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SCIENTIFIC OPINION

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Safety and efficacy of the feed additive Anpro consisting of a mixture of Sepiolite and Kieselguhr (diatomaceous earth) for all terrestrial animal species (Anpario plc)

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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 the mixture of Sepiolite and Kieselguhr (diatomaceous earth) (Anpro) as a technological feed additive for all terrestrial animal species. According to the conventional risk assessment, due to lack of adequate data, the safety of the additive Anpro for the target species cannot be established. Based on current knowledge, there is no indication of substantial absorption of the components of the additive and therefore no concern for the consumer. The additive poses a risk by inhalation. It is not irritant to the skin or eyes but should be considered as skin sensitiser. As no suitable data in line with the requirements of the Guidance on risk assessment of nanomaterials were provided by the applicant, the potential risks associated with the presence of nanoparticles could not be assessed for the target species, the consumer and the user. The additive is safe for the environment. The FEEDAP Panel is not in the position to conclude on the efficacy of the additive for all terrestrial animal species.

KEYWORDS

diatomaceous earth, efficacy, safety, sepiolite, substances for the reduction of mycotoxin contamination, technological additives, terrestrial animals

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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 a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Anpario plc[2] for authorisation of the product preparation of sepiolite and diatomaceous earth, when used as a feed additive for all terrestrial animal species (category: technological additives; functional group: substances for reduction of the contamination of feed by mycotoxins).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 18 June 2019.

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

1.2 | Additional information

The present assessment concerns the feed additive Anpro, a mixture of the additives Sepiolite and Kieselguhr (diatomaceous earth) for all terrestrial animal species. Both components are currently authorised in the EU as technological feed additives (binders, anticaking agents and coagulants) for all animal species/categories: Kieselguhr (diatomaceous earth, E551c)[3] with no maximum use level, and Sepiolite (E562)[4] with a maximum use level of 20,000 mg/kg complete feed.

The term 'sepiolite' is reported throughout the opinion with capital letter when it refers to the feed additive itself and in lower case letter when it refers to the mineral contained in the additive under assessment.

The FEEDAP Panel delivered an opinion on the re-evaluation of Sepiolite in 2022 (EFSA FEEDAP Panel, 2022) and the re-evaluation of Kieselguhr (diatomaceous earth) is currently ongoing.

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[5] in support of the authorisation request for the use of a preparation of sepiolite and diatomaceous earth as a feed additive.

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

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the additive (sepiolite and diatomaceous earth) in animal feed.[6]

2.2 | Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the preparation of sepiolite and diatomaceous earth is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEEDAP Panel, 2017a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on studies

[1]Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2]Anpario plc. Unit 5, Manton Wood Enterprise Park. S80 2RS. Worksop, Nottinghamshire. UK, represented in the EU by Pen & Tec Consulting SLU, Plaza Ausias March 1, 4° planta, D01, 08195, Sant Cugat del Vallès, Barcelona, Spain.

[3]Commission Regulation (EC) No 2439/1999. Currently under re-evaluation.

[4]Commission Regulation (EC) No 2439/1999. Currently under re-evaluation.

[5]FEED dossier reference: FAD-2019-0011.

[6]The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2019-0011_en

[7]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.

concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (EFSA Scientific Committee, 2021a), Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health (EFSA Scientific Committee, 2021b).

3 | ASSESSMENT

The additive Anpro is a mixture of the additives Sepiolite and Kieselguhr (diatomaceous earth) and is proposed to be used in feed for all terrestrial animal species as a technological additive (functional group 'substances for the reduction of the contamination of feed by mycotoxins: aflatoxin B1 (AFB1)').

3.1 | Characterisation

3.1.1 | Characterisation of the additive

Anpro is a mixture of the additives Sepiolite and Kieselguhr (diatomaceous earth), at a ratio of [redacted].

The two components of the additive (Sepiolite and Kieselguhr [diatomaceous earth]) are obtained from mining. The additive Anpro is produced mixing the two components in the proposed ratio sepiolite/diatomaceous earth of [redacted].

Data on mineralogical composition by X-ray diffraction (XRD) was submitted for five batches of the additive Anpro and for five batches of Sepiolite and Kieselguhr (diatomaceous earth) used to formulate the additive (Table 1).[8,9,10]

TABLE 1 Mineralogical composition (%) of Anpro, Sepiolite and Kieselguhr (diatomaceous earth).

Mineral	Anpro (range)	Sepiolite (range)	Kieselguhr (diatomaceous earth) (range)
Sepiolite	36.5 (35.5–38.7)	52.4 (50.3–54.3)	nd
Vermiculite	22.4 (20.5–26.1)	22.1 (20.3–24.8)	nd
Smectite	21.2 (19.0–22.9)	nd	86.5[a] (85.6-87.5)
Alkali Feldspar	5.5 (5.1–6.1)	7.5 (7.0–8.0)	0.6 (0.3–0.7)
Illite	5.5 (5.0–6.4)	7.3 (6.5–8.2)	4.8 (4.2–5.6)
Quartz	3.9 (2.3–4.8)	2.5 (1.8–3.6)	2.6 (2.3–2.8)
Dolomite	2.2 (1.8–2.6)	3.8 (3.5–4.0)	nd
Albite	1.6 (1.2–2.1)	3.1 (2.6–3.5)	3.1 (2.8–3.4)
Jarosite	0.9 (0.4–1.4)	nd	1.5 (1.3–1.8)
Calcite	0.4 (0.3–0.5)	1.1 (0.8–1.9)	nd
Enstatite	nd	0.2 (0–1)	nd
Prehnite	nd	nd	1.0 (0.5–1.3)

Abbreviation: nd, not detected.

