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Safety of vitamin B_{12} (in the form of cyanocobalamin) produced by *Ensifer adhaerens* CNCM-I 5541 for all animal species

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Abstract

The vitamin B_{12} (in the form of cyanocobalamin) under assessment is produced by fermentation with a genetically modified strain of *Ensifer adhaerens* and it is intended to be used as a nutritional additive for all animal species. In 2018, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of EFSA issued an opinion on the safety and efficacy of the product. In that assessment, the Panel could not conclude on the safety of the additive for the target species, consumers and the environment due to uncertainties on the safety of the production strain and the resulting product. Due to high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, the additive was considered to pose a risk to user safety. The applicant provided supplementary data on the identity of the production strain, its susceptibility to antibiotics and toxigenic potential, as well as on the absence of cells and recombinant DNA of the production strain in the final product. The production strain is not expected to produce any toxic compound during fermentation but harbours antimicrobial resistance genes. However, viable cells and recombinant DNA of the strain were not detected in the most concentrated form of the additive. With this new information, the FEEDAP Panel concluded that vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 (identified as SCM 2034 in the previous opinion) is safe for all animal species, the consumers and the environment. The applicant did not provide new evidence that would lead the FEEDAP Panel to reconsider previous conclusions regarding the safety for the user.

Keywords: Nutritional additive, vitamin B_{12}, cyanocobalamin, *Ensifer adhaerens*, safety

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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 rules governing the Community authorisation of additives for animal nutrition and, in particular, Article 9 defines the terms of the authorisation by the Commission.

The applicant, FEFANA asbl -former VITAC EEIG, is seeking a Community authorisation for vitamin B_{12} (in form of cyanocobalamin) for all animal species (Table 1).

Table 1: Description of the substances

Category of additive	Nutritional additive
Functional group of additive	Vitamins, provitamins and chemically well-defined substances having a similar effect
Description	–
Target animal category	All animal species
Applicant	FEFANA asbl -former VITAC-EEIG
Type of request	New opinion

On 12 June 2018, the Panel on Additives and Products or Substances used in Animal Feed of the European Food Safety Authority ("Authority"), in its opinion on the safety and efficacy of the vitamin B_{12} (in the form of cyanocobalamin) when used as a feed additive for all animal species, could not conclude on the safety.

The Commission gave the possibility to the applicant to submit complementary information in order to complete the assessment and allow a revision of Authority's opinion. The new data have been received on 24 April 2019.

In view of the above, the Commission asks the Authority to deliver a new opinion on vitamin B_{12} (in the form of cyanocobalamin) as a nutritional additive for all animal species based on the additional data submitted by the applicant.

1.2. Additional information

The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) issued an opinion on the safety and efficacy of vitamin B_{12} (in the form of cyanocobalamin) produced by three strains of *Ensifer* spp. as a feed additive for all animal species (EFSA FEEDAP Panel, 2018a). One of the products assessed in that opinion was the one produced using the strain *E. adhaerens* CNCM I-5541 (SCM 2034).[2] Due to significant uncertainties on the identity and safety of the production strain *E. adhaerens* CNCM I-5541 and its resulting product, including the presence of antibiotic resistance genes, the absence of viable cells of the production strain or its DNA in the final product, the FEEDAP Panel could not conclude on the safety of the vitamin B_{12} produced by this strain for the target species, consumers and the environment. Additionally, due to high endotoxin content, the potential inhalation exposure when handling premixtures and the reported irritancy for skin and eyes, vitamin B_{12} produced using *E. adhaerens* CNCM I-5541 was considered to pose a risk to users of the additive.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of supplementary information[3] to a previous application on the same product.[4]

[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] Identified by the applicant as SCM 2034 in the previous application, and currently deposited at the Collection Nationale de Cultures de Microorganismes with the deposition number CNCM I-5541.
[3] FEED dossier reference: FAD-2019-0030.
[4] FEED dossier reference: FAD-2010-0199.

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety of vitamin B_{12} (in the form of cyanocobalamin) produced by *E. adhaerens* CNCM I-5541 is in line with the principles laid 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, 2017) and Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018b).

3. Assessment

The additive under assessment is vitamin B_{12} (in the form of cyanocobalamin) produced by fermentation with a genetically modified strain of *E. adhaerens* (CNCM I-5541) and intended to be used as a nutritional additive (functional group: vitamins, pro-vitamins and chemically well-defined substances having a similar effect) in feed for all animal species. The active substance, cyanocobalamin, is diluted with carriers to obtain a feed additive with two concentrations of cyanocobalamin, 0.1 or 1.0%.

