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Safety and efficacy of the feed additive consisting of Vitamin B_2/Riboflavin produced by *Eremothecium ashbyi* CCTCCM 2019833 for all animal species (Hubei Guangji Pharmaceutical Co., Ltd)

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the feed additive Vitamin B_2/Riboflavin produced by *Eremothecium ashbyi* CCTCCM 2019833 for all animal species and categories. The additive contains a minimum content of riboflavin of 5%. The production strain has been characterised and data showed that viable cells are not present in the final additive. The additive is not a skin or eye irritant nor a skin sensitiser, but it is considered to be a respiratory sensitiser. The lack of data on the toxicological profile of the additive, including its genotoxic potential, did not allow the Panel to conclude on the safety of the additive for the target species, consumers and users. The FEEDAP Panel concluded that the use of the product as a feed additive poses no concerns for the environment. The additive under assessment is effective in covering the animals' requirements for vitamin B_2 when administered via feed.

Keywords: Vitamin B_2/Riboflavin, *Eremothecium ashbyi*, nutritional additive, vitamins, safety, efficacy

Requestor: European Commission

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

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 Hubei Guangji Pharmaceutical Co., Ltd represented in the EU by Nutreco Procurement B.V.[2] for authorisation of the product containing Vitamin B_2/Riboflavin produced by *Eremothecium ashbyi* CCTCCM 2019833, when used as a feed additive for all animal 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 8 June 2020.

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 product Vitamin B_2/Riboflavin produced by *Eremothecium ashbyi* CCTCCM 2019833, when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

The product Vitamin B_2 (5%) produced by *Eremothecium ashbyi* CCTCCM 2019833 has not been authorised in the EU.

The FEEDAP Panel has adopted five opinions on the use of vitamin B_2 as a feed additive. Two opinions related to the safety and efficacy of vitamin B_2 (80%) as riboflavin produced with *Bacillus subtilis* KCCM-10445 for all animal species (EFSA FEEDAP Panel, 2014, 2018a), an opinion on the safety and efficacy of vitamin B_2 as riboflavin and riboflavin-5′-phosphate ester monosodium salt, produced by either *Bacillus subtilis* DSM 17339 or *Bacillus subtilis* DSM 23984 (EFSA FEEDAP Panel, 2016), and another opinion on the safety and efficacy of vitamin B_2 (riboflavin) produced by *Ashbya gossypii* (EFSA FEEDAP Panel, 2018b). The last one was related to the safety and efficacy of vitamin B_2 (riboflavin 5′-phosphate ester monosodium salt) for all animal species when used in water for drinking (EFSA FEEDAP Panel, 2018c).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of Vitamin B_2 (5%) produced with *Eremothecium ashbyi* CCTCCM 2019833 as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in Annex A.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the product Vitamin B_2 (5%) produced by *Eremothecium ashbyi* CCTCCM 2019833 is in line with the principles laid

[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] Hubei Guangji Pharmaceutical Co., Ltd represented by Nutreco Procurement B.V. Veerstraat 38, 5831 JN, Boxmeer, The Netherlands.
[3] FEED dossier reference: FAD-2020-0027.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2020-0027_vitb2.pdf

down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018d), 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 the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018e), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The assessment deals with the safety and efficacy of the product vitamin B_2 (5%) produced with *Eremothecium ashbyi* CCTCCM 2019833 as a nutritional additive (functional group: vitamins, pro-vitamins and chemically well-defined substances having similar effect) for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the production organism

The additive is produced with a non-genetically modified strain of *Eremothecium ashbyi* deposited at the China Center for Type Culture Collection with the number CCTCC M 2019833.[6] The strain was developed from a parental strain (origin not given) by UV irradiation and selection for high production of riboflavin.