[a]Diatomaceous earth should not contain a so high amount of smectite. According to the certificates of analysis provided: 'The smectite phase shows a basal spacing of about 14.9 Å (Angstroms), with a very broad peak centred at about 5.9 degrees two theta. The broadness of the peak makes distinguishing the smectite phase difficult without additional work, i.e. heat treatment and glycolation of the mounted clay portion'.

The applicant provided specifications for Anpro based on the elemental composition expressed as oxides: silicon dioxide ≥ 54%, magnesium oxide ≥ 15% and aluminium oxide ≥ 7%.

Quantitative and qualitative analysis to support the elemental composition by X-ray fluorescence (XRF) of five batches of Anpro were submitted.[11] The data confirmed compliance with the specification set by the applicant for the main elements, except for the content of aluminium oxide which was slightly below the specification of 7% in two of the batches tested (6.78% and 6.83%, respectively) (Table 2).

[8]Technical dossier/Section II/ Annex_II_2_1_1_0_Composition_XRD.pdf.
[9]Technical dossier/Supplementary information September 2019/Annex_II_1_3_2_1_Batch_to_batch_XRD.pdf.
[10]Technical dossier/Supplementary information January 2022/0_Appl_24Jan21_Sln_May21_signed.
[11]Technical dossier/Section II/Annex II.1.3.2.

TABLE 2 Average elemental composition of Anpro expressed as respective oxides and loss on ignition.

Element[1]	Mean (%)	Range (%)	Element[1]	Mean (%)	Range (%)
SiO_2	56.3	54.6–57.6	Na_2O	0.6	All batches
Al_2O_3	7.0	6.8–7.2	TiO_2	0.4	0.3–0.4
K_2O	1.7	1.6–1.8	MnO	0.1	All batches
CaO	1.7	1.6–1.8	P_2O_5	0.1	All batches
Fe_2O_3	2.3	2.2–2.3	Loss on ignition	13.4	11.4–16.2
MgO	16.2	15.7–16.6			

[1]Expressed as oxide.

Aflatoxin B1 (AFB1) binding capacity[12] of Anpro, was measured *in vitro* at pH 3 and pH 7.[13] Values of 18.2 and 20.0 g/tonne feed,[14] were found and were taken as specification.

Three batches of Anpro were examined for impurities: levels (in mg/kg additive) of As were 8.3–9.0, Cd 0.19–0.21, Pb 3.8–4.0, Hg < 0.1 and F 311–402. Dioxins (Polychlorinated dibenzo-p-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF)), expressed as World Health Organization (WHO) PCDD/F toxic equivalents (TEQ) ranged from 0.32 to 0.36 ng WHO-PCDD/F-TEQ/kg and the sum of dioxins plus dioxins like polychlorinated biphenyl (PCBs) from 0.46 to 0.49 ng WHO-PCDD/F-PCB-TEQ/kg.[15] The contribution of the fluorine from the additive to complete feed when Anpro would be added at the maximum recommended level of 5000 mg/kg feed would result in 2 mg/kg, which is in compliance with the maximum content foreseen in the legislation.[16]

Data on nickel (Ni) content of three batches of the additive were provided, indicating an average content of 4.97 mg Ni/kg additive (4.81–5.09 mg/kg).[17]

The FEEDAP Panel considers that the amounts of the detected impurities do not raise safety concerns with the exception of nickel, which is addressed in the user safety section (see Section 3.2.4).

3.1.2 | Interference of Anpro with the analysis of mycotoxins in feed

The capacity of Anpro to interfere with the analytical determination of mycotoxins in feed, was studied in a total of five feeds for chickens for fattening (one feed), pigs (three feeds) and laying hens (one feed).[18] Different mycotoxins (AFB1, fumonisins, zearalenone and deoxynivalenol (DON)) were determined in basal and/or mycotoxin spiked feeds and then compared to the mycotoxin content in the Anpro supplemented diets (5000 mg/kg complete feed). Results showed that Anpro, when included in feeds at the maximum level of 5000 mg/kg feed, did not interfere with the analysis of mycotoxins in feed.

3.1.3 | Physical properties of the additive

The additive Anpro is an off-white powder. Three batches of the additive were analysed for bulk density (688 kg/m^3), density (862 kg/m^3) and solid density (2560 kg/m^3).[19]

The dusting potential, analysed in three batches of the additive, resulted in an average of 2260 mg/m^3 (range: 2130–2420 mg/m^3).[20]

The analysis of particle size distribution by laser diffraction (three batches) of Anpro showed that 100% of particles have a diameter < 50 μm and that on average 76.7% (range 75.5%–77.6%) of the particles is < 10 μm.[21] The applicant provided additional data on particle size distribution of three further batches analysed by laser diffraction with a wet dispersion method.[22,23] The results showed that, on average, 0.13% of the particles were below 250 nm, and no particles were identified with ≤ 100 nm.

