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Safety and efficacy of *Bacillus subtilis* PB6 (*Bacillus velezensis* ATCC PTA-6737) as a feed additive for chickens for fattening, chickens reared for laying, minor poultry species (except for laying purposes), ornamental, sporting and game birds

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Maryline Kouba, Mojca Fašmon Durjava, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa, Ruud Woutersen, Pier Sandro Cocconcelli, Boet Glandorf, Lieve Herman, Miguel Prieto Maradona, Maria Saarela, Rosella Brozzi, Jaume Galobart, Lucilla Gregoretti, Gloria López-Gálvez, Maria Vittoria Vettori, Matteo Lorenzo Innocenti and Konstantinos Sofianidis

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 renewal of the authorisation of *Bacillus subtilis* PB6, the extension of use to ornamental, sporting and game birds and a modification on the concentration of the said additive. The product under assessment is based on viable spores of a strain originally identified as *Bacillus subtilis*. During the course of the current assessment, the active agent has been redesignated as *Bacillus velezensis* ATCC PTA-6737. The bacterial species *B. velezensis* is considered suitable for the qualified presumption of safety (QPS) approach to safety assessment. The identity of the active agent is established and the compliance with the other qualifications confirmed. Therefore, *B. velezensis* ATCC PTA-6737 is presumed safe for the target species, consumers of products derived from animals fed the additive and the environment. The additive is not a dermal/eye irritant or a skin sensitiser. Exposure via inhalation is unlikely. In the previous assessments performed by the FEEDAP Panel, the additive showed to be efficacious as a zootechnical additive in feedingstuffs for chickens for fattening and chickens reared for laying at the level of 1×10^7 CFU/kg. Considering that efficacy at the same level has been shown, this conclusion is extrapolated to ornamental, sporting and gaming birds.

Keywords: zootechnical additive, gut flora stabiliser, *Bacillus velezensis*, chickens, poultry, QPS, efficacy

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. Article 13(3) of that Regulation lays down that if the holder of an authorisation proposes changing the terms of the authorisation by submitting an application to the Commission, accompanied by the relevant data supporting the request for the change, the Authority shall transmit its opinion on the proposal to the Commission and the Member States. Moreover, Article 14(1) of that Regulation lays down that an application for renewal shall be sent to the Commission at the latest one year before the expiry date of the authorisation.

The European Commission received a request from Kemin Europa N.V.[2] for renewal of the authorisation of the product *Bacillus subtilis* PB6 (*Bacillus velezensis*[3] ATCC PTA-6737) when used as a feed additive for chickens for fattening, chickens reared for laying, minor poultry species (except for laying purposes), for modification of the concentration of the product from 1.0×10^{10} CFU/g to 8.0×10^{10} CFU/g and for new authorisation when used as feed additive for ornamental, sporting and game birds (category: zootechnical additive; functional group: gut flora stabilisers).

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), under Article 13(3) (modification of the authorisation of a feed additive) and under Article 14(1) (renewal of the authorisation). The particulars and documents in support of the application were considered valid by EFSA as of 5 September 2019.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product *Bacillus subtilis* PB6 (*Bacillus velezensis* ATCC PTA-6737), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

EFSA has issued several opinions on the safety and efficacy of *Bacillus subtilis* PB6 including its use in chickens for fattening (EFSA FEEDAP Panel, 2009), chickens reared for laying, ducks for fattening, quails, pheasants, partridges, guinea fowl, pigeons, geese for fattening and ostriches (EFSA FEEDAP Panel, 2011), weaned piglets and weaned minor porcine species (EFSA FEEDAP Panel, 2012), turkeys for fattening and turkeys reared for breeding (EFSA FEEDAP Panel, 2013a,b), laying hens, other minor laying poultry birds (EFSA FEEDAP Panel, 2015) and sows (EFSA FEEDAP Panel, 2017). An opinion on the compatibility of *Bacillus subtilis* PB6 (*Bacillus subtilis* ATCC PTA-6737) with coccidiostats was also published in 2010 (EFSA FEEDAP Panel, 2010). Furthermore, an opinion on the substantiation of health claims related to non-characterised microorganisms was also published in 2010 by EFSA Panel on Dietetic Products, Nutrition and Allergies (EFSA NDA Panel, 2009).