The whole genome sequence (WGS) of the strain was sequenced and annotated in order to taxonomically classify the production strain and to characterise it in terms of virulence factors and production of secondary metabolites.[7] The total genome was of 12,745,383 bp, (N50 148033) with a total number of 4,746 genes. The result of the gene set assessment for *Eremothecium ashbyi* showed that 84.5% of the 1,711 BUSCO groups searched were successfully found in the annotation, which would show a high quality of the assembly considering the likely lack of data in the databases.

The data were used to perform a phylogenomic analysis which could not be completed due to the lack of genome sequences publicly available for the expected taxonomical unit. Therefore, the ITS marker gene was sequenced and analysed by alignment to the CBS-KNAW database and confirmed the classification as *Eremothecium ashbyi.*

The presence of virulence factors was checked by alignment of the amino acid sequences of the annotated genes against the Database of Virulence Factors in Fungal Pathogens. A cut-off of 0.9 was applied and a total of nine hits were found. Seven of the genes identified are house-keeping genes, the other two are reported to act as a virulence factors in plant pathogenic fungi (1,3-beta-D-glucan-synthase and GDP-mannose pyrophosphorylase).

The WGS-based data were also checked for the presence of pathways for secondary metabolites using the antiSMASH 4.0 tool. Two pathways were identified, a cluster with unknown function and a cluster involved in the biosynthesis of terpene molecules. No hits were found with pathways known to be involved in mycotoxin production. A search was done to check the literature available on PubMed reporting virulence and/or pathogenicity for *Eremothecium ashbyi* or *Eremothecium ashbyii.* According to the report, no publications were identified reporting for virulence or pathogenicity of the strain.

3.1.2. Manufacturing process

[redacted]

[5] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[6] Technical dossier/Section II/Annex II.8 and supplementary information October 2020/Annex A.
[7] Technical dossier/Section II/Annex II.7.

[redacted]

3.1.3. Characterisation of the additive

The product vitamin B_2 (5%) produced with *Eremothecium ashbyi* CCTCCM 2019833 contains riboflavin (International Union of Pure and Applied Chemistry (IUPAC) name: 7,8-dimethyl-10-[(*2S,3S,4R*)-2,3,4,5,-tetrahydroxypentyl]benzo[*g*] pteridine-2,4(*3H,10H*)-dione, synonyms: vitamin B_2, 7,8,-dimethyl-10-(1′-D-ribityl)isoalloxazine; lactoflavin, 1-deoxy-1-(7,8,dimethyl-2,4-dioxo-3,4-dihydrobenzo[*g*]pteridin-10(2H)-yl)-D-ribitol), which is identified by the CAS (Chemical Abstracts Service) number 83–88–5 and the EINECS (European Inventory of Existing Chemical Substances) number 201–507–1. The molecular formula of riboflavin is $C_{17}H_{20}N_4O_6$ and its molecular weight is 376.37. The structural formula of riboflavin is shown in Figure 1.

