Safety and efficacy of feed additives consisting of Vitamin B$_2$ (98%) and Vitamin B$_2$ (80%) as riboflavin produced by *Bacillus subtilis* KCCM 10445 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 safety and efficacy of vitamin B$_2$ 98% and vitamin B$_2$ 80% in the form of riboflavin produced by fermentation with a genetically modified strain of *Bacillus subtilis* (KCCM 10445) as nutritional additives in feed and water for drinking for all animal species. The production strain harbours antimicrobial resistance genes. With the data available the Panel is not in the position to confirm the correspondence between the strain deposited under accession number KCCM 10445 and the strain under the current assessment. The production strain and its recombinant DNA were not detected in the final products. Therefore, the additives do not pose any safety concern associated with the genetic modification of the production strain. The additives are safe for the target species, the consumer and the environment. Vitamin B$_2$ 80% is not hazardous by inhalation. In the absence of data, no conclusions on the possible risk by inhalation of vitamin B$_2$ 98% can be reached. Neither vitamin B$_2$ 98% nor vitamin B$_2$ 80% are irritant to skin or eyes. In the absence of data, no conclusions on the skin sensitisation potential of the additives can be reached. The additives under assessment are effective in covering the animals’ requirements for vitamin B$_2$ when administered via feed and/or water for drinking.

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1. **Introduction**

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

Regulation (EC) No 1831/2003 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 EU by GUANG JI PHARMACEUTICAL BELGIUM SA NV for authorisation of the product Vitamin B2/Riboflavin produced by *Bacillus subtilis* KCCM 10445, when used as a feed additive for all animal species (category: nutritional additives; functional group: vitamins, provitamins and chemically well-defined substances having a 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 5 May 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 B2/Riboflavin produced by *Bacillus subtilis* KCCM 10445, when used under the proposed conditions of use (see Section 3.1.6).

1.2. **Additional information**

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued two opinions on the safety and efficacy of vitamin B2 (80%) produced by *Bacillus subtilis* KCCM 10445 for all animal species (EFSA FEEDAP Panel, 2014, 2018a). In its opinion of 2014 (EFSA FEEDAP Panel, 2014), the Panel concluded that neither the production strain nor its recombinant DNA were detected in the final product, and therefore, the additive did not give rise to any safety concern with regard to the genetic modification of the production strain. In the context of official controls, viable cells and recombinant DNA from the production strain were found in reference samples other than those used to produce the data included in the dossier submitted to EFSA. Based on the new data provided, the FEEDAP Panel issued a second opinion in 2018 (EFSA FEEDAP Panel, 2018a) and concluded that the product vitamin B2 (80%) as riboflavin posed a risk for the target species, consumers, users and the environment due to the presence of viable cells and DNA of a genetically modified strain-harbouring genes coding for resistance to antimicrobials of human and veterinary importance.

The FEEDAP Panel issued an opinion on the safety and efficacy of vitamin B2 as riboflavin and riboflavin-5’-phosphate ester monosodium salt, produced by either *B. subtilis* DSM 17339 or *B. subtilis* DSM 23984 (EFSA FEEDAP Panel, 2016), another opinion on the safety and efficacy of vitamin B2 (riboflavin) produced by *Ashbya gossypii* (EFSA FEEDAP Panel, 2018b) and an opinion on the safety and efficacy of vitamin B2 (riboflavin 5’-phosphate ester monosodium salt) for all animal species when used in water for drinking (EFSA FEEDAP Panel, 2018c). The last one was related to the safety and efficacy of the feed additive consisting of vitamin B2/riboflavin produced by *Eremothecium ashbyi* CCTCCM 2019833 for all animal species (EFSA FEEDAP Panel, 2021).

Riboflavin produced by fermentation using different production strains is currently authorised for its use in all animal species as a nutritional additive. The authorisation of vitamin B2 (80%) produced by *Bacillus subtilis* KCCM 10445 has been denied by Commission Implementing Regulation (EU) 2018/1254.