This product is currently authorised for use as a feed additive in diets for chickens for fattening,[4] chickens reared for laying, ducks for fattening, quails, pheasants, partridges, guinea fowl, pigeons, geese for fattening, ostriches,[5] turkeys for fattening and reared for breeding,[6] for weaned piglets and

[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] Kemin Europa NV, Toekomstlaan 42, 2200, Herentals, Belgium.
[3] Originally designated as *Bacillus subtilis*.
[4] Commission Regulation (EU) No 107/2010 of 8 February 2010 concerning the authorization of *Bacillus subtilis* ATCC PTA-6737 as a feed additive for chickens for fattening (holder of authorization Kemin Europa NV). OJ L 36, 9.2.2010, p. 1.
[5] Commission Implementing Regulation (EU) No 885/2011 of 5 September 2011 concerning authorization of *Bacillus subtilis* ATCC PTA-6737 as a feed additive for chickens reared for laying, ducks for fattening, quails, pheasants, partridges, guinea fowl, pigeons, geese for fattening, ostriches (holder of authorization Kemin Europa NV). OJ L 229, 6.9.2011, p. 3.
[6] Commission Implementing Regulation (EU) No 787/2013 of 16 August 2013 concerning the authorization of a preparation of *Bacillus subtilis* (ATCC PTA-6737) as a feed additive for turkeys for fattening and reared breeding ostriches (holder of authorization Kemin Europa NV). OJ L 220, 17.8.2013, p. 15.

weaned Suidae other than *Sus scrofa domesticus*,[7] for laying hens and minor poultry species for laying,[8] and for sows (4b1823).[9]

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[10] in support of the authorisation request for the use of *Bacillus subtilis* PB6 concentrate green (*Bacillus velezensis* ATCC PTA-6737) as a feed additive.

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

The European Union Reference Laboratory (EURL) considered that the conclusions and recommendations reached in the previous assessment regarding the methods used for the control of the *Bacillus subtilis* PB6 in animal feed are valid and applicable for the current application.[11]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Bacillus subtilis* PB6 concentrate green is in line with the principles laid down in Regulation (EC) No 429/2008[12] and the relevant guidance documents: Guidance on the renewal of the authorisation of feed additives (EFSA FEEDAP Panel, 2013a,b), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a) and Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b).

3. Assessment

Bacillus subtilis PB6 is the trade name for a feed additive based on viable spores of a strain of *Bacillus velezensis*. The product is currently authorised as a zootechnical additive (functional group: gut flora stabilisers) in diets for chickens for fattening, chickens reared for laying, minor poultry species (except for laying purposes). The applicant is asking for the renewal of this authorisation, the extension of use to ornamental, sporting and game birds and a modification of the concentration of the product – after standardisation – from 1×10^{10} CFU/g to 8×10^{10} CFU/g additive.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The active agent *B. velezensis* is a non-genetically modified organism that was isolated from the intestinal tract of a chicken and was deposited in the American Type Culture Collection with the accession number ATCC PTA-6737.[13,14]

The active agent was formerly identified as *Bacillus subtilis* (EFSA FEEDAP Panel, 2009). The data recently provided to support the taxonomic identification allocated the strain to the newly recognised species *Bacillus velezensis*. [redacted]

[7] Commission Implementing Regulation (EU) No 306/2013 of 2 April 2013 concerning the authorization of a preparation of *Bacillus subtilis* (ATCC PTA-6737) as a feed additive for weaned piglets and weaned Suidae other than *Sus scrofa domesticus*. OJ L 91, 3.4.2013, p. 5.

[8] Commission Implementing Regulation (EU) No 2015/1020 of 29 June 2015 concerning the authorization of a preparation of *Bacillus subtilis* (ATCC PTA-6737) as a feed additive for laying hens and minor poultry species for laying. OJ L 163, 30.6.2015, p. 22.