It was noted that the particle size data made available did not allow the risk assessors to exclude the presence of small/nano particles as foreseen in the Guidance on technical requirements for regulated food and feed product applications to

[12]Expressed as the quantity of the additive to be added to 1 tonne of mycotoxin contaminated feed (containing 1 mg mycotoxin/kg) to achieve a 50% reduction of the adsorbable mycotoxin, within a range of pH 3–7.

[13]Technical dossier/Section II/Annex IV.1.1.

[14]The figures represent the quantity of Anpro to be added to 1 tonne of AfB1 contaminated feed (containing 1 mg AfB1/kg) to achieve a 50% reduction of the absorbable mycotoxin. Therefore, at pH 7, 0.02 kg of Anpro should be added to a 1 ton of AfB1 contaminated feed (containing 1 mg AfB /kg) to achieve a 50% reduction of the absorbable AfB1.

[15]Technical dossier/Section II/Annex II.1.4.1 and Annex II.1.4.2.

[16]From 30 to 350 mg F/kg feed, depending on the species/categories for which the feed is manufactured.

[17]Technical dossier/Supplementary information September 2019/Annex_II_1_4_3.

[18]Technical dossier/Section II/Annex II.4.4.1.

[19]Technical dossier/Section II/Annex II.1.5.2. and Annex II.1.5.3.

[20]Technical dossier/Section II/Annex II.1.5.4.

[21]Technical dossier/Section II/Annex II.1.5.1.

[22]Technical dossier/Supplementary information September 2019/Annex_II_1_5_5.

[23]The wet dispersion technique increases the sensitivity of the method and allows smaller particles to be detected (up to 0.01 μm or 10 nm) in comparison to dry dispersion (up to 0.1 μm or 100 nm).

establish the presence of small particles including nanoparticles (EFSA Scientific Committee, 2021a). Therefore, the applicant was requested to provide information by choosing any of the appraisal routes as indicated by the aforementioned guidance document. The applicant submitted[24] analysis of the particle size from five batches of Anpro using [redacted] following the criteria of the Guidance on technical requirements (EFSA Scientific Committee, 2021a). [redacted] Thus, the data confirmed that the additive under assessment contains small particles including nanoparticles (> 10% of the particles of the sub-500 nm fraction have at least one external dimension smaller than 250 nm).

3.1.4 | Stability and homogeneity

Stability studies are normally not required for mineral-based additives. However, studies supporting the shelf life of the additive and its stability in feeds were provided and are described below.

The stability of the additive's ability to bind AFB1 from a phosphate buffer solution at pH 7 was evaluated in three batches of Anpro.[25] The AFB1 adsorption was determined after manufacturing and after 3, 6 and 12 months of storage. No reduction on the AFB1 adsorption values was observed during storage time. The mean values of AFB1 adsorption measured for all batches were within 98.1%–99.2%.

A feed for pigs for fattening, in both mash and pelleted form, supplemented with 0 or 5000 mg Anpro/kg feed, was stored for up to 3 months. No substantial losses of aluminium oxide and silicon dioxide concentration (using XRF method) were observed.

Stability of Anpro was also confirmed measuring AFB1 adsorption in different matrixes over time. A commercial mineral vitamin premixture and a complete feed for pigs for fattening, in both mash and pelleted form, were supplemented with 5000 mg Anpro/kg feed. The AFB1 adsorption was determined after manufacturing (time 0) and after 3 and 6 months of storage for the premixture, and after 1, 2 and 3 months of storage for the feed. No loss of the capacity to bind AFB1 was observed in the premixture. In both forms of the feed a significant difference was observed in the capacity to bind AFB1 after 3 months of storage, compared to the values measured at time 0 (mash feed: time 0: 83.8%, 3 months: 76.4%; pellet feed: time 0: 93.4%, 3 months: 84.5%).

The capacity of Anpro to homogeneously distribute in feed was analysed measuring the recovery of aluminium oxide and silicon dioxide in 10 subsamples of a mash and of a pelleted feed for pigs for fattening, supplemented with 5000 mg Anpro/kg.[26] The results showed coefficients of variation (CV) for aluminium oxide and silicon dioxide of 12.7% and 8.9%, respectively, in the mash feed, and of 0% and 1.2%, respectively, in the pelleted feed.

3.1.5 | Conditions of use

The additive is intended to be used in feed for all terrestrial animal species at the minimum and maximum concentrations of 500 and 5000 mg/kg complete feed, respectively.[27]

3.2 | Safety

The studies submitted in the dossier considering a conventional risk assessment are described below.

The Panel notes that the additive contains nanoparticles and no suitable data in line with the requirements of the Guidance on risk assessment of nanomaterials (EFSA Scientific Committee, 2021b) were provided by the applicant. Considerations on the implication of the presence of nanoparticles on the safety assessment are made at the end of the section.

3.2.1 | Genotoxicity studies

The applicant focused the investigation on the assessment of the potential genotoxicity of the soluble part of the additive (if any) by testing a homogenous suspension of each of the two components of the additive separately. These studies are described below.

[24]Technical dossier/2023-04-11_FAD-2019-0011_Additional Data_(SIn) Reply.

[25]Technical dossier/Section II/Annex II.4.1.1. The analysis was done using the AfB1 adsorption method (HPLC-FLD).

[26]Technical dossier/Section II/Annex II.4.2.

