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## EFSA statement on the risk posed to humans by a vitamin B<sub>2</sub> produced by a genetically modified strain of *Bacillus subtilis* used as a feed additive

European Food Safety Authority

### Abstract

The detection of recombinant DNA in a vitamin B<sub>2</sub> used as feed additive was notified by the Belgian national authorities on 2 October 2018 via the Rapid Alert System for Food and Feed (RASFF). The European Commission requested scientific advice from EFSA on the risk posed to humans by the presence of genetically modified material in the feed additive, particularly with regard to antimicrobial resistance (AMR). EFSA assessed the analytical data from RASFF regarding the presence of AMR genes in both additive and feed. Samples of the additive and feed tested positive for presence of DNA of a genetically modified *Bacillus subtilis*. The results were compatible with, but did not demonstrate the presence of, a full-length chloramphenicol resistance gene. No information was made available on the presence of other AMR genes or viable cells of the *Bacillus subtilis*. The statement provides a risk assessment pathway indicating the events needed to produce adverse human health effects from the presence of AMR genes in feed additives. Data on the likelihood of occurrence of all events are needed to produce an evidence-based estimate of the risk. All the events are theoretically possible, but there are no scientific data available to estimate the probability of each taking place. Moreover, there is no evidence of the presence of full-length AMR gene(s) in the vitamin B<sub>2</sub> additive or feed, thus it is not clear whether the first step towards AMR gene transfer is fulfilled. The sole presence of fragments of AMR genes in a feed additive is not a risk. If a full-length AMR gene were present in a feed additive, it could lead to risks linked to its transmission to pathogens via the food chain and/or to the environmental spread of AMR bacteria/genes, potentially contributing to the environmental reservoir of AMR determinants.

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**Keywords:** RASFF notification, vitamin B<sub>2</sub>, feed additive, antimicrobial resistance, gene transfer, DNA, human health

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## 1. Introduction

### 1.1. Background as provided by the requestor

In accordance with Regulation (EC) No 1831/2003 on additives for use in animal nutrition, vitamin B<sub>2</sub> may currently be placed on the EU market as an “existing product”, pending the adoption of a decision on the applications for re-evaluation submitted under Article 10(2) of the said Regulation<sup>1</sup>.

At present, Vitamin B<sub>2</sub> is produced mainly by fermentation of genetically modified (GM) microorganisms. It may be placed on the market provided that no recombinant DNA or viable cells from the GM micro-organism are present in the feed additive, if no specific authorisation has been obtained for that GM product under Regulation (EC) No 1829/2003 on GM food and feed.

In the framework of official controls, the Belgian authorities have notified on 2 October 2018 under the RASFF system (notification number 371917, with reference 2018.2755) the detection of traces of recombinant DNA in a non-authorised vitamin B<sub>2</sub> to be used as a feed additive, using two analytical methods (*Bacillus subtilis* -558 and -3557). No test was performed to analyse the presence of any viable cells of the specific strain in the product.

According to the Belgian authorities, the producer of the additive stated that the vitamin was produced by strain CGMCC13326 and could not explain the presence of different sequences of recombinant DNA related to other strains (see attached analytical reports). The Belgian authorities also analysed samples of compound feed containing vitamin B<sub>2</sub> from the affected batch (the level of incorporation of vitamin B<sub>2</sub> in compound feed ranges from 1-5 mg/kg of compound feed with 12% of humidity) and the results were negative.

While Member States authorities have been invited by the Commission to withdraw implicated products from the market along the entire feed chain, some compound feed containing the non-authorised vitamin had already been consumed by animals.

Based on the data provided by the Belgian authorities under the RASFF system, the Commission would like to know what is the impact on the safety for the consumers of products derived from animals fed with such feed. In particular in the context of the general situation concerning antimicrobial resistance and on the basis of the findings of the Belgian authorities:

- Is it possible to determine the presence in feed and in derived animal products of genetic material which harbours genes coding for resistance to antimicrobials of human and veterinary importance?
- What is the risk derived from the consumption of product of animal origin deriving from animals fed with this feed additive?