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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 in EU by GUANG JI PHARMACEUTICAL BELGIUM SA NV. Rue laïd Burnait 3, 1348, Louvain-La-Neuve, Belgium.
3. Commission Implementing Regulation (EU) 2019/901 of 29 May 2019 concerning the authorisation of riboflavin produced by *Ashbya gossypii* (DSM 23096), riboflavin produced by *Bacillus subtilis* (DSM 17339 and/or DSM 23984) and riboflavin 5’-phosphate sodium salt produced by *Bacillus subtilis* (DSM 17339 and/or DSM 23984) (sources of vitamin B2) as feed additives for all animal species. OJ L 144, 3.6.2019, p. 41.
4. Commission Implementing Regulation (EU) 2018/1254 of 19 September 2018 concerning the denial of authorisation of riboflavin (80%) produced by *Bacillus subtilis* KCCM-10445 as a feed additive belonging to the functional group of vitamins, pro-vitamins and chemically well-defined substances having similar effect. OJ L 37, 20.9.2018, p. 5-8.
Riboflavin is included in the current edition of the European Pharmacopeia (PhEur) monograph 01/2008:0292 (PhEur, 2021).

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 in support of the authorisation request for the use of vitamin B2 98% and vitamin B2 80% produced by *B. subtilis* KCCM 10445 as nutritional additives in feed and water for drinking.

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.6

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of vitamin B2 98% and vitamin B2 80% produced by *B. subtilis* KCCM 10445 is in line with the principles laid down in Regulation (EC) No 429/20087 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) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

This application deals with vitamin B2 98% and vitamin B2 80% produced by fermentation with a genetically modified strain of *B. subtilis* (KCCM 10445). The product is intended to be used as a nutritional additive (functional group: vitamins, provitamins and chemically well-defined substances having a similar effect) in feed and water for drinking for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the production organism

Riboflavin is produced by a genetically modified strain of *B. subtilis* which is deposited in the Korean Culture Centre of Microorganisms (KCCM) with accession number KCCM 10445.8 The strain *B. subtilis* KCCM 10445 has been assessed in a previous opinion (EFSA FEEDAP Panel, 2014). According to the applicant, the strain KCCM 10445 However, the newly submitted data show differences in the genetic modification steps reported previously, which according to the applicant In the herein assessment, the applicant has provided a comprehensive analysis of the production strain which triggered a new appraisal and allows the analysis of the genetic modification as presented in the dossier under assessment.

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5 FEED dossier reference: FAD-2019-0053.
6 The full report is available on the EURL website: [https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0053_vitb2.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0053_vitb2.pdf)
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.
8 Technical dossier/Section II/Annexes_Section II/Annex_II_33.
9 Technical dossier/Supplementary information August 2020/Annex-Reply to Supplementary Information Request, reply to question 1.
A bioinformatic analysis of the WGS of the production strain confirmed its identity as *B. subtilis*\(^{10}\). This was based on

The susceptibility of the production strain to the antibiotics recommended by the FEEDAP Guidance (EFSA FEEDAP Panel, 2018d) was tested\(^{11}\). Therefore, the production strain *B. subtilis* KCCM 10445 is considered resistant to

The WGS of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes\(^{12}\). Antimicrobial activity was measured in culture supernatants of the production strain.\(^{16}\) No inhibitory activity was detected

The WGS of the production strain was interrogated for the presence of virulence factors\(^{13}\). The toxigenic potential of *B. subtilis* KCCM 10445 was determined in\(^{15}\) and following the requirements of the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018d).\(^{14}\) so *B. subtilis* KCCM 10445 is considered not to be toxigenic.

3.1.1.1. Information related to the genetically modified microorganism

*Characterisation of the parental or recipient microorganism*

*Characteristics of the introduced sequences*
Description of the genetic modification

3.1.1.2. Uncertainty in relation to the deposition number of the production strain

The Panel notes that two different descriptions of the genetic modification of the production strain *B. subtilis* KCCM 10445 were provided for the previous and current assessment, particularly concerning the used in the development of the strain. These differences raise uncertainties on the correspondence of the deposited strain with the strain under assessment. With the data currently available no comparison between the deposited strain and the strain under assessment is possible.

