substance would be the same.

In the study included in Leyva-Jimenez et al. (2019), a challenge was induced to the birds with live coccidiosis vaccine intended to induce intestinal damage. This approach was not considered adequate by the Panel as evidence of the efficacy. Therefore, the study was not considered further for the assessment.

[44] Technical dossier/Section III/Annex_III.3.3.
[45] Technical dossier/Section III/Annex_III.3.1.
[46] Commission Regulation (EC) No 887/2009 of 25 September 2009.

Goodgame et al. (2011) described one bioequivalence trial in which the dietary supplementation of increasing levels of the test item (25-OH-D_3 produced by *P. autotrophica* 10 M213) was compared with 25-OH-D_3 from a different source. One old-day chicks received a basal diet with no vitamin D containing marginal levels of calcium and phosphorus for 7 days. Thereafter, the basal diet was supplemented with the two 25-OH-D_3 sources at increasing levels from 2.5 to 80 μg/kg complete feed and offered to the chickens for 14 days. The study assessed the effect of both sources of 25-OH-D_3 on the zootechnical performance and tibia diameter and breaking force of chickens for fattening. The results showed increases in the tibia ash content with increasing levels of 25-OH-D_3; no statistical differences were found between the two sources of 25-OH-D_3.

The tolerance/efficacy trial assessed the effect of the dietary supplementation of chickens for fattening [redacted]

3.3.1. Conclusions on the efficacy

The FEEDAP Panel concludes that 25-OH-D_3 is an efficient source of vitamin D_3 under the proposed conditions of use.

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[47] and Good Manufacturing Practice.

4. Conclusions

The production strain *P. autotrophica* DSM 32858 is not genetically modified however, uncertainties remain on the possible presence of its viable cells in the final product.

Due to the lack of adequate safety data and uncertainty on the presence of nano particles, the FEEDAP Panel cannot conclude on the safety of the additive for the target species and the consumer.

The additive is not irritant to skin or eyes and is not a skin sensitiser and the exposure through inhalation is unlikely. However, the FEEDAP Panel considers that uncertainties remain on genotoxicity and on the possible presence of viable cells of *P. autotrophica* DSM 32858 in the final product which might have an impact on the safety for the users.

The use of the feed additive is considered safe for the environment.

The additive is regarded as an effective dietary source of the vitamin D_3 under the proposed conditions of use.

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Abbreviations

AMR	Antimicrobial resistance
BW	body weight
CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
dDDH	DNA–DNA hybridization
DM	dry matter
DSMZ	German Collection of Microorganisms and Cell Cultures
EINECS	European Inventory of Existing Chemical Substances
EURL	European Union Reference Laboratory
LOD	limit of detection
MW	molecular weight
NLT	Not less than

NMT	Not more than
OECD	Organisation for Economic Co-operation and Development
RH	relative humidity
TEM	transmission electron microscopy
TLC	thin layer chromatography
SEM	scanning electron microscopy
VFDB	Virulence Factor Database

Appendix A – Calculation of the consumer exposure with FACE model

A.1. Methodology

As described in the Guidance on the safety of feed additives for consumers (EFSA FEEDAP Panel, 2017a,b,c), consumption data of edible tissues and products as derived from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) will be used to assess exposure to residues from the use of feed additives in different EU countries, age classes 35 and special population groups. For each EU country and age class, only the latest survey available in the Comprehensive Database will be used.

While the residue data reported for feed additives refer to organs and tissues (raw agricultural commodities (RAC)), the Comprehensive Database includes consumption data for foods as consumed.

In order to match those consumption data with the available residue data for feed additives, the consumption data reported in the Comprehensive Database have been converted into RAC equivalents. For assessing the exposure to coccidiostats from their use in (non-reproductive) poultry, the following list of commodities is considered: meat, fat, liver, other offals (including kidney).

Depending on the nature of the health-based guidance derived, either a chronic or acute exposure assessment may be required.