[9] Commission Implementing Regulation (EU) No 2017/2276 of 8 December 2017 concerning the authorization of a new use of the preparation of *Bacillus subtilis* (ATCC PTA-6737) as a feed additive for sows (holder of authorization Kemin Europa NV). OJ L 326, 9.12.2015, p. 50.

[10] FEED dossier reference: FAD-2019-0017.

[11] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2008-0039.pdf

[12] 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.

[13] Technical dossier/Section II/Annex II.44.

[14] Technical dossier/Supplementary information December 2019/Annex_SIn_1_Report taxonomy (v2).

[redacted][14]

The susceptibility of the strain was tested against the set of antibiotics recommended by the FEEDAP Panel guidance (EFSA FEEDAP Panel, 2018a). All the minimum inhibitory concentration (MIC) values for the strain were equal to or fell below the cut-off values, with the exception of tetracycline which exceeded by one dilution. Exceedance of the cut-off value by one dilution is considered to fall within the normal range of variation and thus, not a matter of concern.

The whole genome sequence (WGS) of the strain was interrogated for the presence of known genes coding for antimicrobial resistances [redacted][15]

The toxigenic potential of the strain was assessed according to the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a). No lysis of Vero cells was detected. [redacted] Consequently, *B. velezensis* ATCC PTA-6737 [redacted][15] [redacted][17] The results of this analysis, with targets having a $\geq$ 75% similarity score, showed no evidence of gene(s) associated with aminoglycosides production. This search, however, was not considered sufficient as genes within the gene cluster involved in aminoglycoside (butirosin) production in *B. velezensis* show only marginal homology (7%) to those equivalent from the well-characterised butirosin-producing species *Bacillus circulans*.

The antimicrobial activity of three batches of industrial-scale culture supernatants from *Bacillus velezensis* ATCC PTA-6737 was analysed by an agar well diffusion method using five indicator strains: i.e. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633. Aminoglycoside antibiotics were used as positive inhibitory compounds. None of the indicator strains was inhibited, demonstrating the absence of antimicrobial activity in supernatants of the active agent, and thus, of the aminoglycoside production of *B. velezensis* ATCC PTA-6737.

The WGS was also interrogated for the presence of genes coding for virulence factors and plasmids using the Virulence Factors of Pathogenic Bacteria (VFPB) database and the NCBI RefSeq plasmid database. No known genes encoding for virulence factors were found in the genome of the strain. No relevant plasmid sequences were detected in the strain.

3.1.2. Characterisation of the additive

The additive is currently authorised as a preparation of *B. velezensis* ATCC PTA-6737 containing a minimum of 1×10^{10} CFU/g additive.

In the current dossier, the applicant proposes a modification of the specifications of the additive, in particular an increase of the minimum concentration from 1×10^{10} CFU/g to 8×10^{10} CFU/g additive. In addition, the applicant has modified the carrier –which amounts 75–95% of the additive – from maltodextrin to sodium bicarbonate.

The applicant provided five certificates of analysis confirming that the additive meets the specification regarding the minimum concentration of *B. velezensis* (1.2×10^{11} CFU/g, 1.4×10^{11} CFU/g, 1.3×10^{11} CFU/g, 1.5×10^{11} CFU/g and 1.4×10^{11} CFU/g).[18]

The applicant stated that the manufacturing process has not changed, besides the change in the carrier and the increased concentration of the active agent.

The final product is a dry, flowing powder standardised to a minimum declared concentration of 8×10^{10} CFU of spores per gram.

Chemical impurities (heavy metals, i.e. fluoride, arsenic, cadmium, lead and mercury) and biological impurities (aflatoxin B1) were tested in five different batches.[19] The data provided showed that the

[15] Technical dossier/Supplementary information December 2019/Annex_SIn_4_Report antimicrobials.
[16] Technical dossier/Supplementary information December 2019/Annex_SIn_5_Report cytotoxicity.
[17] Technical dossier/Supplementary information March 2020/Annex_SIn_1_Bioinformatic sequence analysis of PB6_conf.
[18] Technical dossier/Section II/Annex_II_8_Certify of analysis report.
[19] Technical dossier/Section II/Annexes_II_14 to II_18.

observed results fall below the limit of detection (LOD),[20] except for fluoride which showed values between 138 to 243 mg/kg. These values do not pose concern.