3.1.2. Manufacturing process

Vitamin B₂ in the form of riboflavin is produced by fermentation with *B. subtilis* KCCM 10445.
According to the applicant, no antimicrobial substances are used in the manufacturing process.\textsuperscript{24}

### 3.1.3. Characterisation of the active substance

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, synonym Vitamin B\textsubscript{2}; 7,8-dimethyl-10-\{(1’-ribo-)isoalloxazine; lactoflavin; 1-deoxy-1-(7,8-dimethyl-2,4-dioxo-3,4-dihydrobenzo [g]pteridin-10(2H)-yl)-\(\beta\)-ribo), a compound identified with the Chemical Abstracts Service (CAS) No 83-88-5, the European Inventory of Existing Commercial chemical Substances (EINECS) No 201-507-1), has a molecular weight of 376.37 g/mol. The chemical formula of Riboflavin is C\textsubscript{17}H\textsubscript{20}N\textsubscript{4}O\textsubscript{6}. The structural formula is given in Figure 1.

\[ \text{Figure 1: Structural formula of riboflavin} \]

Riboflavin produced by fermentation is described in the European Pharmacopoeia (monograph 01/2008:0292) with a purity of \(\geq 97\%\) to \(\leq 103.0\%\) in the dried substance, less than \(0.5\%\) total impurities, less than \(0.025\%\) impurity A (luminflavin), less than \(0.2\%\) impurity B (7,8-dimethylbenzo[g]pteridine-2,4(1H,3H)-dione), C (6,7-dimethyl-8-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]pteridine-2,4(3H,8H)-dione) and D (8-(hydroxymethyl)-7-methyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4(3H,10H)-dione), respectively, and less than \(0.1\%\) for each unspecified impurity. Specifications are also set for loss on drying \(< 1.5\%\), sulphated ash \(< 0.1\%\), specific optical rotation between \(-115^\circ\) and \(-135^\circ\) and absorbance ratios \(A\textsubscript{373}/A\textsubscript{267}\) between 0.31 – 0.33 and \(A\textsubscript{444}/A\textsubscript{267}\) between 0.36 – 0.39.

### 3.1.4. Characterisation of riboflavin containing products

The applicant intends to place in the market two preparations containing riboflavin, one at 98\% and one at 80\%.

#### 3.1.4.1. Vitamin B\textsubscript{2} 98\%

Vitamin B\textsubscript{2} 98\% produced with \textit{B. subtilis} KCCM 10445 is specified to contain \(\geq 98\%\) riboflavin, \(\leq 1.5\%\) loss on drying and \(\leq 0.3\%\) residue on ignition.

Analysis of five batches showed compliance with the proposed specifications, resulting in an average content of 98.9\% riboflavin (range 98.9–99\%), loss on drying 0.2\% (range 0.2–0.3\%), residue on ignition 0.1\% (in all batches tested).\textsuperscript{25} In the same batches luminflavin was on average 0.0045\% (range 0.0041–0.0047\%), impurity B 0.076\% (range 0.05–0.09\%), impurity C 0\% in all

\textsuperscript{24} Clarification received by e-mail on 20 April 2021.
\textsuperscript{25} Technical dossier/Section II/Annexes Section II/Annex II 4.
batches tested, impurity D 0.14% (range 0.12–0.16%) and total impurities 0.37% (range 0.35–0.45%). The additive under assessment complies with the description and purity criteria set by the European Pharmacopoeia (monograph 01/2008:0292).

The specific optical rotation measured in five batches ranged from −129° to −127°. This is within the range of the reference values established in the European Pharmacopoeia (range between −115° and −135°).