For chronic exposure assessments, the total relevant residues will be combi ned for each individual with the average daily consumptions of the corresponding food commodities, and the resulting exposures per food will be summed in order to obtain total chronic exposure at individual level (standardised by using the individual body weight). The mean and the higher percentile (usually the 95th percentile) of the individual exposures will be subsequently calculated for each dietary survey (country) and each age class separately.

As opposed to the chronic exposure assessments, acute exposure calculation will be carried out foreach RAC value separately. The higher percentile (usually the 95th percentile) exposures based on the consuming days only will be calculated for each food commodity, dietary survey and age class separately.

As described in the Guidance on the safety of feed additives for consumers (EFSA FEEDAP Panel, 2017a,b,c), consumption data of edible tissues and products as derived from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) will be used to assess exposure to residues from the use of feed additives in different EU countries, age classes[48] and special population groups. For each EU country and age class, only the latest survey available in the Comprehensive Database will be used.

While the residue data reported for feed additives refer to organs and tissues (raw agricultural commodities (RAC)), the Comprehensive Database includes consumption data for foods as consumed. In order to match those consumption data with the available residue data for feed additives, the consumption data reported in the Comprehensive Database have been converted into RAC equivalents. For assessing the exposure to vitamin E from their use in (non-reproductive) poultry, the following list of commodities is considered: meat, fat, liver, other offals (including kidney).

Depending on the nature of the health-based guidance derived, either a chronic or acute exposure assessment may be required.

For chronic exposure assessments, the total relevant residues will be combined for each individual with the average daily consumptions of the corresponding food commodities, and the resulting exposures per food will be summed in order to obtain total chronic exposure at individual level (standardised by using the individual body weight). The mean and the higher percentile (usually the 95th percentile) of the individual exposures will be subsequently calculated for each dietary survey (country) and each age class separately.

As opposed to the chronic exposure assessments, acute exposure calculation will be carried out foreach RAC value separately. The higher percentile (usually the 95th percentile) exposures based on the consuming days only will be calculated for each food commodity, dietary survey and age class separately.

[48] Infants: < 12 months old, toddlers: ≥ 12 months to< 36 months old, other children: ≥ 36 months to< 10 years old, adolescents: ≥ 10 years to< 18 years old, adults: ≥ 18 years to< 65 years old, elderly: ≥ 65 years to< 75 years old, and very elderly: ≥ 75 years old.

Appendix B – Detailed results on chronic exposure calculation

Chronic dietary exposure per population class, country and survey (μg/kg bw per day) of consumers to 25-OH-D_3 expressed as Vitamin D_3 equivalents based on residue data in birds, mammals, eggs and milk (Table B.1).

Table B.1: Chronic dietary exposure per population class, country and survey (μg/kg bw per day) of consumers to 25-OH-D_3 expressed as Vitamin D_3 equivalents based on residue data

Population class	Survey's country	Number of subjects	HRP value	HRP description
Infants	Bulgaria	523	0.7450022317	95th
Infants	Germany	142	0.3850803016	95th
Infants	Denmark	799	0.5491984875	95th
Infants	Finland	427	0.3678252621	95th
Infants	Italy	9	0.1509555380	50th
Infants	United Kingdom	1,251	0.4563871791	95th
Toddlers	Belgium	36	0.6522598489	90th
Toddlers	Bulgaria	428	0.8760772253	95th
Toddlers	Germany	348	0.6445679806	95th
Toddlers	Denmark	917	0.6724526521	95th
Toddlers	Spain	17	0.7039212780	75th
Toddlers	Finland	500	0.6725109581	95th
Toddlers	Italy	36	0.5864118170	90th
Toddlers	Netherlands	322	0.6446175566	95th
Toddlers	United Kingdom	1,314	0.6298049012	95th
Toddlers	United Kingdom	185	0.5823670692	95th
Other children	Austria	128	0.7444387911	95th
Other children	Belgium	625	0.7077684235	95th
Other children	Bulgaria	433	0.8390611726	95th
Other children	Germany	293	0.6103110597	95th
Other children	Germany	835	0.5259762448	95th
Other children	Denmark	298	0.5867147761	95th
Other children	Spain	399	0.6574173213	95th
Other children	Spain	156	0.8287025673	95th
Other children	Finland	750	0.6949466037	95th
Other children	France	482	0.6897141476	95th
Other children	Greece	838	0.6470680441	95th
Other children	Italy	193	0.6453611288	95th
Other children	Latvia	187	0.5796069006	95th
Other children	Netherlands	957	0.5258476099	95th
Other children	Netherlands	447	0.5333055335	95th
Other children	Sweden	1,473	0.6032802317	95th
Other children	Czechia	389	0.7053451398	95th
Other children	United Kingdom	651	0.5253527249	95th
Adolescents	Austria	237	0.4023532622	95th
Adolescents	Belgium	576	0.3087887923	95th
Adolescents	Cyprus	303	0.2791427244	95th
Adolescents	Germany	393	0.4166515877	95th
Adolescents	Germany	1,011	0.3005758954	95th
Adolescents	Denmark	377	0.3428633016	95th
Adolescents	Spain	651	0.4332848472	95th
Adolescents	Spain	209	0.5616639524	95th
Adolescents	Spain	86	0.4179358905	95th
Adolescents	Finland	306	0.3280495137	95th

