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Safety and efficacy of EB15 10 (*Bacillus subtilis* DSM 25841) as a feed additive for weaned piglets and minor porcine species

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Abstract

Following a request from the European Commission, the EFSA 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 EB15 10 for weaned piglets and minor porcine species. The additive is a preparation containing viable spores of a strain of *Bacillus subtilis* intended for use in feed at the proposed dose of 5×10^8 CFU/kg complete feedingstuffs and in water for drinking at 1.7×10^8 CFU/L. The additive exists in two forms, EB15 and EB15 10, which contain the bacterium in concentrations of 1.25×10^9 CFU/g additive and 1.25×10^{10} CFU/g additive, respectively. The two formulations are considered equivalent when used to deliver the same dose. *B. subtilis* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to establishing safety. The active agent fulfils the requirements of the QPS approach to the assessment of safety. Consequently, the additive can be presumed safe for the target animals, consumers of products from treated animals and the environment. Given the proteinaceous nature of the active agent, the additive should be considered a potential respiratory sensitiser. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy potential of the additive to skin and eyes or its dermal sensitisation. Insufficient evidence was provided to conclude on the efficacy of the additive in weaned piglets or minor weaned porcine species.

Keywords: zootechnical additive, EB15 10, *Bacillus subtilis*, safety, QPS, efficacy, weaned piglets and minor porcine species

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Table of contents

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.

The European Commission received a request from Chr. Hansen A/S[2] for authorisation of the product EB15 10 (*Bacillus subtilis* DSM 25841), when used as a feed additive for weaned piglets and weaned minor porcine species (category: zootechnical additives; 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). The particulars and documents in support of the application were considered valid by EFSA as of 13 January 2017.

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 EB15 10 (*Bacillus subtilis* DSM 25841), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The additive EB15 10 is a preparation containing viable spores of *Bacillus subtilis* DSM 25841. It has not been previously authorised as a feed additive in the European Union.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[3] in support of the authorisation request for the use of EB15 10 as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008[4] and the applicable EFSA guidance documents.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agent in animal feed. The Executive Summary of the EURL report can be found in Annex A.[5]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of EB15 10 is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on zootechnical additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014) and Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012c).

[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] Chr. Hansen A/S, 10-12 Boege Allé, 2970 Hoersholm, Denmark.
[3] FEED dossier reference: FAD-2016-0070.
[4] 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.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0070-baci_subtilis.pdf

3. Assessment

The additive is a preparation containing viable spores of *B. subtilis* DSM 25841 intended to be used in feed and water for drinking for weaned piglets and minor weaned porcine species to improve growth.

3.1. Characterisation

3.1.1. Characterisation of the active agent

B. subtilis DSM 25841 was isolated from the faeces from healthy adult pigs and has not been genetically modified.[6] The strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen with the accession number DSM 25841.[7]

Taxonomical identification of the product strain as *B. subtilis* was achieved using nearly complete 16S rRNA gene sequencing and multilocus sequence typing of partial sequences of the genes *groEL, gyrA, polC, purH* and *rpoB* and comparison with reference databases. Strain-specific identification was based on the use of pulsed-field gel electrophoresis after cleavage with restriction enzymes *Not*I and *Spe*I used individually.[8]

B. subtilis DSM 25841 was tested for antibiotic susceptibility using two-fold broth dilutions. The battery of antibiotics tested was that recommended by EFSA (EFSA FEEDAP Panel, 2012c).[9] All minimum inhibitory concentration values fell below the corresponding cut-off values defined by the FEEDAP Panel, therefore the strain is considered to be susceptible to relevant antibiotics.

The toxigenic potential of *B. subtilis* DSM 25841 was assessed according to the Technical Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition (EFSA FEEDAP Panel, 2014).[10] No lysis of Vero cells was detected, so *B. subtilis* DSM 25841 is considered to be not toxigenic.

3.1.2. Characterisation of the additive[11]

The manufacturing process of the additive is detailed in the dossier.[12] EB15 10 is produced with a minimum guaranteed concentration of 1.25×10^{10} colony forming units (CFU) per gram of additive. Batch-to-batch variation was measured in five batches of the additive and found to be consistently compliant with specifications (mean: 1.54×10^{10} CFU/g, range: $1.5–1.6 \times 10^{10}$ CFU/g).[13] The applicant mentions in the dossier a second formulation called EB15 and with a minimum concentration of 1.25×10^{9} CFU/g additive. This formulation was used in some stability and efficacy studies.