Three batches of vitamin B2 98% were analysed for chemical and microbiological contamination. Mycotoxins (fumonisin B1 and B2; aflatoxin B1, B2, G1 and G2; deoxynivalenol; ochratoxin A; zearalenone) and heavy metals (mercury, lead, cadmium) and arsenic were all below their corresponding LOQs. Yeasts, moulds, Enterobacteriaceae, E. coli and Bacillus cereus were <10 colony forming units (CFU)/g and Salmonella spp. was not detected in 25 g.

Three batches of vitamin B2 98% were also analysed for the potential presence of chloramphenicol and erythromycin in the final product. In all batches tested, chloramphenicol and erythromycin were below their corresponding LOQs.

The presence of viable cells and spores of the production strain in the final product was tested in three batches. Samples of 10 g of each batch (triplicate samples) were dissolved in 90 mL of buffered peptone water. Then a total of 10 mL (corresponding to 1 gram of the original sample) was plated in a total of 10 PCA plates + chloramphenicol (5 mg/L) before and after heat treatment. The plates were incubated at 37°C for 72 h. Positive controls were included. No viable cells nor spores of the production strain were detected in the final product.

The presence of DNA from the production strain was tested in three batches of the final product by quantitative polymerase chain reaction (qPCR) analysis with an internal fluorescent probe. The starting material was 1 gram, and the primers targeted the junction of the 16S rRNA gene with an amplicon size of 76 bp. The protocol included a lysis step. Positive and negative controls were included. The limit of detection (LOD) in samples spiked with genomic DNA of the production strain was 0.1 ng/g of additive. No DNA of the production strain was detected.

The additive under assessment is a yellow to orange-yellow crystalline powder. It has a density of approximately 620 kg/m³, a bulk density of 0.40–0.50 g/mL, a melting point of 280°C and a Kow of −1.46. The pH of a 20 g/L solution at 20°C is 5.63. It is slightly soluble in water (0.06–0.08 g/L at 25°C) and not soluble in ether, alcohol and chloroform.

The dusting potential was measured in three batches of the additive according to Stauber–Heubach and the values ranged between 180 and 420 mg/m³. The same three batches were analysed for particle size distribution and the fraction of particles below 10 µm and 52 µm were in the ranges of 39.0–39.9% and 87.7–88.9%, respectively.

### 3.1.4.2. Vitamin B2 80%

Vitamin B2 80% produced with B. subtilis KCCM 10445 is specified to contain ≥80% riboflavin, ≤20% maltodextrin, ≤3% loss on drying and ≤5% residue on ignition.

Analysis of five batches showed compliance with the proposed specifications, resulting in an average content of 81.5% riboflavin (range 80.5–82.5%), maltodextrin 16% (range 15.5–16.6%), loss on drying 1.4% (range 1–2%) and residue on ignition 0.5% (range 0.1–1%).

Three batches of Vitamin B2 80% were analysed for chemical and microbiological contamination. Mycotoxins (fumonisin B1 and B2; aflatoxin B1, B2, G1 and G2; deoxynivalenol; T2 and HT-2 toxins; ochratoxin A; zearalenone), lead, cadmium and arsenic were all below their corresponding LOQs.

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26 Technical dossier/Section II/Annexes_Section II/Annex II_10 and Annex II_11. LOQ in µg/kg were 20 for fumonisin B1 and B2 and deoxynivalenol; 0.5 for aflatoxin B1, B2, G1 and G2; 10 for HT-2 toxin, T-2 toxin and zearalenone; 1 for ochratoxin A. LOQ in mg/kg were 0.005 for mercury; 0.05 for lead; 0.1 for arsenic and 0.01 for cadmium.

27 Technical dossier/Section II/Annexes_Section II/Annex II_46. LOQs were 0.3 µg/kg for chloramphenicol and 0.01 mg/kg for erythromycin.