[27]Technical dossier/Supplementary information September 2019.

3.2.1.1 | *Sepiolite*

In order to investigate the potential of Sepiolite to induce gene mutations in bacteria, an Ames test was performed according to OECD Test Guideline (TG) 471 (1997) and claiming compliance with Good Laboratory Practice (GLP) in Salmonella Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102.[28] Based on the results of a cytotoxicity assay, a uniform homogenous suspension of Sepiolite in Milli-Q water was tested at five concentration levels ranging from 312.5 to 5000 μg/plate. Two independent experiments were performed applying the plate incorporation and preincubation methods both in the presence and absence of metabolic activation. No cytotoxicity was observed. No increase in the mean number of revertant colonies was observed at any tested condition in any tester strain. Therefore, the FEEDAP Panel concludes that the soluble part (if any) of a water suspension of the test item, did not induce gene mutations in bacteria under the experimental conditions applied in the study.

An *in vitro* micronucleus assay was carried out according to OECD TG 487 and claiming compliance with GLP to evaluate the potential of Sepiolite to induce chromosome damage in human peripheral blood lymphocytes.[29] Based on the results of a preliminary cytotoxicity test and the occurrence of precipitation interfering with the analysis, three concentrations of Sepiolite suspended in growth medium were selected (62.5, 125 and 250 μg/mL) for the short treatment (3 + 23 h recovery), in the absence and presence of metabolic activation, and for the continuous treatment (26 + 0 h recovery), in the absence of metabolic activation. Cytochalasin B, blocking cytokinesis to obtain binucleated cells for the analysis of micronuclei, was added at the end of treatment for the short-term exposure cultures and along with treatment for the continuous exposure cultures. Cytotoxicity up to 28% relative to vehicle control (growth medium) cultures was observed at the highest concentration after continuous treatment in the absence of metabolic activation. No increase in the frequency of micronuclei was observed in binucleated cells after treatment with Sepiolite. The FEEDAP Panel concludes that the soluble part (if any) of a water suspension of the test item in culture medium did not induce structural and numerical chromosome aberrations in cultured human peripheral blood lymphocytes under the experimental conditions employed in the study.

3.2.1.2 | *Kieselguhr (diatomaceous earth)*

An Ames test was performed to assess the potential of Kieselguhr to induce gene mutations in bacteria.[30] The study was performed in *S.*Typhimurium strains TA98, TA100, TA1535, TA1537, TA102 in accordance with OECD TG 471 and claiming compliance with GLP. The test item was suspended homogeneously in water. Five concentrations ranging from 312.5 to 5000 μg/plate were tested by plate incorporation and preincubation methods in the absence and presence of metabolic activation. No precipitation and cytotoxicity were observed. No biologically relevant increase in the number of revertant colonies was detected in any strain and treatment condition. The FEEDAP Panel concludes that the soluble part (if any) of a water suspension of the test item did not induce gene mutations in bacteria both in the presence and absence of metabolic activation.

The *in vitro* mammalian cell micronucleus test with human lymphocytes was used to evaluate the potential of Kieselguhr to induce chromosome damage, in compliance with the OECD TG 487 and claiming compliance with GLP.[31] Based on the results of the preliminary cytotoxicity test and the occurrence of precipitation interfering with the analysis, three concentrations of the test item suspended in growth medium (i.e. 31.25, 62.5 and 125 μg/mL) were selected for short treatment (3+ 23 h of recovery), both in the presence and absence of metabolic activation, and long treatment (26 + 0 h of recovery), in the absence of metabolic activation. Cytochalasin B, blocking cytokinesis to obtain binucleated cells for the analysis of micronuclei, was added at the end of short-term exposure cultures and along with treatment for continuous exposure treatment cultures. Cytotoxicity up to 16.9% relative to the vehicle control (growth medium) cultures was observed at the highest concentration tested after long treatment in the absence of metabolic activation, while levels ranging from 11.59% to 15.94% were detected after short exposure with and without metabolic activation. No increase of the frequency of micronuclei was observed in binucleated cells after treatment with the test item in any of the experimental conditions. The FEEDAP Panel concludes that the soluble part (if any) of a suspension of the test item in culture medium did not induce structural and numerical chromosome aberrations in human lymphocytes both in the presence and absence of metabolic activation under the experimental conditions employed in the study.

Based on these results, the FEEDAP Panel concludes that the soluble part (if any) of the two components of the additive shows no genotoxic potential under the tested conditions. No conclusions can be drawn from the test results on the genotoxic potential of the particulate fraction of the suspension, since in the Ames test, particles do not penetrate the bacterial cell wall and the *in vitro* mammalian cell micronucleus test was performed without specific adaptations of the test design for the assessment of the particulate fraction.

[28]Technical dossier/Supplementary information January 2021/Annex 2_Reverse_mutation_test_report and Supplementary information January 2022/ 0_Appl_24Jan21_SIn_May21.

[29]Technical dossier/Supplementary information January 2021/Annex 3_Micronucleus_test_report and Supplementary information January 2022/ 0_Appl_24Jan21_SIn_May21.

[30]Technical dossier/Supplementary information January 2022/ 4_Annex_3.

[31]Technical dossier/Supplementary information January 2022/ 5_Annex_4.