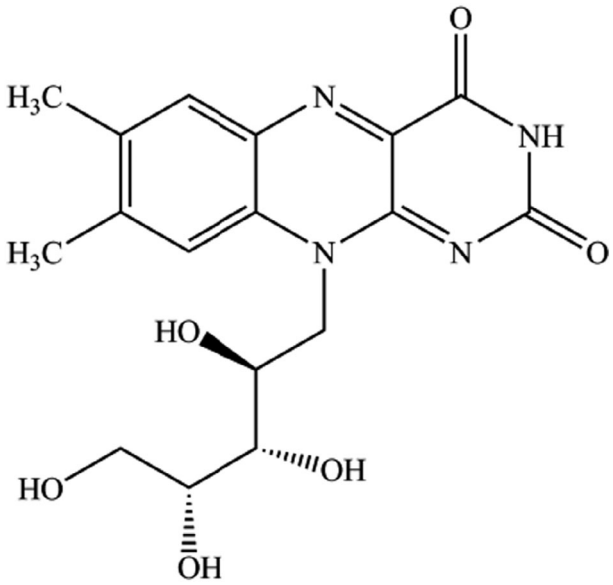

Figure 1: Structural formula of riboflavin

The additive is the dried fermentation product and it is a brown powder that contains a minimum of 5% of riboflavin and less than 7% of moisture. The batch to batch variation as regards to the riboflavin, measured in five batches of the additive, showed a mean value of 6.9% (ranging from 6.5% to 7.4% riboflavin) with a moisture content of 4.1%.[9] These batches were also analysed to determine the composition of the additive.[10] The product contains (on as is basis) 38–41% crude protein, 18–20% non-starch polysaccharides, 13–16% crude ash (< 0.2–5% of insoluble ash), 5.9–8.3% carbohydrates (2.9–3.4% glucose), 4.5–6.2% crude fat, 3.4–3.5% organic acids, 3.2–3.4% potassium, 2.3% sodium, 0.6–1.8% starch and less than 1% of crude fibre and lactose.

The amount of microbial biomass was measured in the final additive and in the product obtained right after the fermentation. The results showed that the product contains 20–26% of dry microbial biomass.[11] Considering the composition of fungi, this microbial biomass is estimated to contain protein (20–50%), glucans (20–30%), lipids (5–10%), DNA (10%), chitin (5–10%) and ash (5%).[12] Therefore,

[8] Technical dossiers/Section II and Supplementary information October 2020. Depending on the parameter the number of results varies from 3 to 6 analysis.
[9] Technical dossier/Section II/Annex II.1.
[10] Technical dossier/Section II/Annexes II.1 and II.2 and Supplementary information October 2020 Annex E.
[11] Technical dossier/Supplementary information October 2020.
[12] Technical dossier/Supplementary information October 2020/Annex F.

the amount of protein present in the final product from the microorganism would amount to 8–10% of the total protein present.

Data on the chemical and microbiological contamination were provided for five batches.[13] The chemical contamination included lead (0.25–1.3 mg/kg), mercury (< 0.01 mg/kg (detection limit, LOD) to 0.04 mg/kg), arsenic (0.3–0.4 mg/kg), cadmium (0.06–0.10 mg/kg) and fluorine (8–12 mg/kg), polychlorinated dibenzo-p-dioxins and furans (≤ 0.73 ng WHO-PCDD/F-TEQ/kg in three batches and 1.63 and 1.77 ng WHO-PCDD/F-TEQ/kg in the other two),[14] dioxin-like polychlorinated biphenyls (≤ 0.09 ng WHO-PCB-TEQ/kg) and non-dioxin-like polychlorinated biphenyls (nDL-PCBs ≤ 0.13 μg/kg in three batches and < 3 μg/kg in the other two). The FEEDAP Panel notes that the levels of PCDD/F in two batches of the additive are above the maximum content defined in Commission Regulation (EU) No 277/2012 for feed materials of plant, mineral and animal origin.[15] The following mycotoxins were analysed: fumonisin B_1 (852–2,000 μg/kg), fumonisin B2 (59–240 μg/kg), deoxynivalenol (< 150 (LOD) to 470 μg/kg), aflatoxin B_1 (< 1.0 μg/kg, LOD), T2 toxin (< 10 μg/kg or < 20 μg/kg, LOD, depending on the method used), HT2-toxin (< 5 μg/kg or < 200 μg/kg, LOD, depending on the method used), ochratoxin A (< 1 μg/kg, LOD) and zearalenone (< 20 μg/kg, LOD). The microbial contamination included, moulds (100 colony-forming units (CFU)/g, one sample showed 700 CFU/g), yeasts (< 100 CFU/g), aerobic colony counts (13,000–18,000 CFU/g), Enterobacteriaceae and coliforms (< 50 CFU/g two batches and < 10 CFU/g in three batches), *Salmonella* (absent in 25 g, quantitative polymerase chain reaction (qPCR)), and *Bacillus cereus* (1,400–86,000 CFU/g). Further batches were analysed for the presence of *B. cereus.* In total, nine new batches were analysed, all were analysed by two different laboratories, and showed values < 50 CFU/g to 200 CFU/g in laboratory 1 and values from 30 to 85 CFU/g in laboratory 2 (four aggregated samples of the nine batches).[11] The data in the second batch showed a considerably lower number of counts of *B. cereus,* the applicant explained that the first data set were pilot batches prepared with a spray drying process in an open area while the newly submitted ones were prepared in a better equipped area for the process ensuring a lower presence of *Bacillus cereus* in the final product.