28 Technical dossier/Section II/Annexes_Section II/Annex II_13.

29 Technical dossier/Section II/Annexes_Section II/Annex II_14.

30 Technical dossier/Section II/Annexes_Section II/Annex II_21 and Annex II_22.

31 Technical dossier/Section II/Annexes_Section II/Annex II_23.

32 Technical dossier/Section II/Annexes_Section II/Annex II_20.

33 Technical dossier/Section II/Annexes_Section II/Annex II_6.

34 Technical dossier/Section II/Annexes_Section II/Annex II_7 and Annex II_9.

35 Technical dossier/Section II/Annexes_Section II/Annex II_18 and Annex II_19. LOQ in µg/kg were 20 for fumonisin B1 and B2 and deoxynivalenol; 0.5 for aflatoxin B1, B2, G1 and G2; 10 for HT-2 toxin, T-2 toxin and zearalenone; 1 for ochratoxin A. LOQ in mg/kg were 0.05 for lead and arsenic and 0.01 for cadmium.
Mercury was on average 0.003 mg/kg (range 0.002–0.004 mg/kg). Yeasts, moulds, Enterobacteriaceae and E. coli were < 10 CFU/g and Salmonella spp. was not detected in 25 g.

The same batches were also analysed for the potential presence of antimicrobials in the final product and the values were below their corresponding LOQs.

Neither the production strain nor its recombinant DNA were detected in three batches of vitamin B2 80%, each analysed in triplicate, under the same conditions as for vitamin B2 98%.

The additive under assessment appears in the form of yellow-orange to yellow-brown fine granules with weak to no odour. It has a density of approximately 540 kg/m³, a bulk density of 0.40–0.50 g/mL, a melting point of 280°C and a Kow of –1.46. The pH of a 20 g/L solution at 20°C is 5.63. It is slightly soluble in water (0.06–0.08 g/L at 25°C) and not soluble in ether, alcohol and chloroform.

The dusting potential was measured in three batches of the additive according to Stauber–Heubach and the values ranged between 460 and 920 mg/m³. The same three batches were analysed for particle size distribution and the fraction of particles below 10 μm and 52 μm were in the ranges of 3.6%–3.9% and 12.3%–13.4%, respectively.

3.1.5. Stability and homogeneity

Three batches of vitamin B2 98% and vitamin B2 80% were stored in polyethylene bags/fibre drum system at 25°C for 36 months (vitamin B2 98%) and 9 months (vitamin B2 80%) or at 40°C for 6 months. No losses were observed under both conditions.

The stability of both formulations (one batch each) in a vitamin premixture for laying hens (without choline chloride) was studied when added at 0.061% (vitamin B2 98%) or 0.075% (vitamin B2 80%) and stored at 25°C for 6 months (tight containers). Losses of 2.8 and 3.5% of riboflavin content were observed in vitamin B2 98% and in vitamin B2 80%, respectively, in the premixture up to 6 months.

The stability of both formulations (one batch each) in compound feed for laying hens (based on maize gluten meal) was studied when added at 6 mg/kg feed and stored at 25°C for 6 months (tight containers). Losses of 3.7 and 4.4% of riboflavin content were observed in vitamin B2 98% and in vitamin B2 80%, respectively, in compound feed up to 6 months.

The stability of the additive in water for drinking of one batch of vitamin B2 98% was tested. A total of 10 mg of vitamin B2 98% was suspended in 1 L of tap water and stored at 27°C. The content of riboflavin was measured in a subsample after 0, 24 and 48 h. No losses were observed after 48 h at 27°C.

The capacity of one batch of vitamin B2 98% to distribute homogeneously was studied in ten subsamples in compound feed for pigs and water for drinking. The homogeneity in water for drinking showed a coefficient of variation (CV) of 0.05%. Samples of the compound feed showed a CV of 8.7%.

3.1.6. Conditions of use

The additives, vitamin B2 98% and vitamin B2 80%, are intended to be added to feed or water for drinking of all animal species and categories. They can be incorporated into feed directly or via premixtures with no maximum content. The supplementation rate is directed to meet the animal’s needs. Considering the limited water solubility of the additives, the maximum supplementation of water for drinking should not exceed 0.07 g riboflavin/L (at 20°C).