The additive was characterised in a previous opinion of the FEEDAP Panel (EFSA FEEDAP Panel, 2018a), but the information provided regarding the production strain and the final additive did not allow the Panel to characterise it in full. In particular, the production strain was not taxonomically identified, no data on its sensitivity to antimicrobials relevant for human or veterinary medicine were provided (except for tetracycline), and its capacity to produce toxins or to harbour virulence factors was not established. Moreover, uncertainty remained regarding the presence of viable cells of the production strain and its DNA in the additive. Therefore, the FEEDAP Panel could not conclude on the safety aspects regarding the use of vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 for the target species, consumers and the environment.

In that assessment, the Panel concluded also that due to the high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 was considered to pose a risk to users.

The applicant has provided supplementary information to address the limitations regarding the characterisation of the production strain and the additive and the safety for the user.

3.1. Characterisation

3.1.1. Characterisation of the production strain

The production strain has been deposited at the French Collection Nationale de Cultures de Microorganismes under the accession number CNCM I-5541.[6]

The production strain was identified as *E. adhaerens* [redacted] confirming the identification of the production strain as *E. adhaerens.*[8]

The production strain was developed from [redacted] The assessment of the genetic modification of the production strain was performed in the EFSA FEEDAP Panel opinion of 2018 (EFSA FEEDAP Panel et al., 2018a) and the Panel concluded that the production strain showed increased production of vitamin B_{12} and [redacted].

The applicant provided new data regarding the susceptibility of the production strain to 14 antimicrobials of human and veterinary importance (including ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, chloramphenicol, erythromycin, tylosin, vancomycin, clindamycin, nalidixic

[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/Supplementary information September 2020/Annexes/Annex_Qi and Annex_Qii.
[7] Technical dossier/Supplementary information April 2020/Annexes/Annex_Qi.
[8] Technical dossier/Supplementary information September 2020/Annexes/Annex_Qiii.

acid, sulphonamide, trimethoprim and apramycin). *E. adhearens* CNCM I-5541 was shown to be resistant to aminoglycoside antimicrobials kanamycin and streptomycin and to tetracycline.[9] The WGS-based data of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes [redacted].

The WGS based data of *E. adhaerens* CNCM I-5541 was interrogated for the presence of toxin and virulence factor genes [redacted].[10] No relevant hits were identified. Moreover, the applicant conducted a new literature search using PubMed databases, including the search terms: *Ensifer adhaerens*, *Agrobacterium radiobacter*, *Pseudomonas denitrificans* (former names and classification of the production strain CNCM I-5541), toxin, virulence gene, virulence factor, pathogen and safety.[11] The search did not identify relevant hits.

3.1.2. Absence of production strain in the additive

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

The presence of DNA from the production strain was analysed [redacted] No recombinant DNA was detected in the nine analysed samples [redacted]

Therefore, it can be concluded that no viable cells or DNA of the production strain were detected in the additive containing 1% vitamin B_{12}. The results of the analysis with vitamin B_{12} 1% can be extended to vitamin B_{12} 0.1%.

3.1.3. Stability

No data on the stability of the additive in premixtures and feedingstuffs were provided in the original submission (EFSA FEEDAP Panel, 2018a).

The applicant has provided new studies on the stability of the additive formulated at 1% vitamin B_{12} in premixtures, feedingstuffs and at different pelleting temperatures.

The three batches of vitamin B_{12} 1% used for stability studies showed an average loose and tapped density of 1.0 g/cm^3 (range 0.96–1.02 g/cm^3) and 1.4 g/cm^3 (range 1.39–1.47 g/cm^3), respectively.[14] The dusting potential measured in the same three batches (Heubach I test) ranged from 10 to 11 g/m^3 and the fractions of particles (measured by laser diffraction) with diameters < 50 μm and < 100 μm ranged 68–69.5% and 90.8–91.2%, respectively.

The stability of the additive containing 1% vitamin B_{12} (three batches) in two vitamin–mineral premixtures (one with 38 g/kg choline chloride, and the other one without choline chloride) was studied when added at 2.9 mg/kg premixture and stored at 25°C for 6 months in plastic bags.[14] After 6 months, losses of vitamin B_{12} in the premixture with choline chloride ranged from 0 to 13% depending on the batch considered, and from 5 to 15% in the premixture without choline chloride.

The stability of vitamin B_{12} 1% (three batches) during feed processing was studied when added at 0.044 mg/kg feed to two different mash feeds for chickens for fattening (one based on maize and soybean meal and the other on maize, soybean meal and wheat, none having choline chloride), one pelleted at 84°C and the other at 93°C.[14] When pelleting at 84°C, the losses ranged from 6 to 12% depending on the batch considered. Pelleting at 93°C resulted in losses ranging from 0 to 4%.