### 1.2. Terms of Reference as provided by the requestor

In accordance with Article 31(1) of Regulation (EC) No 178/2002, the European Commission asks the European Food Safety Authority to provide a scientific statement on the risk posed to humans derived from the presence in the vitamin B<sub>2</sub> notified under the RASFF (ref. 2018.2755) of genetic modified material.

In particular, in the context of the situation concerning antimicrobial resistance, the question is posed of the presence in feed containing that vitamin B<sub>2</sub> and in products derived from animals fed with such feed, of gene(s) coding for resistance to antimicrobials of human and veterinary importance.

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<sup>1</sup> One of the applications for re-evaluation was subject to 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 237, 20.9.2018, p.5).

### 1.3. Interpretation of the Terms of Reference

The assessment will be limited to determining the presence of antimicrobial resistance (AMR) genes in the feed additive and the AMR-derived public health risks.

## 2. Data

The assessment will be based on the information provided to EFSA by the European Commission. The documents are related to RASFF notification number 371917, from 2 October 2018 and reference 2018.2755, and follow up notifications received until 7 January 2019. The European Commission specifically referred to the analytical reports linked to notifications 371917 (vitamin B<sub>2</sub> samples) and follow up 38 for notification 371917 (ref 379444, referring to feed<sup>2</sup>). Additional information regarding the analysis linked to notification 371917 from the laboratory that performed the analysis and a scientific publication regarding the methods used to analyse the samples (Paracchini et al., 2017) were also made available to EFSA. EFSA also considered the publication from Barbau-Piednoir et al. (2015) that regards one of the analytical methods used.

In the RASFF notifications received until 7 January, follow up 35, analyses of samples from a compound feed for piglets were notified. Samples tested negative, these findings were not considered in this assessment.

## 3. Assessment

### 3.1. Analysis of the information provided

The RASFF notification with reference 2018.2755, initiated by the Belgian Authorities, relates to 'unauthorised genetically modified (*Bacillus subtilis*) bacteria in vitamin B<sub>2</sub> 80% from China, via the Netherlands'. The Belgian Reference Laboratory analysed, in the frame of an official control, samples from a vitamin B<sub>2</sub> from the market and identified the presence of 'unauthorised genetically modified *Bacillus subtilis*'. Later, the same laboratory analysed feed samples in which the concerned vitamin B<sub>2</sub> was used and the samples tested positive.

The samples were analysed for the detection of recombinant DNA of *B. subtilis* with two different methods which are described in two publications Paracchini et al. (2017) and Barbau-Piednoir et al. (2015), respectively. Both methods were developed to detect the presence of a non-authorised genetically modified strain of *B. subtilis* found in 2014 by the French competent authorities in samples of vitamin B<sub>2</sub> produced by another company, and for which a RASFF notification was raised at that time (RASFF 2014.1249). This strain was characterised by whole genome sequence analysis by the German authorities and the JRC (Paracchini et al., 2017). Those detection methods are based on real-time PCR (limit of detection<sub>95%</sub> = 10 copies) using primers which target the following fragments:

- a 76-bp chromosomal fragment covering the junction between the *B. subtilis* *recA* gene and the chloramphenicol resistance gene (called method 558),
- a 128-bp fragment of plasmid origin covering the junction between the *B. subtilis* riboflavin biosynthesis operon and the pSM19035 vector backbone (called method 3357).

From the analytical reports provided to EFSA and linked to the RASFF notification, it can be concluded that in the vitamin B<sub>2</sub> samples analysed amplification was found above the limit of detection for both fragments (two positives for 558 and one positive for 3357). The feed samples tested positive only using method 558. Neither of the analyses included the calculation of the amount of DNA present per weight of sample.

These results indicate that recombinant DNA from a genetically modified *B. subtilis* strain containing at least part of the chloramphenicol resistance gene and plasmid pSM19035 is present in the vitamin B<sub>2</sub> and feed tested.