The FEEDAP Panel notes that the values found regarding the polychlorinated dibenzo-p-dioxins and furans, fumonisins and 'presumptive' *B. cereus* are high and deserve attention/monitoring during the production process.

The analysis of the absence of viable cells of the production strain was submitted in two different data sets. In the first,[16] five batches were analysed, and for each batch, a total of 2 g of sample were diluted into 100 mL, 1 mL of the dilution was plated in PCA plates (with or without ampicillin (10 mg/L)) and cultured at 30°C for 120 h or 1 mL added to the plates and then 15–20 mL of liquid PCA (with or without ampicillin) poured and cultured. A positive control with the production strain but not spiked to the product and a negative control was also considered. The results indicated no growth of the production strain, however, the amount of sample analysed was too little to be in line with the requirements of the FEEDAP Panel Guidance (EFSA FEEDAP Panel, 2018c). In the second data set,[17] three batches of the additive were analysed. For each batch, three subsamples of 1 gram were obtained and each sample was dissolved in 4 mL of water. The suspension (4 mL) obtained for each sample was distributed in 15–20 GYPA/PS (penicillin and streptomycin) plates by spread plating 0.3–0.4 mL into each plate. The plates were incubated for 14 days at 25°C. A strain of *E. ashbyi* (a solution of ascospores) was spiked into subsamples as positive control. Viable cells of the production strain were not detected in the samples analysed, while positive controls showed growth.

Three batches were analysed for the presence of antimicrobial activity.[18] A sample from each batch was diluted with distilled water to a stock solution of 128 mg product/L. From there, twofold dilutions were prepared. In the broth, the concentrations were half, so the test started with 64 mg/L. The reference strains were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633. No inhibition was detected, but the levels tested do not allow the Panel to conclude on the levels of use of the product (see below, estimated 0.06–1.6 g additive/kg feed).

[13] Technical dossier/Section II/Annex II.1 and II.2.
[14] Values expressed as relative to a feed with a moisture content of 12% (three samples) and on original matter (two samples).
[15] Commission Regulation (EU) No 277/2012 of 28 March 2012 amending Annexes I and II to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels and action thresholds for dioxins and polychlorinated biphenyls. OJ L 91, 29.3.2012, p. 1.
[16] Technical dossier/Section II/Annex II.3.
[17] Technical dossier/Section II/Annex II.4 and supplementary information October 2020.
[18] Technical dossier/Supplementary information October 2020/Annexes J, K and L.

Three batches of the additive were analysed for the particle size, dusting potential and bulk density.[19] The dusting potential was measured with the Stauber-Heubach test in duplicate and showed a dusting potential ranging from 550 to 635 mg/m^3. The particle size of the additive was measured by laser diffraction and showed that 80% of the particles are below 50 μm and 12% below 10 μm. The bulk density of the product is of 535 kg/m^3.

3.1.4. Stability and homogeneity[20]

The shelf-life of the additive was studied in three batches. In an ongoing study, samples were stored at 25 or at 40°C for 26 weeks in closed jars. Recovery values showed losses of riboflavin content from 4% to 13% when stored at 25°C and from 11% to 16% when stored at 40°C.

Stability in a premixture was tested for three batches of the additive in a premixture that contained basically calcium carbonate (75%), other minerals and vitamins and the additive added at a level of 3.3% (representing 0.20 g riboflavin/100 g). The premixture did not contain choline chloride. The samples were stored at 25 or at 40°C for 26 weeks. Recoveries of the initial levels were 94% and 78% at 25 and 40°C, respectively.

