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Safety and efficacy of an additive consisting of *Bacillus subtilis* DSM 32325 for all animal species (Chr. Hansen A/S)

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the additive consisting of *Bacillus subtilis* DSM 32325 when used as technological additive (hygiene condition enhancer) in feed for all animal species. The product is intended for use in dry feeds at a minimum inclusion level of 1×10^8 colony forming unit (CFU)/kg complete feedingstuff. The bacterial species *Bacillus subtilis* DSM 32325 is considered by EFSA to be eligible for the qualified presumption of safety approach. As the identity of the strain has been clearly established and it did not show acquired resistance to antibiotics of human and veterinary importance, the use of the strain in animal nutrition is considered safe for the target species, consumers and the environment. No conclusions can be drawn on the skin/eye irritancy or skin sensitisation potential of the product. Exposure of users by inhalation is likely and the product should be considered a respiratory sensitiser. The Panel is not in the position to conclude on the efficacy of *Bacillus subtilis* DSM 32325 when used in animal nutrition as hygiene condition enhancer due to lack of data. *Bacillus subtilis* DSM 32325 is compatible with diclazuril, decoquinate and halofuginone. The data provided do not allow to conclude on the compatibility of the additive with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

Keywords: technological additive, hygiene condition enhancer, *Bacillus subtilis* DSM 32325, safety, efficacy

Requestor: European Commission

Question number: EFSA-Q-2017-00745

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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 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 *Bacillus subtilis* DSM 32325, when used as a feed additive for all animal species, (category: technological additive; functional group: hygiene condition enhancer).

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 8 January 2018.

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* DSM 32325, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The product under assessment is based on viable spores of *Bacillus subtilis* DSM 32325. The active agent is currently authorised as a feed additive (category: zootechnical additive) for all porcine species in the European Union.

The microorganism *Bacillus subtilis* DSM 32325 is authorised as a feed additive in the European Union as part of a zootechnical additive composed of *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840.

EFSA delivered an opinion on the safety and efficacy of the microorganism *B. subtilis* DSM 32325 as part of the additive GalliPro® Fit (*Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840) (EFSA FEEDAP Panel, 2020).

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 *Bacillus subtilis* DSM 32325 as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agents in animal feed. The Executive Summary of the EURL report can be found in Annex A.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Bacillus subtilis* DSM 32325 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, 2017a), Guidance on the characterisation of microorganisms

[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-2017-0059.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0059-baci_subtilis.pdf
[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.

used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The additive is composed of spores of *Bacillus subtilis* DSM 32325 and is intended to be used as a technological additive (functional group: hygiene condition enhancer) in feed for all animal species. The applicant states that the additive improves hygienic characteristics of the feed by reducing the microbiological contamination by *Escherichia coli.*

3.1. Characterisation

3.1.1. Characterisation of the active agent

Bacillus subtilis DSM 32325 was isolated from faeces collected from healthy adult pigs in Germany. It is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen with the accession number DSM 32325.[6] The strain has not been genetically modified.

Taxonomic identification of strain DSM 32325 as *B. subtilis* was established by bioinformatic analysis of the whole genome sequences (WGS).[7] This was based on multi-locus sequence analysis (16S rRNA gene and partial groEL, *gyrA*, *polC*, *purH* and *rpoB* gene sequences) and average nucleotide identity (ANI). The ANI value obtained from the comparison with the type strain (NCTC 3610^T) genome based on MUMmer (ANIm) was 98.6%.[8]

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,b). No lysis of Vero cells was detected. Therefore, *B. subtilis* DSM 32325 is considered to be non-toxigenic.[9]

The susceptibility of the strain to the antimicrobials recommended by the FEEDAP Panel was tested [redacted] according to the Clinical and Laboratory Standards Institute (CLSI) and all of the minimum inhibitory concentration (MIC) values determined fell below the FEEDAP cut-off values (EFSA FEEDAP Panel, 2018a,b).[10] The WGS of the active agent was interrogated for the presence of antimicrobial resistance genes (AMR) [redacted][11] No relevant hits were identified.