Microbial contamination (i.e. *E. coli*, total coliforms, *Salmonella* sp., yeasts, moulds, Enterobacteriaceae) was tested on five different batches. Results showed no microbial contamination of concern.[21] Three additional batches of the additive were tested for the presence of *Bacillus cereus*; no *B. cereus* was detected.[21]

Particle size distribution of *Bacillus subtilis* PB6 analysed by laser diffraction[22] and dusting potential analysed by Stauber–Heubach method[23] were tested in three batches. Measurements indicated that 30–32% of the particles had a diameter < 10 μm; 89–97% < 50 μm and 97–99.9% < 100 μm. The dusting potential ranged from 0.02 to 0.03 g/m^3.

3.1.3. Stability and homogeneity

The applicant submitted studies to investigate the stability and homogeneous distribution of the additive with the new formulation.

The shelf-life of *Bacillus subtilis* PB6 was studied in three batches stored in heat-sealed container at 5°C and at 25°C.[24] No reduction in spore counts (< 0.5 log) was observed after one year of storage.

The stability of the additive in premixtures and in mash feed was studied for a period of six months when stored at room temperature.[25] No significant decrease in bacterial counts (< 0.5 log) was observed during the storage period.

Four samples of a complete feed (inclusion level in the mash feed 2.5 × 10^7 kg) were examined for stability during pelleting at different temperatures (ranging from 70 to 95°C).[26] The viability of *B. velezensis* ATCC PTA-6737 was not affected by the highest pelleting temperatures (90–95°C). Moreover, bacilli spore counts remained stable for three months of storage after pelleting.

The capacity of the additive to homogeneously distribute in premixtures and mash feedingstuffs feed was tested, based on 10 subsamples of one batch. The coefficients of variation were 13% and 8% for premixture and feed, respectively.[25]

3.1.4. Conditions of use

The additive is currently authorised as a zootechnical additive, functional group 'gut flora stabilisers', for chickens for fattening, chickens reared for laying and minor poultry species (except for laying purposes) at a minimum content of 1 × 10^7 CFU/kg feedingstuffs.

Under other provisions in the authorisation the following are listed:

1) In the directions for use of the additive and premixture, indicate the storage temperature, storage life and stability to pelleting.
2) The product is currently authorised for simultaneous use with permitted coccidiostats diclazuril, decoquinate, salinomycin sodium, narasin/nicarbazin, lasalocid A sodium, maduramycin ammonium, monensin sodium, narasin or robenidine hydrochloride on condition that these coccidiostats are authorised for the relevant species.

The applicant proposes to keep the same conditions in the application for renewal of the authorisation.

In addition, the applicant is proposing the extension of use to ornamental, sporting and game birds with the same conditions of use.

3.2. Safety

The bacterial species *B. velezensis* is considered by EFSA to be suitable for the qualified presumption of safety approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strain to be conclusively established and evidence that it does not show acquired resistance to relevant antimicrobials, lacks toxigenic potential and does not produce

[20] As: < 0.20 ppm, Cd: < 0.05 ppm, Pb: < 0.20 ppm, Hg: < 0.01 ppm, Aflatoxin B1: not detected (0.05 ppb).
[21] Technical dossier/Section II/Annexes_II_32 to II_34.
[22] Technical dossier/Section II/Annex_II_36_Particle size analysis report.
[23] Technical dossier/Section II/Annex_II_35_Dusting potential analysis report.
[24] Technical dossier/Section II/Annex_II_88_ Stability of the Bacillus subtilis.
[25] Technical dossier/Section II/Annex_II_89_Stability and homogeneity in premix & feed.
[26] Technical dossier/Section II/Annex_II_90_Heat stability pelleting.

aminoglycosides. In the view of the FEEDAP Panel, the identity of the active agent is established and the compliance with the other qualifications confirmed. Therefore, *B. velezensis* ATCC PTA-6737 is presumed safe for the target species, consumers of products derived from animals fed the additive and the environment. As the other component of the additive is a feed material which does not raise safety concerns, the FEEDAP Panel concludes that the additive remains safe for the target species, consumers and the environment.