The additive is routinely monitored for microbial and chemical contamination. Limits are set for total coliforms ($< 10^{3}$ CFU/g), yeasts and filamentous fungi ($< 10^{3}$ CFU/g), *Escherichia coli* (< 10 CFU/g), *Salmonella* (absent in 25 g), aflatoxin B1 (< 0.005 mg/kg), lead (< 5.0 mg/kg), cadmium (< 0.5 mg/kg), mercury (< 0.1 mg/kg) and arsenic (< 2.0 mg/kg). Analyses of five batches showed compliance with the limits set for microbial impurities[13] and absence of *Bacillus cereus* contamination.[14,15] Analyses of three batches of the additive for chemical contamination showed values compliant with specifications or levels not giving rise to concerns (lead ≤ 0.161 mg/kg, cadmium ≤ 0.012 mg/kg, mercury ≤ 0.014 mg/kg, arsenic ≤ 0.059 mg/kg and aflatoxin B1 < 0.64 μg/kg, dioxins and polychlorinated biphenyls (PCBs): octachlorodibenzo-*p*-dioxin (OCDD) ≤ 0.153 ng/kg, octachlorodibenzofuran (OCDF) ≤ 0.161 ng/kg, WHO-polychlorinated dibenzodioxins/dibenzofurans-toxic equivalent (WHO-PCDD/F-TEQ) ≤ 0.196 ng/kg and WHO-PCDD/F-PCB-TEQ ≤ 0.208 ng/kg.[16]

The particle size distribution of EB15 10 was tested in three batches by laser diffraction. Results showed that 9% (v/v) of the additive consists of particles with diameters less than 50 μm and 4% less

[6] Technical dossier/Section II/Annex II.1.3a.
[7] Technical dossier/Section II/Annexes II.2.1.2a.
[8] Technical dossier/Section II/Annexes II.2.1.2b and c.
[9] Technical dossier/Section II/Annex II.2.2.2c.
[10] Technical dossier/Section II/Annex II.2.2.2a and Supplementary information July 2017.
[11] This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[12] Currently under re-evaluation according to Article 10(2) of 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.
[13] Technical dossier/Section II/Annex II.1.3b.
[14] Technical dossier/Supplementary information July 2017/Annex EM15_1_*B. cereus*.
[15] Limit of detection: 1,000 CFU/g.
[16] Technical dossier/Section II/Annex II.1.4.1.

than 10 μm.[17] The dusting potential of the same three batches of the additive, tested with a Heubach Dustmeter, showed a mean value of 2 g/m^3.[18]

3.1.3. Stability and homogeneity

The stability of three batches of EB15 10 packed in impermeable bags was monitored at 25, 30 and 37°C for 12 months.[19] No viability losses were observed at any temperature during this period.

Three batches of EB15 mixed with two types of vitamin–mineral premixture (not containing choline chloride) for piglets according to the conditions of use were stored for 6 months at 20–25°C.[20] No viability losses were detected.

To test stability at pelleting conditions, three batches of the EB15 were mixed with a pig mash feed, in accordance with the conditions of use, and subjected to pelleting temperatures of 75, 85 and 95°C.[21] Total counts of bacilli showed recovery equal or higher than 90% at the lowest temperatures and of 78% at 95°C.

To test stability in feed for piglets, three batches of EB15 were mixed with mash and pelleted (at 95°C) feed in accordance with the conditions of use. Samples were stored for 6 months at 20–25°C.[22] Total counts of bacilli showed recovery equal or higher than 90% in the mash feed and 80% in the pelleted feed.

The stability of three batches of EB15 suspended in water for drinking at 20–25°C was tested after one and two days, respectively. No viability losses were detected.[23]

The capacity of EB15 10 to homogeneously distribute in pelleted feed for piglets (based on 10 subsamples) according to the conditions of use was investigated in one study. Analyses of total counts showed a coefficient of variation of 0.9%.[24]

3.1.4. Conditions of use

The product is proposed for use in feed for weaned piglets and weaned minor porcine species at a dose of 5×10^8 CFU/kg complete feedingstuffs and of 1.7×10^8 CFU/L of drinking water.

3.2. Safety

3.2.1. Safety for the target species, consumers and environment

The bacterial species *B. subtilis* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2017). This approach requires the identity of the strains to be conclusively established and evidence that the strains lack toxigenic potential and do not show resistance to antibiotics of human and veterinary importance.

In the view of the FEEDAP Panel, the identity of the active agent is established as *B. subtilis* and the toxigenic potential and the antibiotic resistance qualifications have been met. Therefore, the strain is presumed safe for the target species, consumer and the environment. EB15 10 is also considered safe for target animals, consumers and the environment.

3.2.2. Safety for the user

No information was provided on the inhalation toxicity of the additive. The dustiness of the preparations tested indicated a potential for users to be exposed via inhalation. A significant fraction of the product consists of fine particles that have the potential to reach the alveoli when inhaled. Given the proteinaceous nature of the active agent, the additive should be considered to be a potential respiratory sensitiser. No data are available on skin/eye irritation or skin sensitisation. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of EB15 10 to skin and eyes or on its dermal sensitisation.