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36 Technical dossier/Supplementary information November 2020/Annex_A. LOD was 15 µg/kg.
37 Technical dossier/Section II/Annexes_Section II/Annex II_19. LOQ was 0.1 µg/kg for chloramphenicol and 1 µg/kg for clindamycin, erythromycin, josamycin, kitasamycin, lincomycin, roxithromycin, tilmicosin and tylosin.
38 Technical dossier/Section II/Annexes_Section II/Annex II_13 and Annex II_14.
39 Technical dossier/Section II/paragraphs 2.1.5, 2.2.1 and 2.2.2 and Annex II_21 and Annex II_22.
40 Technical dossier/Section II/Annexes_Section II/Annex II_25.
41 Technical dossier/Section II/Annexes_Section II/Annex II_24.
42 Technical dossier/Section II/Annexes_Section II/Annex II_62 and Annex II_63.
43 Technical dossier/Section II/Annexes_Section II/Annex II_64 and Annex II_66.
44 Technical dossier/Section II/Annexes_Section II/Annex II_65 and Annex II_67.
45 Technical dossier/Section II/Annexes_Section II/Annex II_68.
46 Technical dossier/Section II/Annexes_Section II/Annex II_69 and Annex II_69.
47 The applicant refers to the recommendations of FEFANA publication – Vitamins in Animal Nutrition.
3.2. Safety

3.2.1. Safety of the production organism

The parental strain of the production strain *B. subtilis* KCCM 10445 belongs to a species considered to be suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strain to be unequivocally established and evidence provided that the strain lacks toxigenic potential and does not show acquired resistance to antibiotics of human and veterinary importance and for genetically modified strains the safety of the genetic modification needs to be established. The identification of the strain has been conclusively established and lack of toxigenic potential has been confirmed. The strain is resistant to 

As a result of the genetic modifications, the production strain harbours genes for resistance to 

However, viable cells and recombinant DNA of this production strain were not detected in the final products. Therefore, the use of *B. subtilis* KCCM 10445 in the production of riboflavin contained in the final products does not raise safety concerns as regards the genetic modification of the production strain.

3.2.2. Safety for the target species, consumer and environment

Safety concerns from the additives may derive either from riboflavin or from the residues of the fermentation process/production strain remaining in the final product. The active substance is produced by a genetically modified microorganism for which the recipient strain is considered by EFSA to qualify for the QPS approach to safety assessment and for which the genetic modification raises no toxicological concerns. The genetic modification introduced a however, this is not expected to have an impact on the toxicological profile of the production strain.

The nutrient requirements/recommendations of the target species for vitamin B2, the background levels of vitamin B2 in feed materials and the tolerance to overdoses of vitamin B2 were reviewed by the FEEDAP Panel in previous opinions (EFSA FEEDAP Panel, 2014, 2016, 2018b). The Panel concluded that the use levels based on the requirement/background levels would pose no safety concerns to the target species. The Panel is not aware of any more recent findings which would modify the above conclusion. The inclusion rate of riboflavin would usually not exceed 10 mg/kg complete feed, corresponding to 10.2 mg vitamin B2 98%. The sum of identified substances (see Section 3.1.4.1) is as the mean of 5 batches of vitamin B2 98% at least 99.2%. Moreover, the production strain is considered safe from the toxicological point of view. Therefore, it can be concluded that no safety concerns for the target animal would rise from the fermentation residues that may be present in the final additives and the product used to formulate them (i.e. maltodextrin). The FEEDAP Panel concludes that vitamin B2 produced by *B. subtilis* KCCM 10445 is considered safe for the target species when used in feed or water for drinking.

The safety of riboflavin and consumer exposure to riboflavin were reviewed in previous opinions (EFSA ANS Panel, 2013; EFSA FEEDAP Panel, 2014, 2016). The FEEDAP Panel concluded that the supplementation of feed with riboflavin would not be of concern for the consumers. The Panel is not aware of any more recent findings which would modify the above conclusion.

The active substance riboflavin occurs in nature. Its use in animal nutrition is not expected to substantially increase the concentration in the environment. Considering that viable cells and recombinant DNA of the production strain *B. subtilis* KCCM 10445 were not detected in the final products, a risk for the environment resulting from the use of the additives under assessment in animal nutrition is not foreseen.