The safety for the user was assessed by the FEEDAP Panel in the first opinion delivered for this additive (EFSA FEEDAP Panel, 2009). The Panel concluded that the additive is non-irritant to skin and eyes or a dermal sensitiser and that the data on dusting potential do not raise concerns via respiratory route. The Panel considers that the nominal increase in the minimum concentration of the active agent in the additive and the change of the carriers will not have an impact on the conclusions previously reached. Therefore, the Panel reiterates its previous conclusions on the safety of the additive for the users.

3.3. Efficacy

The efficacy of the additive was established at the level of 1×10^7 CFU/kg feed in chickens for fattening, in chickens reared for laying, ducks for fattening, quails, pheasants, partridges, guinea fowl, pigeons, geese for fattening and ostriches (EFSA FEEDAP Panel, 2009, 2011). The conditions of use for these target species have not been modified and therefore no further assessment of efficacy is needed for the renewal of the authorisation for these target species.

The applicant has requested to extend the use of the additive at the level of 1×10^7 CFU/kg feed to the following species and categories: ornamental, sporting and gaming birds. The efficacy data from chickens for fattening can be extrapolated to the new species/categories (EFSA FEEDAP Panel, 2018b). Therefore, the Panel concludes that the additive has the potential to be efficacious in these new species and categories under the proposed conditions of use.

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[27] and Good Manufacturing Practice.

4. Conclusions

The applicant has provided evidence that the additive currently in the market complies with the conditions of authorisation.

The identity of the active agent has been established as *Bacillus velezensis* ATCC PTA-6737 and the strain does not show acquired antimicrobial resistance determinants for antibiotics of human and veterinary interest. Following the QPS approach to safety assessment, the use of the strain as zootechnical additive is considered safe for the target species, consumers and the environment. The FEEDAP Panel concludes that the additive is also safe for the new target species/categories including ornamental, sporting and game birds.

The additive is non-irritant to skin and eyes and is not a dermal sensitiser. The exposure via inhalation is unlikely.

There is no need for assessing the efficacy of *Bacillus velezensis* ATCC PTA-6737 in the context of the renewal of the authorisation (chickens for fattening, chickens reared for laying, minor poultry species (except for laying purposes)). The efficacy data from chickens for fattening can be extrapolated to the new species/categories. The FEEDAP Panel concludes that the additive at minimum inclusion level of 1×10^7 CFU/kg feed has the potential to be efficacious in ornamental, sporting and game birds.

[27] 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.

5. Documentation as provided to EFSA/Chronology

Date	Event
28/02/2019	Dossier received by EFSA. *Bacillus subtilis* PB6. Submitted by Kemin Europa N.V
06/05/2019	Reception mandate from the European Commission
05/09/2019	Application validated by EFSA – Start of the scientific assessment
31/10/2017	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 of the stain, safety for the additive*
05/12/2019	Comments received from Member States
17/12/2017	Reception of supplementary information from the applicant - Scientific assessment re-started
18/02/2020	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 of the stain*
19/03/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
30/09/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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EFSA FEEDAP Panel (Panel on Additives and Products or Substances used in Animal Feed), 2013b. Scientific Opinion on the safety and efficacy of *Bacillus subtilis* PB6 (*Bacillus subtilis*) as a feed additive for turkeys for fattening and turkeys reared for breeding. EFSA Journal 2013;11(4):3176, 10 pp. https://doi.org/10.2903/j.efsa.2013.3176

EFSA FEEDAP Panel (Panel on Additives and Products or Substances used in Animal Feed), 2015. Scientific Opinion on the safety and efficacy of *Bacillus subtilis* PB6 as a feed additive for laying hens and minor poultry species for laying. EFSA Journal 2015;13(1):3970, 10 pp. https://doi.org/10.2903/j.efsa.2015.3970

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Abbreviations

ATCC	American Type Culture Collection
EURL	European Union Reference Laboratory
EFSA FEEDAP Panel	EFSA Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
MIC	minimum inhibitory concentration
VFPB	Virulence Factors of Pathogenic Bacteria
WGS	whole genome sequence