[17] Technical dossier/Section II/Annex II.1.5a.
[18] Technical dossier/Section II/Annex II.1.5b.
[19] Technical dossier/Section II/Annexes II.4.1a and Annex_EB15_2_12mths.
[20] Technical dossier/Section II/Annexes II.4.1d.
[21] Technical dossier/Section II/Annex II.4.1b.
[22] Technical dossier/Section II/Annexes II.4.1c.
[23] Technical dossier/Section II/Annexes II.4.1e.
[24] Technical dossier/Section II/Annexes II.4.2

3.3. Efficacy

3.3.1. Efficacy for weaned piglets[25]

Six efficacy studies were performed in three Member States and are described in the dossier.[26]

In all four studies considered, supplementation of EB15 led to a numerical greater weight gain, however, reaching significance only in one case (EB15 = 466 g/day vs control = 446 g/day, $p < 0.05$). Feed to gain ratio was significantly improved in two studies (EB15 = 1.56 g/g vs control = 1.64 g/g in one study and EB15 = 1.52 g/g vs control = 1.65 g/g in the other study; $p < 0.05$). Mortality in the treatment group was also significantly lower in one study (EB15 = 2% vs control = 5.6%, $p < 0.05$).

Significant differences on faecal scores[27] were observed only in one study (EB15 = 7.42 vs control = 7.23, $p < 0.05$).

Significant effects were found only in two studies. Therefore, there is insufficient evidence to conclude on the efficacy of EB15 in weaned piglets.

3.3.2. Efficacy for weaned minor porcine species

In the absence of a demonstration of efficacy for weaned piglets, no conclusions can be drawn for minor porcine species.

3.4. Post-market monitoring

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

4. Conclusions

The active agent fulfils the requirements of the QPS approach to the assessment of safety, consequently, EB15 10 is presumed safe for the target animals, consumers of products from treated animals and the environment.

The additive should be considered a potential respiratory sensitiser. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy potential of the additive to skin and eyes or its dermal sensitisation.

Insufficient evidence was provided to conclude on the efficacy of the additive in weaned piglets or minor weaned porcine species.

Documentation provided to EFSA

1) EB15 (*Bacillus subtilis* DSM 25841) Zootechnical feed additive for weaned piglets and other weaned minor porcine species + use in drinking water. November 2016. Submitted by Chr. Hansen A/S
2) EB15 (*Bacillus subtilis* DSM 25841) Zootechnical feed additive for weaned piglets and other weaned minor porcine species + use in drinking water. Supplementary information. July 2017. Submitted by Chr. Hansen A/S.
3) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for EB15 10.
4) Comments from Member States.

[25] This section has been amended following the provisions of Article 8(6) and Article 18 of Regulation (EC) No 1831/2003.
[26] Technical dossier/Section IV/Annexes IV.3.1-6 and Supplementary information July 2017/Annex_EB15_5_vii.
[27] Using a 10-point scale with 1 denoting severe diarrhoea, 9-10 denoting overly dry faeces and with 8 as optimal score.
[28] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Girones R, Herman L, Koutsoumanis K, Lindqvist R, Nørrung B, Robertson L, Ru G, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlström H, Cocconcelli PS, Klein G (deceased), Prieto Maradona M, Querol A, Peixe L, Suarez JE, Sundh I, Vlak JM, Aguilera-Gomez M, Barizzone F, Brozzi R, Correia S, Heng L, Istace F, Lythgo C and Fernández Escámez PS, 2017. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA Journal 2017;15(3):4664, 177 pp. https://doi.org/10.2903/j.efsa.2017.4664

EFSA (European Food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal 2007;5(12):587, 16 pp. https://doi.org/10.2903/j.efsa.2007.587

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Technical guidance: tolerance and efficacy studies in target animals. EFSA Journal 2011;9(5):2175, 15 pp. https://doi.org/10.2903/j.efsa.2011.2175

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012a. Guidance for the preparation of dossiers for zootechnical additives. EFSA Journal 2012;10(1):2536, 19 pp. https://doi.org/10.2903/j.efsa.2012.2536

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012c. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal 2012;10(6):2740, 10 pp. https://doi.org/10.2903/j.efsa.2012.2740

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014. Guidance on the assessment of the toxigenic potential of Bacillus species used in animal nutrition. EFSA Journal 2014;12(5):3665, 10 pp. https://doi.org/10.2903/j.efsa.2014.3665

Abbreviations

CFU	colony forming unit
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
OCDD	octachlorodibenzo-*p*-dioxin
OCDF	octachlorodibenzofuran
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzodioxin/dibenzofuran
QPS	qualified presumption of safety
TEQ	toxic equivalent

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Methods of Analysis for EB15 10

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis DSM 25841* under the category/functional group 4(b) 'zootechnical additives'/'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for weaned piglets and weaned minor porcine species.

According to the Applicant, the *feed additive* contains as active substance viable spores of the non-genetically modified strain *Bacillus subtilis DSM 25841*. The *feed additive* is to be marketed as a powder containing a minimum *Bacillus subtilis DSM 25841* content of 1.3×10^{10} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used in *drinking water* at a minimum dose of 1.7×10^{8} CFU/L, and directly in *feedingstuffs* or through *premixtures* at a minimum dose of 5×10^{8} CFU/kg complete *feedingstuffs*.

For the identification of *Bacillus subtilis DSM 25841*, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of *Bacillus subtilis DSM 25841* in *feed additive*, *drinking water*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread plate CEN method EN 15784. Based on the performance characteristics available, the EURL recommends this method for official control.

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) is not considered necessary.