The information provided regarding the additive and strain that produces the vitamin (quoted in the mandate received as strain CGMCC13326) does not allow the full molecular characterisation of the

<sup>2</sup> According to a communication from European Commission these were complimentary feed for horses and the samples are not the same as those referred to in the mandate sent by European Commission.

production strain. The only information available is that the strain CGMCC13326 shares the insertion of the chloramphenicol resistance gene in the chromosome and the riboflavin operon in a pSM19035-derived vector with the strain characterised and sequenced by Paracchini et al., 2017. However, it is not possible to ascertain if it is the strain sequenced in the publication from Paracchini et al. 2017 or any other existing *B. subtilis* strain carrying those specific genetic modifications. The data available are compatible with, but are not sufficient to demonstrate the presence of transferable full-length AMR genes in the strain CGMCC13326.

### 3.2. Answer to the terms of reference

#### 3.2.1. Presence of AMR genes in feed and food

The detection of AMR genes in feed and food products derived from animals is technically possible, provided adequate methodology is used and the genetic material is present in amounts above the limit of detection. Such methodology could be designed to target the full-length AMR gene(s) or fragments of recombinant DNA of a similar length or to be able to detect viable cells of a production strain that harbours those AMR genes.

In the specific case of the RASFF notification 2018.2755, the methodologies applied to the samples analysed cannot ascertain whether DNA is present in fragments long enough to contain complete coding sequences, in this particular case the chloramphenicol resistance gene (*cat*). In conclusion, the results are compatible with, but do not demonstrate, the presence of a full-length chloramphenicol resistance gene in the vitamin B<sub>2</sub> or in the feed samples tested without further analysis with methods able to detect full-length gene. No information was provided to EFSA on the possible presence of other AMR genes and on the possible presence of *B. subtilis* viable cells in the samples analysed.

#### 3.2.2. Assessment of the risk posed to humans

In the case of chemical substances present in a feed additive and for which there is an established health-based guidance value, consumer exposure to the substance resulting from the consumption of products from food-producing animals fed with a feed additive can be estimated. The estimation would only require data on the food consumption and on the occurrence of the chemical substance in tissues and animal products resulting from the use of the additive. The presence in a feed additive of genes coding for resistance to an antibiotic of human and veterinary importance represents a hazard of biological origin that may result, when taken up by a bacterium, in an overall environmental increase of antimicrobial resistant bacteria.

This statement provides a risk assessment pathway indicating the steps necessary to produce adverse effects in human health due to the presence of genetic material coding for AMR present in feed additives.

Table 1 summarises the list of main steps of a risk pathway that would need to take place to allow the transmission of recombinant DNA carrying AMR genes from feed to human-borne and/or environmental bacteria, including pathogens, and also indicates the main factors that may affect the likelihood of such steps taking place. Finally, it gives an indication on the availability of data on such factors in the specific case described in RASFF notification 2018.2755.

The series of events that should occur is long and complex and with the limited data available it is not possible to provide an evidence-based estimation of the risk that the specific finding described in the RASFF notification would pose to human health. Data on the RASFF notification do not allow concluding on the presence of specific full-length chloramphenicol or any other AMR gene(s) or on the presence of viable cells from the production strain in vitamin B<sub>2</sub> or in feed.

When considering the theoretical/potential risk more generally, a few issues should be taken into consideration:

- All the steps in Table 1 are theoretically possible.
- Different scenarios should be considered in relation to the genetic material present. The likelihood of transfer of AMR genes to bacteria in animal intestines is dependent on the genetic material present. In particular, there is an increasing likelihood of this transfer in the

presence of full-length genes, genes with flanking regions, genes located on mobile genetic elements, and finally viable cells of the production strain carrying AMR genes.

- Once AMR genes/bacteria reach the animal intestinal environment in adequate quantities, there are ample opportunities for interaction with the resident microbiota and subsequent transfer of AMR genes to other bacteria.
- Possible human health risks are not only linked to the direct transmission of AMR genes along the feed and food chain. One should also consider the possibility that AMR genes present in the vitamin B<sub>2</sub> can spread to the environment at various stages of the feed and food chain (e.g. from the feed, from the food-producing animals, from the food, etc.). This environmental contamination would result in an overall increase in the likelihood that AMR genes could spread further in environmental bacterial populations and finally end up in human pathogens; this would constitute a long-term risk at human population level rather than at the single food consumer level. Humans can be exposed to bacteria carrying relevant AMR genes in different ways, e.g. through direct contact with humans or animals, through food, through contaminated environment.