[9] Technical dossier/Annex_4.
[10] Technical dossier/Supplementary information April 2020/Annexes/Annex_Qii.
[11] Technical dossier/Annex_8.
[12] Technical dossier/Supplementary information April 2020/Annexes/Annex_Qiii.
[13] Technical dossier/Supplementary information April 2020/Annexes/Annex_Qiv.
[14] Technical dossier/Annex_7.

The stability of the additive containing 1% vitamin B_{12} was evaluated when added to a mash and pelleted feed for chickens for fattening (three batches, intended level 0.044 mg/kg) and for piglets (one batch, intended level 0.077 mg/kg, diet based on maize, soybean meal and rapeseed) after storage in plastic bags at room temperature for 3 months.[14] Pelleting of piglets feed (83°C) represented a loss of 5% vitamin B_{12}. At the end of the storage period, losses observed in mash feed for chickens for fattening ranged from 15 to 28% depending on the batch considered, and from 0 to 19% in pelleted feed for chickens for fattening. No losses were observed in mash and pelleted feed for piglets after 3 months storage.

3.2. Safety of the additive for target species, consumers and environment

In the previous opinion, the FEEDAP Panel could not conclude on the safety of the use of vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 (SCM 2034) in animal nutrition for the target species, consumers and the environment due to significant uncertainties on the identity and safety of the production strain *E. adhaerens* CNCM I-5541 (SCM 2034), including the presence of antibiotic resistance genes, the absence of viable cells of the production strain or their DNA in the product.

The identity of the production strain as *E. adhaerens* has been confirmed. Based on the WGS data provided, the production strain is not expected to produce any toxic compound during fermentation [redacted]. However, viable cells and recombinant DNA of the strain were not detected in the most concentrated form of the additive. The supplementary information submitted by the applicant allows the Panel to conclude that vitamin B_{12} does not raise safety concerns as regards the production strain. The newly submitted data allow to address the limitation expressed by the Panel in the previous assessment regarding the production strain.

Considering the above, the FEEDAP Panel concludes that the additive is safe for the target species, consumers of products derived from animals fed the additive and for the environment.

3.3. Safety for user

The safety for the user was evaluated by the FEEDAP Panel in a previous assessment (EFSA FEEDAP Panel, 2018a). The Panel concluded that 'Due to high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 (SCM 2034) is considered to pose a risk to user safety'.

In particular, the endotoxin activity measured in three batches of the additive formulated at 1% vitamin B_{12} ranged from [redacted] (EFSA FEEDAP Panel, 2018a). Based on the calculation of the potential endotoxin content in dust (Wallace et al., 2016), and on the dusting potential and respirable particle fraction, the inhalation exposure was calculated to be up to [redacted] endotoxin IU per 8-h working day, indicating a risk from the exposure to endotoxins for people handling the additive (EFSA FEEDAP Panel, 2018a). The Panel notes that in the current dossier the applicant has provided data on the dusting potential of the three batches of the additive formulated at 1% vitamin B_{12} used in stability studies.[14] The dusting potential in those batches was up to 11 g/m^3, which is higher than the one reported in the previous opinion (EFSA FEEDAP Panel, 2018a) and used for the calculations of the potential endotoxin content in dust (3 g/m^3). Therefore, the exposure of the users to endotoxins could potentially be higher.

The applicant has provided a report on inhalation exposure to vitamins (vitamin A and vitamin D_3) and minerals (Co, Se) in a producing plant in Korea which is considered not relevant for the product under assessment and was not further considered in the assessment. Therefore, the applicant did not provide new evidence that would lead the FEEDAP Panel to reconsider previous conclusions that vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 poses a risk to user safety.

4. Conclusions

No viable cells and recombinant DNA of the strain were detected in the most concentrated form of the additive. The production of vitamin B_{12} does not raise safety concerns as regards the production strain. The FEEDAP Panel concludes that vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 is safe for all animal species, the consumers and the environment.

Due to high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, vitamin B_{12} produced by *E. adhaerens* CNCM I-5541 is considered to pose a risk to user safety.

5. Documentation as provided to EFSA/Chronology

Date	Event
26/04/2019	Dossier received by EFSA. Vitamin B12 produced by *Ensifer adhaerens* SCM 2034 for all animal species. Submitted by FEFANA asbl.
10/05/2019	Reception mandate from the European Commission
22/05/2019	Application validated by EFSA – Start of the scientific assessment
11/09/2019	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*
02/04/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
19/05/2020	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*
29/09/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
18/11/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

AMR	antimicrobial resistance
CNCM	Collection Nationale de Cultures de Microorganismes
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
WGS	whole genome sequence