3.2.2 | Safety for the target species

The applicant provided three tolerance trials (one with chickens for fattening,[32] one with weaned pigs[33] and another one with lambs[34]) and a literature search to support the safety for the target species.

The maximum intended overdose level applied in the two trials with chickens and pigs was 4× the proposed maximum concentration, while in the study in lambs it was 5x the proposed maximum concentration; however, the gross pathology examination of the organs of the animals at the end of the studies was not performed in any of the studies. In the absence of such data, the FEEDAP Panel cannot perform a complete assessment of the safety of the additive for chickens for fattening, weaned piglets and lambs; therefore, these studies cannot be further considered in the assessment.

A structured literature review was also performed, covering the period 1996–2018, to identify studies investigating the safety of sepiolite and/or diatomaceous earth in ruminants, pigs, horses and poultry. Search terms used included several keywords, covering the target species, toxicological and efficacy terms. The databases consulted were CAB Abstracts, Medline on PubMed and the Veterinary Sciences Database. In total, 370 publications were retrieved, of which 12 were considered relevant. Six studies were related to the use of Sepiolite in feed for poultry (three with chickens for fattening and one with laying hens) and in piglets (two studies), and six were related to the use of Kieselguhr (diatomaceous earth) in feed for chickens for fattening. In these studies, no adverse effects were observed when sepiolite was supplemented at concentrations up to 20,000 mg/kg feed and diatomaceous earth up to 60,000 mg/kg feed. However, none of these studies was designed to meet the characteristics of a tolerance study showing several deficiencies (e.g. no overdose of the additive, lack of complete gross pathology, histopathology and haematology). Therefore, none of these studies would support the safety of the two components of the additive at the proposed use level in feed.

No study was identified for the use of both clays, Sepiolite plus Kieselguhr (diatomaceous earth), representing the additive under assessment (Anpro). The FEEDAP Panel considered the results of the studies identified of low relevance for the assessment of the safety of the additive for the target species.

3.2.2.1 | *In vivo interactions*

An *in vivo* interaction/digestibility study was conducted as part of the tolerance study in chickens for fattening, in order to evaluate the interactions of Anpro with other components of the diet.

A total of 300 male 1-day-old chickens for fattening (Ross 308) were randomly allocated to 30 replicate pens, each containing 10 birds.[35] Five dietary treatments (six pens each) were included. For the *in vivo* interaction part of the study, the relevant treatments consisted in a control group, fed the basal diet with no supplementation and a treated group fed the basal diet supplemented with 5000 mg Anpro/kg complete feed. The basal diet was based on wheat and soybean; for starter and grower diets, the analysed crude protein was 23.2 and 20.7%, respectively, crude fat - 6.1 and 6.3%, respectively and energy - 12.38 and 12.81 MJ/kg, respectively. The starter diet (1–14 days) was fed as crumbs and the grower diet (14–35 days) as pellets. The diets were offered ad libitum and contained narasin as a coccidiostat.

Faecal samples were collected between days 28 and 35 from the birds of the control and the treated groups to calculate the nitrogen retention, and the utilisation of zinc, vitamin A, thiamine and narasin disappearance from the gut lumen. No differences were observed in the utilisation of zinc, vitamin A and thiamine. For narasin, a higher recovery in the excreta of the birds fed the additive than the control diet (7.5% vs. 3.2%, $p = 0.001$) was observed.

3.2.2.2 | *Conclusions on safety for the target species*

In the absence of adequate data, the FEEDAP Panel cannot conclude on the safety of the additive for the target species.

3.2.3 | Safety for the consumer

Based on current knowledge there is no indication of substantial absorption of the components of the additive. Therefore, the FEEDAP Panel concludes that the use of the additive in animal nutrition assessed under the conventional approach and according to the conditions of use is of no concern for the consumer.

3.2.4 | Safety for the user

Based on the dusting potential data (> 2000 mg/m^3), the FEEDAP Panel considers that the exposure by inhalation is likely.

The additive contains crystalline silica (up to 4.8%). Inhalation of silica is known to be hazardous and is associated with increased risk of lung cancer and the industrial disease, silicosis. The European Directive 2017/2398[36] set an occupational

[32]Technical dossier/Section II/Annex III.1.1.1.

[33]Technical dossier/Section II/Annex III.1.1.2.

[34]Technical dossier/Section II/Annex III.1.1.3.

[35]Technical dossier/Section II/Annex III.1.1.1.

[36]Directive (EU) 2017/2398 of the European Parliament and of the Council of 12 December 2017 amending Directive 2004/37/EC on the protection of workers from the risks related to exposure to carcinogens or mutagens at work (Text with EEA relevance). OJ L 345, 27.12.2017, p. 87–95.

exposure limit (OEL) of 0.1 mg/m^3 of air for respirable crystalline silica dust. The applicant submitted data on dusting potential on three batches of the additive however, data on respirable fraction of the dust were not available. Therefore, the FEEDAP Panel used as a worst-case scenario the highest dusting potential data to calculate the content of silica present in the dust. The highest measured dust fraction of Anpro was 2420 mg/m^3, corresponding to 116 mg crystalline silica/m^3 dust, exceeding the OEL.