The stability in feed was tested for one batch of the additive in two batches of feed for piglets when added at 4 or 6 mg/kg diet. The samples were stored at 25 or at 40°C for 26 weeks. Recoveries showed no losses of riboflavin.

The capacity to homogeneously distribute of the product under assessment in premixtures and feed was studied in the samples used for the stability study. Ten subsamples were measured and the coefficient of variation was 1.2% in premixtures and 3.4% in the feed.

3.1.5. Conditions of use

The additive is to be used for all animal species, and according to the applicant, the additive can be administered to the animals via feed materials, premixtures, complete feed and/or complementary feed with recommended levels of use from 3 to 80 mg riboflavin/kg complete feed (Table 1).[21]

Table 1: Recommended use levels of riboflavin for the target species

Target species	Riboflavin (mg/kg complete feed)
Poultry	
Chickens, starter	8–10
Chickens, grower-finisher	7–9; 6–8
Chickens, breeders	12–16
Laying hens	5–7
Turkey, starter	15–20
Turkey, grower	10–15
Turkey, finisher	8–10
Turkey, breeder	15–20
Ducks/geese	7–9
Partridges/Quails	5–7
Pigs	
Piglets pre-starter	10–15
Piglets starter	10–15
Grower phase	7–10
Finisher phase, gilts, sows and boars	6–10
Horses	
Foals*	20–30
Leisure horses*	30–40
Race and breeding horses*	70–85

[19] Technical dossier/Section II/Annex II.9.
[20] Technical dossier/Section II/Annex II.11 and Supplementary information October 2020.
[21] Technical dossier/Section II/References 5.01 – no recommentation given for young ruminants.

Target species	Riboflavin (mg/kg complete feed)
Other species	
Dogs	13–22
Cats	22–27
Rabbits	3–6
Minks & Foxes	10–20
Trout and salmon	20–30
Warm-water fish	15–20
Sea bream and sea bass	20–30
Shrimp	40–80

*: Dosage recommendation in mg/head per day.

Assuming a minimum content of 5% of riboflavin in the additive, the recommended levels of riboflavin would be achieved by inclusion levels of the additive in the feed from 0.06 to 1.6 g additive/kg feed.

3.2. Safety

The product under assessment is the result of fermentation with the production strain and it is a dried microbial biomass which contains the remnants of the strain and fermentation broth as well as the products from the fermentation by the production strain, with a riboflavin content of at least 5%. The applicant provided a set of toxicological studies which is described below.

3.2.1. Toxicological studies

3.2.1.1. Genotoxicity

No test addressing the potential of the additive to induce gene mutation was submitted.

An *in vivo* chromosomal aberration test was performed in bone marrow cells of KM mice according to OECD TG 475 to evaluate the potential of the additive to induce chromosomal damage.[22] Animals (5 males and 5 females) were daily given by gavage a single dose of 2,000 mg additive/kg bw for 14 days. Mitomycin C (MMC) was used as positive control at 1.5 mg/kg bw per day. No changes in body weight, feed intake or clinical signs of toxicity were observed after treatment with the test item compared to the negative control group. Two hundred metaphases per mice were scored for the analysis of structural and numerical chromosome aberrations. Non-conventional measures of chromosome aberrations were recorded, i.e. loops, monomer swap, tiny fragments in addition to gaps and aneuploidy. The positive control induced a statistically significant increase in the frequency of aberrations. No evidence of exposure of the target tissue was reported, since no toxicity was observed in the bone marrow by the analysis of the mitotic index. The frequency of chromosome aberrations was comparable between treated and negative control groups. The Panel notes some study limitations, such as testing one dose only, no evidence of target tissue exposure, and application of unusual parameters for the analysis of structural chromosome damage, which do not allow to consider conclusive the results of the study.

Due to the lack of data on the potential of the additive to induce gene mutations and the limitations identified in the *in vivo* chromosomal aberration test, the Panel cannot conclude on the genotoxic potential of the additive under assessment.