The FEEDAP Panel concludes that the use of vitamin B2 produced by *B. subtilis* KCCM 10445 is safe for the target species, for the consumer and for the environment.

3.2.3. Safety for the user

3.2.3.1. Effect on respiratory system

The dusting potential indicates that exposure by inhalation is likely for both forms of the additive.

An acute inhalation toxicity study was performed with vitamin B2 80% according to OECD TG 403 (not good laboratory practice (GLP) compliant).48 Exposure of rats to 2,000 mg vitamin B2 80%/m³

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48 Technical dossier/Section III/Annexes_Sect.III/Annex_III.1 and Supplementary information November 2020/Annex_B and Annex Reply to Supplementary Information Request FAD-2019-0053 SIn 201020, reply to question 4.
aerosol for 4 h did not cause mortalities, signs of toxicity, effects on body weight or gross changes. Vitamin B2 80% did not induce acute inhalation toxicity under the experimental conditions of the study. No data for inhalation toxicity of vitamin B2 98% were provided. In the absence of data, no conclusions on the possible risk by inhalation of vitamin B2 98% can be reached.

3.2.3.2. Effect on eyes and skin

The acute dermal irritation/corrosion of vitamin B2 98 and 80% was tested in two valid, GLP compliant, studies performed according to OECD TG 404, which showed that the additives are not skin irritants.49 The acute eye irritation/corrosion of vitamin B2 98 and 80% was tested in two valid, GLP compliant, studies performed according to OECD TG 405, which demonstrated that the additives are not eye irritants.50

The applicant has provided a limited reported skin sensitisation study with vitamin B2 80% produced with a different manufacturing process which was already submitted with a previous application (EFSA FEEDAP Panel, 2014).51 The study is considered not relevant for the products under assessment and was not further considered in the assessment. Therefore, in the absence of data, no conclusions on the skin sensitisation potential of the additives can be reached.

3.2.3.3. Conclusions on safety for the user

Vitamin B2 80% is not hazardous by inhalation. In the absence of data, no conclusions on the possible risk by inhalation of vitamin B2 98% can be reached. Neither the vitamin B2 98% nor the vitamin B2 80% are irritant to skin or eyes. In the absence of data, no conclusions on the skin sensitisation potential of the additives can be reached.

3.3. Efficacy

Data on requirements, allowances and recommendations for riboflavin levels in feed are easily accessible in the standard literature on animal nutrition. Riboflavin (vitamin B2) has been used world-wide in animal nutrition for decades. Owing to the long history of use and its established nutritional role in domestic animals, riboflavin when administered orally is regarded as effective in covering the animal's requirement for vitamin B2.

The FEEDAP Panel considers that vitamin B2 produced by *B. subtilis* KCCM 10445 is an effective source in covering the animals' requirements when administered via feed and/or water for drinking.

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 Regulation52 and Good Manufacturing Practice.

4. Conclusions

The production strain harbours genes for resistance to KCCM 10445. With the data available, the Panel is not in the position to confirm the correspondence between the strain deposited under accession number KCCM 10445 and the strain under the current assessment. The production strain and its recombinant DNA were not detected in the final products. Therefore, the additives do not pose any safety concern associated with the genetic modification of the production strain.

The use of vitamin B2 produced by *B. subtilis* KCCM 10445 is safe for the target species, the consumer and the environment.

Vitamin B2 80% is not hazardous by inhalation. In the absence of data, no conclusions on the possible risk by inhalation of vitamin B2 98% can be reached. Neither vitamin B2 98% nor vitamin B2 80% are irritant to skin or eyes. In the absence of data, no conclusions on the skin sensitisation potential of the additives can be reached.

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49 Technical dossier/Supplementary information January 2021/Annex_B and D.
50 Technical dossier/Supplementary information January 2021/Annex_C and E.
51 Technical dossier/Supplementary information November 2020/Annex_C.
52 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.
The additives under assessment are effective in covering the animals’ requirements for vitamin B₂ when administered via feed and/or water for drinking.