In a study (Wagner et al., 1987) evaluating the inhalation effects of sepiolite dust, a group of 20 male and 20 female Fischer 344 rats, 6 weeks of age, were exposed to 10 mg/m^3 sepiolite dust for 6 h per day, 5 days per week for 12 months. The respirable dust contained 1.15×10^6 fibres/µg. After 3, 6 and 12 months, two animals per sex per group were killed and their lungs examined for the presence and severity of fibrosis. After 12 months exposure ceased, and the remaining rats were maintained for their normal life span (except two males and two females killed after 24 months). A full necropsy was performed on all animals and lungs, liver, spleen, kidneys and any other relevant organs (not further specified) were examined for histopathology. Animals killed up to 24 months of age showed a grade 3 of fibrosis (early interstitial reaction). Based on the presence of pulmonary early interstitial reaction, the FEEDAP Panel concludes that sepiolite is a respiratory irritant.

The highest Ni content measured in the additive was 5.09 mg Ni/kg. The highest dusting potential of the additive measured amounted to 2420 mg/m^3, corresponding to about 0.01 mg Ni/m^3. This value would not exceed the transitional limit value of 0.1 mg Ni/m^3 for the inhalable fraction and 8 hours' time-weighted average (8h TWA) exposure established in Directive (EU) 2022/431.[37] However, owing to the presence of Ni in the additive, it should be considered as a respiratory sensitiser.

3.2.4.1 | *Effects on the eyes and skin*

The skin irritation potential of Anpro was tested in an *in vitro* skin irritation study using human derived epidermis model EpiDerm™ following the OECD TG 439.[38] The results of the study showed that the additive is not a skin irritant.

The eye irritation potential of Anpro was tested in an *in vitro* eye irritation study using the Epi-Ocular™ Reconstructed human Cornea-like Epithelium (RhCE) test method following the OECD TG 492.[39] The results of the study showed that the additive is not an eye irritant.

Due to the content of nickel, the additive is considered as a skin sensitiser.

3.2.4.2 | *Conclusions on safety for the user*

The additive poses a risk by inhalation. It is not irritant to the skin or eyes but should be considered as skin sensitiser.

3.2.5 | Safety for the environment

Sepiolite and diatomaceous earth are naturally occurring minerals widely distributed in the environment. Therefore, it is not expected that the use of the additive in animal nutrition would adversely affect the environment.

3.2.6 | Specific considerations on presence of nanoparticles and the safety assessment of the additive

Based on particle size data generated according to the Guidance on technical requirements (EFSA Scientific Committee, 2021a), the additive under assessment contains small particles including nanoparticles and should follow a nano-specific risk assessment as indicated in the EFSA SC Guidance on risk assessment of nanomaterials to be applied in the food and feed chain, human and animal health (EFSA Scientific Committee, 2021b).

The FEEDAP Panel noted that no suitable data in line with the requirements of the Guidance on risk assessment of nanomaterials (EFSA Scientific Committee, 2021b) were provided by the applicant. Therefore, an assessment on the potential risks associated with the presence of nanoparticles could not be undertaken for the safety of target animals, consumers and users.

3.2.7 | Conclusions on the safety of the additive

According to the conventional risk assessment, due to lack of adequate data, the safety of the additive Anpro for the target species cannot be established. Based on current knowledge, there is no indication of substantial absorption of the components of the additive and therefore no concern for the consumer. The additive poses a risk by inhalation. It is not irritant to the skin or eyes but should be considered as skin sensitiser.

[37]Directive (EU) 2022/431 of the European Parliament and of the Council of 9 March 2022 amending Directive 2004/37/EC on the protection of workers from the risks related to exposure to carcinogens or mutagens at work. PE/89/2021/REV/2. OJ L 88, 16.3.2022, p. 1–14.

[38]Technical dossier/Supplementary information January 2022/6_Annex_5.

[39]Technical dossier/Supplementary information January 2022/7_Annex_6.

As no suitable data in line with the requirements of the Guidance on risk assessment of nanomaterials (EFSA Scientific Committee, 2021b) were provided by the applicant, the potential risks associated with the presence of nanoparticles could not be assessed for the target species, the consumer and the user.

The additive is safe for the environment.

3.3 | Efficacy

3.3.1 | *In vitro* studies

One study assessed the *in vitro* efficacy of Anpro to adsorb aflatoxin B1 (AFB1).[40] Two adsorption isotherms were derived, one at pH 3 and the other at pH 7, both at 37°C, with varying AFB1 concentrations from 1 to 10 μg/mL to have six to seven experimental points. After preliminary studies to determine the appropriate Anpro/mycotoxin ratio, the optimal adsorbent concentrations were established at 0.05 mg/mL at pH 3 and 0.2 mg/mL at pH 7. The Anpro/mycotoxin suspensions were incubated for 90 min in a thermostatically controlled shaker (37°C, 250 rpm). After the incubation, samples were centrifuged. Then, supernatants were analysed for AFB1 by High-performance liquid chromatography with fluorescence detection (HPLC-FLD). AFB1 adsorption (as percentage of initial AFB1) varied in the ranges 24%–87% at pH 3 and 54%–90% at pH 7. Maximum adsorption capacity was estimated to be 51.2 ± 5.5 mg AFB1/g Anpro at pH 3, and 28.4 ± 2.0 mg AFB1/g Anpro at pH 7.