3.2.1.2. Other studies[23]

The applicant submitted a subchronic oral toxicity study claimed to comply with OECD Guideline 408. Six replicates of three SPF Sprague Dawley rats per sex (18 rats per group) were given diets containing 0 (control) or 1,000 mg additive/kg bw per day for 90 consecutive days. Body weight, food and water intake were recorded daily. Clinical observations were made twice daily throughout the study. The FEEDAP Panel noted some limitations and deviations to the OECD TG 408: (i) only six rats per sex per group were used for ophthalmoscopy measurements, haematology and necropsy at the end of study, (ii) no histopathology was conducted, (iii) functional observations monitored were limited

[22] Technical dossier/Supplementary information October 2020/Annex O.
[23] Technical dossier/Section III/Annex III.4 and supplementary information October 2020/annexes M and N.

and/or insufficiently reported, (iv) organ weights were provided for liver, kidney, adrenal, thyroid, uterus/testis, ovary/epididymis, thymus, spleen, brain and heart (six animals per sex per group) but summarised only as relative to body weight, and (v) there were no data on sperm parameters.

The applicant submitted a prenatal developmental toxicity study claimed to comply with OECD Guideline 414. Ten-week-old adult Sprague Dawley rats (32 unmated females and 16 unmated males) received the additive by oral gavage at dose level of 0 (control) or 1,000 mg additive/kg bw per day. Pregnant rats were randomly divided into two groups (control and test group) of 16 rats each. The test group was treated from days 6 to 19 post-coitum. The FEEDAP Panel noted some limitations and deviations to the OECD TG 414: (i) Number of animals used was borderline (16 in each group), (ii) some of the results were presented in a summarised form, individual values are given only for body weight and feed intake for dams and developmental effects for fetuses, (iii) lack of data on the body weight gain and implantations, resorptions and corpora lutea in dams and external malformations in fetuses, (iv) missing detailed information on mortality, morbidity, pertinent behavioural changes and clinical signs in dams, and on the types of malformations.

Owing to the limitations identified in the studies' design and reporting, the FEEDAP Panel did not consider them further in the assessment.

3.2.1.3. Conclusion on the toxicological data

Owing to the lack of relevant data, the FEEDAP Panel is not in the position to conclude on the toxicological profile of the additive under assessment.

3.2.2. Safety for the target species

In previous assessments, the FEEDAP Panel concluded that riboflavin is safe for the target animals with a wide margin of safety, of about 20–60 compared to the supplementation levels in commercial feed (EFSA FEEDAP Panel, 2014, 2016, 2018b). Therefore, the additive under assessment would not represent a concern for the target species considering the levels of riboflavin proposed to be added to the feeds.

However, owing to the characteristics/nature of the additive, which contains only a small fraction of active substance (riboflavin) and with remnants from the fermentation process and biomass largely present, the FEEDAP Panel considers that in order to be able to conclude on the safety for the target animals, studies performed with the additive under assessment in target animals would be required to support the safety of the additive.

No studies are currently available to the FEEDAP Panel that could support the safety for the target species of the additive under assessment. Therefore, the FEEDAP Panel cannot conclude on the safety of the additive for the target species.

3.2.3. Safety for the consumer

In previous assessments, the FEEDAP Panel concluded that the supplementation of feed with riboflavin could not be of concern for the consumers (EFSA FEEDAP Panel, 2014, 2016, 2018b). However, apart from riboflavin, the toxicological profile of the other components of the additive, which is a fermentation product, has not been established, and therefore, uncertainties remain as regards to the safety for the consumers of food products obtained from animals receiving the additive under assessment.

3.2.4. Safety for user

An acute inhalation toxicity study[24] was performed according to OECD TG 403 (not good laboratory practice (GLP) compliant) with the additive. Exposure of rats to an aerosol with 2,000 mg additive/m^3 for 4 h did not cause mortality, signs of toxicity or changes in the gross pathology inspection. The additive did not induce acute inhalation toxicity under the experimental conditions of the study.