In conclusion,

- Data on the likelihood of a long and complex chain of events are needed to produce an evidence-based estimate of the risk that a feed additive containing recombinant DNA/recombinant bacteria carrying AMR genes could pose to humans through the feed and food chain. All these steps leading to AMR gene transfer are theoretically possible, but there are no scientific data available to estimate the probability of each event taking place. It is important to highlight that, from the data currently available, there is no evidence of the presence of full-length AMR gene(s) in the vitamin B<sub>2</sub> or feed, and hence it cannot be concluded that the first step towards AMR gene transfer is fulfilled.
- It is not possible to produce an evidence-based estimate of the risk that the specific event described in the RASFF notification 2018-2755 would pose to human health through the consumption of food derived from animals fed with the vitamin B<sub>2</sub> feed additive under consideration.
- The sole presence of fragments of AMR genes in a feed additive is not a risk. The risk would be incremental in scenarios that consider the presence of full-length AMR genes, genes with flanking regions, genes located on mobile genetic elements, and finally viable cells carrying AMR genes.
- If a full-length AMR gene were present in a feed additive, it could lead to risks not only linked to their possible direct transmission all along the feed and food chain to pathogens, but also to the environmental spread of AMR bacteria/genes that could increase/contribute to the environmental reservoir of AMR determinants.

**Table 1:** Risk assessment pathway on AMR gene transfer from a feed additive to human-associated bacteria and information available on the specific finding notified under RASFF regarding a vitamin B<sub>2</sub> and subject of this statement\*

Step	Factors influencing the step		Data availability in relation to RASFF notification 2018-2755
<b>1</b>	<b>Production of the feed additive</b>		
1.1	Presence of genetic material originating from production strain	Quantity of feed additive produced and placed on the market. Levels of presence of genetic material from production strain in the feed additive.	Amount of DNA per weight of sample not calculated.
1.2	The genetic material contains AMR genes originating from the production strain	Type and number of AMR genes present (antimicrobials for which they encode resistance). Type of AMR mechanism (mutations, genes). Linkage of the AMR genes present to mobile genetic elements (insertion sequences, transposons, integrons, plasmids). Completeness of genes present (AMR gene fragments, full-length genes, genes with flanking regions, genes located on mobile genetic elements, viable cells).	Data do not allow concluding on the <i>B. subtilis</i> strain from which DNA material identified originates, which does not allow knowing all AMR genes possibly present. Data support the presence of part of chloramphenicol resistance gene and plasmid pSM19035. Data do not allow concluding on the presence of DNA fragments long enough to contain complete coding sequences. Data do not allow concluding on the presence of other resistance genes. No data available on testing performed for the presence of viable cells.
<b>2</b>	<b>Addition of the additive to the feed</b>		
2.1	Feed additive containing AMR genes is incorporated into feed during feed formulation	Dilution factor of the feed additive in the final feed (quantity of feed produced, % feed additive used). This could be specific for every different animal species and production system and for each type of feed produced and feed company.	For vitamin B <sub>2</sub> inclusion rates can be retrieved from feed manufacturers.
2.2	AMR genes survive the feed production process	Feed production method (feed additive included in feed at the moment of feeding or included before further treatments are applied during feed processing). For example, heat treatment, pressure, milling, chemical treatments could have an impact on the integrity of DNA and on the viability of bacteria present in the feed additive incorporated in the feed. This could be specific for every different animal species and production system and for each type of feed produced and feed company.	No data on the stage when/how vitamin B <sub>2</sub> is incorporated into the feed and on the treatments to which the feed is submitted before feeding.
<b>3</b>	<b>Feed administered to food-producing animals</b>		