Three classical models (Langmuir, Freundlich and Sips equations) were fitted to the experimental data outlining the adsorption isotherms. The two-parameter model Langmuir provided the best goodness-of-fit to the observed AFB1 adsorption data. According to this model, the maximum AFB1 adsorption capacities were 49.7 ± 2.2 and 21.2 ± 0.6 mg/g at pH 3 and pH 7, respectively. The energy (affinity) of AFB1 adsorption to Anpro product was affected by the medium pH with greater adsorption rates at acidic pH. In addition, the Langmuir model was also fitted to the data relating the percentage of AFB1 adsorbed to the ratio adsorbent to AFB1. From the kinetic parameters of the model, it was estimated that approximately 20 mg of Anpro should be added to 1 kg of contaminated feed containing 1 mg AFB1/kg to achieve a 50% of AFB1 adsorption.

3.3.2 | Short-term *in vivo* efficacy studies

Four short-term *in vivo* studies, conducted in two different Member States, were submitted to support the efficacy of Anpro to reduce the absorption of AFB1 from feed in chickens for fattening (two studies), dairy cows (one study) and pigs for fattening (one study).

In both chickens for fattening studies – study 1 (combined tolerance/efficacy study)[41] and study 2 (efficacy study)[42] the efficacy of the additive was tested using feeds contaminated with AFB1. However, the concentrations of AFB1 in the feeds used for chickens after 14 days of age (0.015–0.017 mg AFB1/kg feed in study 1 and 0,020–0,022 mg AFB1/kg feed in study 2) exceeded the limits set for young poultry animals (chickens of less than 6 weeks) indicated in the Directive on undesirable substances in animal feed (i.e. 0.005 mg AFB1/kg feed).[43] According to the FEEDAP Guidance for efficacy assessment (EFSA FEEDAP Panel, 2018), 'The target mycotoxin content in feed used in studies should not exceed the values given in Directive 2002/32/EC for aflatoxin B1 […]'. Besides the lack of accomplishment of the regulatory levels, the overdose of AFB1 in feed may raise some uncertainties regarding the extrapolation of the results obtained to a feed with lower mycotoxin level contamination within the acceptable limits. Therefore, these two studies were not further considered for the assessment of the efficacy of the additive.

3.3.2.1 | *Efficacy study in dairy cows*

Twenty-four healthy Holstein dairy cows were divided into two groups balanced for parity and days in milk (DIM).[44] The two groups were designated as 'control' (no additive) or 'Anpro' (receiving daily 20 g Anpro per head). All cows received a daily maize/water drench spiked with AFB1 immediately after the morning milking, throughout the study (22 days). The amount of AFB1 dosed was calculated to be below the EU maximum permitted concentration in complete feed. Each cow received daily 50 g spiked maize (as feed) containing 0.129 mg of AFB1, which was administered mixed with water, via oesophageal tube. Assuming a dry matter intake (DMI) of ca. 23 kg DM/day, the AFB1 concentration if feed would be 5.6 μg AFB1/kg DM (4.9 μg AFB1/kg of complete feed on 12% moisture basis). Cows consumed about 20.4 kg of DM per day of the total diet, delivering 6.34 μg AFB1/kg DM (5.6 μg AFB1/kg of complete feed on 12% moisture basis), which was close to the calculated dose. In addition, all cows received daily a feed supplement in mash form, 10–15 min after the spiked maize/water drench, either alone (100 g of feed supplement per cow in the Control group) or with Anpro (80 g of feed supplement +20 g Anpro for the cows in the Anpro group). This would correspond, considering an average DMI of about 20 kg, to 1000

[40]Technical dossier/Section IV/Annex IV.1.1.

[41]Technical dossier/Section III/ Annex_III_1_1_1_Broiler_TAS_Efficacy_Interaction.

[42]Technical dossier/Spontaneous submission September 2019/ 2_Annex_IV_2_3_broiler_efficacy_report.

[43]Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 30.5.2002, p. 10.

[44]Technical dossier/Section IV/Annex IV.2.1 and Technical dossier/Supplementary information November 2019/3_Annex_IV_2_1_dairy_cow_efficacy.

mg Anpro/kg DM or to approximately 870 mg/kg complete feed. Individual body weight (BW) was recorded at each milking (twice a day). Milk production was recorded daily, milk quality at every milking, somatic cell counts on day 1, 15 and 21. Individual feed supplement intake and group Total Mixed Ration (TMR) intake were recorded daily.

Data were subjected to ANOVA by using a mixed-effects model with the fixed effect of treatment and day of study and the random effect of animal. Day entered the model as a repeated measure. The cow was the experimental unit and statistical significance was declared at $p < 0.10$.

General health and performance during the study was good with no mortality. No relevant differences were observed in the zootechnical parameters observed (control group results: milk yield: 36.7 kg/day; milk fat: 3.98%; milk protein: 3.09%, somatic cell count: 512×10^3/mL) At the end of the study (22 days), the concentration of aflatoxin M1 (AFM1) in milk was significantly ($p < 0.01$) lower in the group that received Anpro (27.1 ng/kg milk) compared to the control group (62.8 ng/kg milk), representing a reduction of 57% in the concentration of AfM1. Blood AFB1 concentration was significantly ($p = 0.08$) lower in the Anpro treated group (0.85 µg/L) compared to the control group (1.26 µg/L), representing a reduction of 33% (Table 3).