3.1.2. Characterisation of the additive

[redacted]

Compliance with the guaranteed minimum total concentration of viable spores of *B. subtilis* DSM 32325 in the product of 1.3×10^{10} CFU/g additive was demonstrated in five batches of the additive (2.0×10^{10} CFU/g additive, range $1.8–2.1 \times 10^{10}$ CFU/g additive).[12]

Three batches of the additive were analysed for chemical contamination (lead (0.377–0.535 mg/kg; cadmium (0.054–0.085 mg/kg); mercury (0.0102–0.0104 mg/kg); arsenic (0.433–0.720 mg/kg)), dioxins (< 0.186 WHO-PCDD/F-TEQ ng/kg), dioxins like PCBs (< 0.198 WHO-PCDD/F-PCB-TEQ ng/kg) and aflatoxin B1 (< LOQ) and they were in compliance with the maximum levels specified.[13] Analysis of microbial contamination in five batches indicated *Bacillus cereus*, coliforms and yeasts and moulds

[6] Technical dossier/Section II/Annex.II.2.1.2a.
[7] Technical dossier/Supplementary information August 2020/Annex Q1_3.
[8] Technical dossier/Supplementary information August 2020/Annex Q1_2.
[9] Technical dossier/Section II/Annex.II.2.2.2a.
[10] Technical dossier/Section II/Annex.II.2.2.2b.
[11] [redacted]
[12] Technical dossier/Section II/Annex.II.1.3b.
[13] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_4_1; LOQ for aflatoxin B1 was 0.64 μg/kg.

were < 10^3 CFU/g, *Escherichia coli* was < 10 CFU/g, while *Salmonella* spp. was absent in 25 g.[14] Chemical and microbiological analyses did not raise any concern.

The additive is a slightly hygroscopic, free-flowing powder with a white to yellowish colour. The dusting potential of the additive was measured in the same three batches following the Stauber-Heubach method and the mean value was 2.9 g/m^3 (range 2.1–3.7 g/m^3).[15] The particle size was measured in the same three batches using laser diffraction, particles below 100 μm amounted up to 14.9%, particles below 10 μm amounted up to 7.8%.[16]

3.1.3. Stability and homogeneity

The shelf-life of the additive was determined by monitoring three batches stored at 25°C, 30°C and 37°C for a period of 6 months.[17] Negligible losses (< 0.5 Log difference) were observed after 6 months.

Three batches of the additive were individually mixed into a standard chicken mineral premixture (without choline chloride) at a concentration of 1.7 × 10^7 CFU/g premixture and samples were stored for 6 months at 20–25°C.[18] Negligible losses (< 0.5 Log difference) were observed after 6 months.

Stability in complete feed was investigated using three batches of the additive incorporated into a typical mash feed for chickens (corn and soybean) and into a pelleted feed of the same composition (pelleting conditions 95°C) at the intended concentration of 1.0 × 10^8 CFU/kg feed.[19] Samples of the mash and pelleted feed were stored for up to 6 months at 20–25°C. Counts of total bacilli were made at the start and after 6 months. The results showed that negligible losses (< 0.5 Log difference) occurred in both mash and pelleted feed.

The stability studies of the additive during pelleting of poultry feed (pelleting temperature: 75°C, 85°C and 95°C) showed a negligible loss of bacilli (< 0.5 Log difference).[20]

A total of 10 subsamples were taken from the pelleted feed and analysed for total bacilli counts. Based on the 10 samples, the coefficient of variation was 11%.[21]

3.1.4. Conditions of use

The product is intended to be used as a technological additive (functional group: hygiene condition enhancer) in dry feed for all animal species, alone or in combination with other hygiene condition enhancer strains, at a minimum inclusion level of 1.0 × 10^8 CFU/kg complete feedingstuffs. The applicant requests for the simultaneous use of the additive with the following coccidiostats: decoquinate, diclazuril, halofuginone, monensin sodium, maduramicin ammonium, narasin, robenidine and salinomycin sodium.

3.2. Safety

3.2.1. Safety for the target species, the consumer and the environment

The strain *B. subtilis* DSM 32325 belongs to a species considered to qualify for the QPS approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strain to be conclusively established and evidenced that the strain does not show acquired resistance to antibiotics of human and veterinary importance and lack of toxigenic potential.

The identity of the strain has been unambiguously established. Evidence was provided on the lack of toxigenic potential of the strain and on the absence of acquired antimicrobial resistance genes. Therefore, the FEEDAP Panel concluded that the additive does not raise safety concerns for the target species, consumers of products derived from animals fed the additive and the environment.

[14] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_3b.
[15] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_5b.
[16] Technical dossier/Section II/Annexes Sect.II/Annex_II_1_5a.
[17] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1a.
[18] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1d.
[19] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1c.
[20] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_1b.
[21] Technical dossier/Section II/Annexes Sect.II/Annex_II_4_2.

3.2.2. Safety for user

No studies, other than those relating to dust formation, were provided by the applicant on the safety of the additive for users. Therefore, no conclusions can be drawn on the skin/eye irritancy or skin sensitisation of the additive.

The high dusting potential (up to 3.7 g/m^3) indicates that exposure by inhalation is likely to occur. Due to the proteinaceous nature of the active agents, the additive should be considered a respiratory sensitiser.