An acute dermal irritation study[25] and an acute eye irritation study[26] were done in rabbits following OECD TG 404 (non GLP compliant) and OECD 405 (non GLP compliant), respectively. The additive did not induce irritation or corrosive effects in the skin or in the eyes, respectively.

[24] Technical dossier/Section III/Annex III.7 and Supplementary information October 2020/Annex P.
[25] Technical dossier/Section III/Annex III.9 and Supplementary information October 2020/Annex Q.
[26] Technical dossier/Section III/Annex III.8 and Supplementary information October 2020.

A skin sensitisation study[27] in guinea pigs, conducted following the OECD TG 406 (GLP compliant), showed that the additive did not induce skin sensitisation to the animals.

The dusting potential is of 600 mg/m^3 and, therefore, the exposure by inhalation is likely. Owing to the proteinaceous nature of the additive, the FEEDAP Panel considers the additive to be a respiratory sensitiser.

3.2.4.1. Conclusion on the safety for the users

The additive is not a skin/eye irritant nor a skin sensitiser, but it is considered to be a respiratory sensitiser.

The lack of data regarding the toxicological profile of the additive does not allow the Panel to conclude on the safety of the additive for the users.

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 production strain is a potential plant pathogen, but no viable cells were detected in the additive. Therefore, a risk for the environment resulting from the use of the additive under assessment in animal nutrition is not foreseen.

3.3. Efficacy

Riboflavin (vitamin B_2) has been used worldwide in animal nutrition for decades. Dietary requirements are set for domestic animals except for ruminants, owing to microbial synthesis of riboflavin in the rumen (GfE, 1995, 2001, 2003; NRC, 1996, 2001, 2007). Owing to the long history of use and its established nutritional role in domestic animals, riboflavin is regarded as effective in covering the animal's requirement. Data on requirement, allowances and recommendations for feed supplementation are easily accessible in the standard literature on animal nutrition.

The FEEDAP Panel considers that the additive under assessment is effective in covering the animal's requirement when administered via feed.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[28] and Good Manufacturing Practice.

4. Conclusions

In the absence of appropriate toxicological data with the additive, the Panel cannot conclude on the safety of vitamin B_2 (5%) produced with *Eremothecium ashbyi* CCTCCM 2019833 for the target animals and the consumers.

The additive is not a skin or eye irritant nor a skin sensitiser, but it is considered to be a respiratory sensitiser. However, the lack of data on the toxicological profile of the additive does not allow the Panel to conclude on the safety of the additive for the users.

The FEEDAP Panel concludes that the additive poses no concerns for the environment.

The additive under assessment is effective in covering the animals' requirements for vitamin B_2 when administered via feed.

5. Recommendation

The FEEDAP Panel notes that the content of polychlorinated dibenzo-p-dioxins and furans, fumonisin B_1 and B_2 and the counts of *B. cereus* (presumed) are high and would deserve attention/ monitoring during the production process.

[27] Technical dossier/Supplementary information October 2020/Annex R.

[28] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

6. Documentation as provided to EFSA/Chronology

Date	Event
21/04/2020	Dossier received by EFSA. VitaminB2/Riboflavin produced by fermentation with non/genetically modified *Eremothecium ashbyii* CCTCC M 2019833. Submitted by Hubei Guangji Pharmaceutical Co. Ltd. Represented in EU by Nutreco Procurement B.V.
20/04/2020	Reception mandate from the European Commission
08/06/2020	Application validated by EFSA – Start of the scientific assessment
08/09/2020	Comments received from Member States
14/07/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: Characterisation and safety*
08/09/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: Methods of analysis*
13/11/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
10/02/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