5. **Documentation as provided to EFSA/Chronology**

| Date            | Event                                                                                                                                 |
|-----------------|---------------------------------------------------------------------------------------------------------------------------------------|
| 05/08/2019      | Dossier received by EFSA. Vitamin B₂ (riboflavin) for all animal species. Submitted by Hubei Guangji Pharmaceutical Co. Ltd. represented in the EU by GUANG JI PHARMACEUTICAL BELGIUM SA NV. |
| 22/01/2020      | Reception mandate from the European Commission                                                                                       |
| 05/05/2020      | Application validated by EFSA – Start of the scientific assessment                                                                      |
| 01/07/2020      | 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** |
| 05/08/2020      | Comments received from Member States                                                                                                  |
| 21/08/2020      | Reception of supplementary information from the applicant - Scientific assessment re-started                                           |
| 01/09/2020      | Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives                                           |
| 20/10/2020      | 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, user safety** |
| 06/11/2020      | Reception of supplementary information from the applicant - Scientific assessment re-started                                           |
| 10/12/2020      | 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, user safety** |
| 29/01/2021      | Reception of supplementary information from the applicant - Scientific assessment re-started                                           |
| 05/05/2021      | Opinion adopted by the FEEDAP Panel. End of the Scientific assessment                                                                      |

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Abbreviations

AMR  antimicrobial resistance
CAS  Chemical Abstracts Service
CFU  colony forming unit
CRM  certified reference materials
CV   coefficient of variation
EINECS European Inventory of Existing Commercial chemical Substances
EURO European Union Reference Laboratory
FEEDAP EFSA Panel on Additives and Products or Substances used in Animal Feed
FLD  fluorescence detection
GLP  good laboratory practice
HPLC-UV high performance liquid chromatography coupled to UV detection
IUPAC International Union of Pure and Applied Chemistry
KCCM Korean Culture Centre of Microorganisms
LOD  limit of detection
LOQ  limit of quantification
OECD Organisation for Economic Co-operation and Development
PhEur European Pharmacopeia
qPCR quantitative polymerase chain reaction
QPS  qualified presumption of safety
RSDR relative standard deviation for reproducibility
WGS  whole genome sequence
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)

In the current application authorisation is sought under Article 4 for vitamin B₂ (riboflavin) 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 genetically modified Bacillus subtilis KCCM-10445 as active substance with a minimum purity (mass fraction) of 98%. According to the Applicant the product is sought for authorisation containing minimum 98% of the active substance or formulated in a preparation constituted by a minimum of 80% of riboflavin and a maximum of 20% of maltodextrin.

The Applicant proposes inclusion levels of the active substance ranging from 3 to 80 mg/kg and specifically for ruminants and horses between 20 and 85 mg/head/day complete feedingstuffs. The feed additive is intended to be added directly into feedingstuffs (or through premixtures) and water for drinking.

For the determination of riboflavin in the feed additive the Applicant proposed the methods presented within the European Pharmacopoeia riboflavin monograph where quantification is based on spectrophotometry at 444 nm. The EURL recommends this method for official control to quantify 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 High Performance Liquid Chromatography coupled to UV detection (HPLC-UV). 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: a relative standard deviation for repeatability (RSDᵣ) ranging from 2.4 to 4.7%; a relative standard deviation for reproducibility (RSDᵦ) ranging from 4.2 to 7.3%; and a recovery rate (Rᵦₑ₇) ranging from 86 to 100%. 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 premixtures within the concentration range covered by the collaborative study.

For the determination of riboflavin (as total vitamin B₂) in feedingstuffs and water 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 coupled to fluorescence detection (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: a RSDᵣ ranging from 1.7 to 3.2%; a RSDᵦ ranging from 7.3 to 7.9%; and a Rᵦₑᵦ 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 and water samples thus confirming the extension of scope of the CEN method to these matrices. 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 and water.

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.