3.3. Efficacy

3.3.1. Efficacy in feedingstuffs

The additive is intended to be used to improve the hygienic characteristics of the feed by reducing the microbiological contamination by *E. coli*.

To prove the effects as a hygiene condition enhancer, an *in vitro* study [redacted] was conducted.[22]

The Panel notes the following limitations in the study design: [redacted] These limitations prevent the assessment of the efficacy of the additive under practical use conditions to be done.

3.3.2. Compatibility with coccidiostats

The compatibility of *B. subtilis* DSM 32325, with diclazuril, decoquinate and halofuginone, monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium[26] was assessed in the context of a previous application (EFSA FEEDAP Panel, 2020). The Panel concluded that *B. subtilis* DSM 32325 is compatible with diclazuril, decoquinate and halofuginone. The data provided do not allow to conclude on the compatibility of the additive with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium. The same information was submitted in the present application, and therefore, the same conclusions apply.

3.3.3. Conclusions on efficacy

In the absence of valid studies, the Panel cannot conclude on the efficacy of *B. subtilis* DSM 32325 as hygiene condition enhancer.

Bacillus subtilis DSM 32325 is compatible with diclazuril, decoquinate and halofuginone. The data provided do not allow to conclude on the compatibility of the additive with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

[22] Technical dossier/section IV/Annex IV_1_1_Efficacy_Conf. [redacted]

[26] Maximum authorised levels: diclazuril 1 mg/kg (chickens for fattening, guinea fowls, chickens reared for laying and turkey for fattening); decoquinate 40 mg/kg; (chickens for fattening); halofuginone 3 mg/kg (chickens for fattening and turkeys); monensin sodium 125 mg/kg (chickens for fattening and chickens reared for laying) and 100 mg/kg (turkeys); salinomycin sodium 70 mg/kg (chickens for fattening) and 50 mg/kg (chickens reared for laying); narasin 70 mg/kg (chickens for fattening); robenidine hydrochloride 36 mg/kg (chickens for fattening and turkeys); maduramicin ammonium 6 mg/kg (chickens for fattening) and 5 mg/kg (turkeys).

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

Based on the QPS approach to safety assessment, *Bacillus subtilis* DSM 32325 is presumed safe for the target species, consumers of products from animals fed the additive and the environment.

Due to the absence of data, no conclusions can be drawn on the skin/eye irritancy or skin sensitisation of the product under assessment. Given the proteinaceous nature of the active agent, *Bacillus subtilis* DSM 32325 should be considered a respiratory sensitiser. Exposure of users by inhalation is likely.

In the absence of valid studies investigating relevant and specific endpoints, no conclusion can be drawn on the efficacy of *Bacillus subtilis* DSM 32325 as hygiene condition enhancer. *Bacillus subtilis* DSM 32325 is compatible with diclazuril, decoquinate and halofuginone. The data provided do not allow to conclude on the compatibility of the additive with monensin sodium, salinomycin sodium, narasin, robenidine hydrochloride and maduramicin ammonium.

5. Documentation as provided to EFSA/Chronology

Date	Event
26/10/2017	Dossier received by EFSA. *Bacillus subtilis* DSM 32325 for all animal species. Submitted by Chr. Hansen A/S.
16/11/2017	Reception mandate from the European Commission
08/01/2018	Application validated by EFSA – Start of the scientific assessment
28/03/2018	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
09/04/2018	Comments received from Member States
17/06/2020	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 additive, characterisation of the strain, purity of the additive*
26/08/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
17/03/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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[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.

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Abbreviations

AMR	antimicrobial resistance genes
ANI	average nucleotide identity
BIOHAZ	EFSA Panel on Biological Hazards
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
QPS	Qualified Presumption of Safety
WHO	World Health Organization

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Bacillus subtilis* DSM 32325

In the current application, authorisation is sought under Article 4(1) for *Bacillus subtilis* DSM 32325 under the category/functional group 1(n) 'technological additives'/'hygiene condition enhancers', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the feed additive for all animal species. According to the Applicant, the feed additive contains as active substance viable spores of the non-genetically modified strain *Bacillus subtilis* DSM 32325. The feed additive is to be marketed as a powder containing a minimum *Bacillus subtilis* DSM 32325 content of 1.3×10^{10} colony forming unit (CFU)/g. The feed additive is intended to be used directly in feedingstuffs or through premixtures at a minimum dose of 1.0×10^{8} CFU/kg of complete feedingstuffs. For the identification of *Bacillus subtilis* DSM 32325, 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 32325 in the feed additive, 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.