TABLE 3 Effect of Anpro on aflatoxin concentration in milk and blood plasma of dairy cows at the end of the study (22 days).

Treatment	Milk, AFM1 (ng/kg)	Blood, AFB1 (µg/L)
Control	62.8	1.26
Anpro (20 g/head per day)	27.1[a]	0.85[a]

[a]Significantly different from the control at $p < 0.1$.

Aflatoxin B1 (AFB1) in feed is absorbed to blood and AfM1 transferred to milk. Anpro, when administered in feed daily at 20 g/cow (~ 870 mg Anpro/kg complete feed), would reduce the AFB1 absorption, as evidenced by the significant decrease observed for AFB1 in blood and AfM1 in milk.

The results from this study indicate that Anpro significantly reduced AFB1 absorption from feed in dairy cows. However, the lack of information on individual feed intake, does not allow the Panel to identify the effective concentration of the additive in complete feed.

3.3.2.2 | *Efficacy study in pigs for fattening*

Thirty-two healthy growing male pigs (Duroc crossbreed, ~ 36 kg average initial BW) were allocated to 16 pens of two pigs per pen and randomly allocated to two treatment groups (eight pens per treatment): Control (AFB1 spiked feed with no additive) and Anpro (AFB1 spiked feed with 5 g Anpro/kg feed).[45] The target concentration of AFB1 in the diets was 20 µg/kg; this concentration was analytically confirmed, being 19–21 µg/kg in control and 15–16 µg/kg in Anpro diets (in compliance with the limit set in Directive 2002/32/EC). Pigs were offered the corresponding diets ad libitum for 21–22 days (from day 7 to day 28–29 of the trial). The study ended on day 28 for 50% of the pigs (four pens per treatment) and on day 29 for the pigs in the other four pens per treatment. Zootechnical performance was measured from 1 to 7 days (acclimation period) and 8–28/29 days (study period). Blood and liver samples were collected from all pigs at the end of the study to determine AFB1 and AfM1 concentrations.

The study followed a completely randomised design, with treatment levels (control or Anpro) assigned to experimental units (pens) at random. Data were subjected to one-way ANOVA with treatment as the only fixed factor. Results were reported as least squares means, the Tukey test was used for the comparison between treatment groups and statistical significance was declared at $p < 0.05$.

General health and performance during the study was good with no mortality. No relevant differences were observed in the zootechnical parameters observed (control group results: body weight gain: 23.0 kg; feed intake: 104.5 kg/pen; feed to gain ratio: 2.28). AFB1 and AfM1 concentrations in blood were significantly reduced in pigs fed the Anpro diet compared to those fed the control diet by –78% and –81%, respectively ($p < 0.001$). Moreover, AFB1 and AfM1 concentrations in liver tissue were significantly reduced in pigs fed the Anpro diet compared to those fed the control diet by –70% and –77%, respectively ($p < 0.001$). Results are shown in Table 4.

TABLE 4 Effect of Anpro on aflatoxin concentration (ng/kg) in blood and liver of pigs at the end of the study (28/29 days, after 3 weeks of feeding the Anpro supplemented diet to the treated group).

	Blood		Liver	
Treatment	AFB1	AFM1	AFB1	AFM1
Control	9.92	8.61	62.1	100.4
Anpro (5000 mg/kg feed)	2.17[a]	1.63[a]	18.5[a]	23.4[a]

[a]Significantly different from the control at $p < 0.01$.

[45]Technical dossier/Section IV/Annex IV.2.2.

The results from this study indicate that Anpro, when administered at 5000 mg/kg complete feed, significantly reduced AFB1 absorption from feed in fattening pigs, as evidenced by significantly reduced liver and blood AFB1 and AfM1 concentrations.

3.3.2.3 | *Conclusions on efficacy*

Although positive effects were shown in reducing aflatoxin B1 absorption in dairy cows and pigs for fattening, in the absence of a positive study in chickens for fattening, the FEEDAP Panel cannot conclude on the efficacy of the additive for all terrestrial target species.

4 | CONCLUSIONS

According to the conventional risk assessment, due to lack of adequate data, the safety of the additive Anpro for the target species cannot be established. Based on current knowledge, there is no indication of substantial absorption of the components of the additive and therefore no concern for the consumer. The additive poses a risk by inhalation. It is not irritant to the skin or eyes but should be considered as skin sensitiser.

As no suitable data in line with the requirements of the Guidance on risk assessment of nanomaterials were provided by the applicant, the potential risks associated with the presence of nanoparticles could not be assessed for the target species, the consumer and the user.

The additive is safe for the environment.

The FEEDAP Panel is not in the position to conclude on the efficacy of the additive for all terrestrial animal species.

5 | REMARK

The FEEDAP Panel notes that the iron content of the product (average 2.3%) would limit the use of this additive in compound feedingstuffs, for which a maximum content for iron is set by EU legislation.

ABBREVIATIONS

AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
ANS	EFSA Scientific Panel on Additives and Nutrient Sources added to Food
BW	body weight
CV	coefficient of variation
DM	dry matter
DMI	dry matter intake
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
OECD	Organisation for Economic Co-operation and Development
SEM	scanning electron microscopy
WHO	World Health Organization
XRF	X-ray fluorescence
XRD	X-ray diffraction

CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

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