Population class	Survey's country	Number of subjects	HRP value	HRP description
Adolescents	France	973	0.4010288412	95th
Adolescents	Italy	247	0.4125892773	95th
Adolescents	Latvia	453	0.4241660620	95th
Adolescents	Netherlands	1,142	0.3872767043	95th
Adolescents	Sweden	1,018	0.3840512494	95th
Adolescents	Czechia	298	0.5490984304	95th
Adolescents	United Kingdom	666	0.3052960909	95th
Adults	Austria	308	0.3215677844	95th
Adults	Belgium	1,292	0.2816339890	95th
Adults	Germany	10,419	0.2869111740	95th
Adults	Denmark	1,739	0.2404671619	95th
Adults	Spain	981	0.3637672293	95th
Adults	Spain	410	0.3424286610	95th
Adults	Finland	1,295	0.2937718706	95th
Adults	France	2,276	0.2917429461	95th
Adults	Hungary	1,074	0.4023400493	95th
Adults	Ireland	1,274	0.2886443846	95th
Adults	Italy	2,313	0.2580146491	95th
Adults	Latvia	1,271	0.3539020823	95th
Adults	Netherlands	2,055	0.2908355980	95th
Adults	Romania	1,254	0.4115642830	95th
Adults	Sweden	1,430	0.3240267418	95th
Adults	Czechia	1,666	0.4242910131	95th
Adults	United Kingdom	1,265	0.2371597300	95th
Elderly	Austria	67	0.2930368984	95th
Elderly	Belgium	511	0.2538984079	95th
Elderly	Germany	2,006	0.2592497619	95th
Elderly	Denmark	274	0.2205050348	95th
Elderly	Finland	413	0.2333541790	95th
Elderly	France	264	0.2497210432	95th
Elderly	Hungary	206	0.3086132945	95th
Elderly	Ireland	149	0.2898186061	95th
Elderly	Italy	289	0.2301052798	95th
Elderly	Netherlands	173	0.2485388531	95th
Elderly	Netherlands	289	0.2226365625	95th
Elderly	Romania	83	0.3216829788	95th
Elderly	Sweden	295	0.2884723420	95th
Elderly	United Kingdom	166	0.1982912679	95th
Very elderly	Austria	25	0.2121490534	75th
Very elderly	Belgium	704	0.2684282211	95th
Very elderly	Germany	490	0.2509591739	95th
Very elderly	Denmark	12	0.1667834988	75th
Very elderly	France	84	0.2565200868	95th
Very elderly	Hungary	80	0.3117110068	95th
Very elderly	Ireland	77	0.2898678536	95th
Very elderly	Italy	228	0.1947195642	95th
Very elderly	Netherlands	450	0.2320893908	95th
Very elderly	Romania	45	0.3335350357	90th
Very elderly	Sweden	72	0.3230392579	95th
Very elderly	United Kingdom	139	0.2119689750	95th