3.1	Feed carrying AMR genes is fed to animals	Feed storage conditions/time, which could have an impact on the DNA degradation. Quantity of contaminated feed fed to animals.	No data on feed storage conditions/time. No data on quantity of feed fed to animals.
3.2	AMR genes survive passage through the digestive tract	Degradation rates during the passage through the intestinal tract. Although this could vary amongst animal species and animal production system.	No data available. Estimations to be done based on experimental studies or from scientific literature. Collection of relevant available data would need precise information on AMR genes, animal species and production system under consideration.
3.3	Transfer of AMR genes occurs in the animal intestine		
3.3.A	Presence of AMR genes (no viable cells) in feed		
3.3.A.1	Transformation - uptake of DNA by bacterial cells** in the animal intestine	Presence of competent bacterial cells. This could vary amongst animal species, production system, diet and health status of animals. Transformation frequencies.	No data available. Estimations to be carried out based on experimental studies or from scientific literature. Collection of relevant available data would need precise information on AMR genes, animal species, production system, diet and respective microbiome under consideration.
3.3.A.2	Transduction - DNA transfer to bacterial cells** by prophages in the animal intestine	Presence of the specific phages.	
3.3.A.3	Transformation/transduction - integration in genetic material (chromosome/plasmid) of host bacterial cell**	Frequency of integration in host bacterial cell.	
3.3.B	Presence of viable cells carrying AMR genes in feed		No data available. Estimations to be carried out based on experimental studies or from scientific literature. Collection of relevant available data would need precise information on AMR genes, animal species, production system, diet and respective microbiome under consideration.
3.3.B.1	Conjugation/mobilization (plasmid horizontal transfer) into bacterial cell**	Plasmid type (Inc type, broad or narrow host range). Conjugation rate.	
<b>4</b>	<b>Food contamination</b>		
4.A	Food of animal origin		
4.A.1	AMR bacteria (commensals or zoonotic pathogens) contaminate food of animal origin (meat, milk, eggs)	Hygiene conditions during animal production. Hygiene conditions during processing of food of animal origin. Cross-contamination opportunities during food processing, storage and preparation.	No data available. Estimations to be carried out based on experimental studies or from scientific literature. Collection of relevant available data would need precise information on AMR genes, animal species, animal/food production systems under consideration.
4.B	Food of non-animal origin		

4.B.1	AMR bacteria (commensals, animal pathogens or zoonotic pathogens) spread to the environment through animal-derived products or farm effluents (e.g. manure, water)	Hygiene conditions during animal production.	No data available. Estimations to be done based on experimental studies or from scientific literature. Collection of relevant available data would need precise information on AMR genes, animal species and animal/food production systems under consideration.
4.B.2	AMR bacteria (commensals, animal pathogens or zoonotic pathogens) from the environment contaminate food of non-animal origin (fruits and vegetables)	Hygiene conditions during food production. Cross-contamination opportunities during food processing, storage and preparation.	
<b>5</b>	<b>Food-borne transmission to human-associated bacteria</b>		
5.1	Ingestion of AMR bacteria (commensals or zoonotic pathogens) through food	Quantity of food of animal origin derived from animals fed the feed additive containing AMR genes. Level of contamination of food with AMR bacteria. General food consumption data and consumption habits.	No data available. Estimations to be done based on scientific literature and general food consumption data. Collection of relevant available data would need precise information on AMR bacteria and food under consideration.
5.2	Colonisation of human intestine by food-borne AMR bacteria (commensals)	Human microbiome. Human health and immune status.	
5.3	Infection with AMR bacteria (zoonotic pathogen)	Dose-response relationship for the zoonotic pathogen in question. Transfer of resistance from commensals to human pathogens within human intestine. Human microbiome.	
5.4	Failure of treatment with antimicrobials	Phenotypic AMR profile expressed by AMR bacteria.	

\* The table only considers direct transmission of AMR genes/bacteria along the food chain but, apart from food of non-animal origin, does not include the environmental compartment which can contribute to the spread of the AMR genes/bacteria, \*\* receptor bacteria could be either commensals (reservoir of AMR genes), animal pathogens (reservoir of AMR genes) or zoonotic bacteria (direct threat for human health)

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