CAS	Chemical Abstracts Service
EINECS	European Inventory of Existing Chemical Substances
EURL	European Union Reference Laboratory
GLP	good laboratory practice
HPLC-FLD	high-performance liquid chromatography coupled to fluorescence detection HPLC-UVhigh-performance liquid chromatography coupled to UV detection
Rrec	Recovery rate
RSDip	Relative standard deviation for intermediate precision
RSDr	Relative standard deviation for repeatability

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Vitamin B2/Riboflavin produced by *Eremothecium ashbyii* CCTCCM 2019833

In the current application, an authorisation is sought under Article 4 for vitamin B2/Riboflavin produced by *Eremothecium ashbyii* CCTCCM 2019833 as feed additive under the category/functional group 3(a) 'nutritional additive'/'vitamins, provitamins and chemically well-defined substances having a similar effect' according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the feed additive for all animal species.

The product presented by the Applicant contains riboflavin produced by fermentation with non-genetically modified *Eremothecium ashbyii* CCTCC M 2019833 as active substance with a minimum mass fraction of 5%. The Applicant proposes inclusion levels of the active substance ranging from 3 to 80 mg/kg complete feedingstuffs. The feed additive is intended to be added directly into feedingstuffs (or through premixtures).

For the determination of riboflavin in the feed additive, the Applicant proposed a method based on high-performance liquid chromatography coupled to fluorescence detection (HPLC-FLD). The method has been single-laboratory validated for the determination of vitamin B2 (as riboflavin) in premixtures and feedingstuffs. On request of the EURL, the method has been further verified by the Applicant in order to extend the scope to the analysis of the active substance in the feed additive under assessment. The following performance characteristics were reported: a relative standard deviation for repeatability (RSDr) of 2.4%; a relative standard deviation for intermediate precision (RSDip) of 2.7%; and a recovery rate (Rrec) of 98%. Based on these performance characteristics, the EURL recommends for official control the single-laboratory validated and further verified method based on HPLC-FLD to determine riboflavin in the feed additive.

For the determination of the riboflavin in premixtures, the Applicant proposed the ring-trial validated method by the Association of German Agricultural Analytical Research Institutes (VDLUFA - Bd. III, 13.9.1) based on ion-pair reversed phase HPLC coupled to UV detection (HPLC-UV). The VDLUFA method is intended for the analysis of vitamin B2 in the feed additive, premixtures and mineral feeds.

The following performance characteristics were reported for the quantification of vitamin B2 in premixture samples with a content ranging from 868 to 15990 mg/kg: an RSDr ranging from 2.4 to 4.7%; an RSDR ranging from 4.2 to 7.3%; and an Rrec ranging from 86 to 100%. Furthermore, the following performance characteristics were reported for the quantification of vitamin B2 in blends of various vitamins with a content of the feed additive of 37.5 g/kg: an RSDr of 1.2% and an RSDR of 5.4%.

Based on these performance characteristics, the EURL recommends for official control the ring-trial validated VDLUFA method (Bd. III, 13.9.1) to determine riboflavin in the feed additive and in premixtures within the concentration range covered by the collaborative study.

For the determination of riboflavin (as total vitamin B2) in feedingstuffs, the Applicant proposed a ring-trial validated CEN method intended for foodstuffs (EN 14152). The analytical method is based on acidic hydrolysis followed by HPLC-FLD. The CEN method was ring-trial validated using milk powder and pig liver certified reference materials (CRM). The following performance characteristics for the determination of the total vitamin B2 content ranging from 145 to 1055 mg/kg were reported: an RSDr ranging from 1.7 to 3.2%; an RSDR ranging from 7.3 to 7.9%; and an Rrec of ca. 100%. Furthermore, as described in a former EURL report, similar performance characteristics have been obtained by applying the CEN method to the analysis of total vitamin B2 in feedingstuffs samples, thus confirming the extension of scope of the CEN method to this matrix. Based on these performance characteristics, the EURL recommends for official control the ring-trial validated CEN method (EN 14152:2003) to determine riboflavin (as total vitamin B2) in feedingstuffs.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.