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**Evaluation Report on the Analytical Methods submitted
in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

**Preparation of *Bacillus licheniformis* DSM 33806
and *Weizmannia faecalis* DSM 32016
(FEED-2023-18810; CRL/230046)**



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in connection with the Application for Authorisation of a
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Dossier related to: **FEED-2023-18810 - CRL/230046**

Name of Product: ***Preparation of Bacillus licheniformis
DSM 33806 and Weizmannia faecalis
DSM 32016***

Active Agent (s): **Bacillus licheniformis DSM 33806 and
Weizmannia faecalis DSM 32016**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
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Date: **16/04/2024**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for a preparation of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilizers', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for piglets (suckling and weaned), pigs for fattening, sows and physiologically related minor growing and reproductive porcine species.

According to the Applicant, the product is a preparation containing viable spores of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 at a minimum amount of 2.4×10^{10} and 6.0×10^9 Colony Forming Unit (CFU) / g *feed additive*, respectively. The *feed additive* is intended to be added to *compound feed* and *water* at a recommended dosage of 1.5×10^9 CFU / kg *feed* and 0.75×10^9 CFU / l *water*, respectively.

For the enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive*, *premixtures* and *compound feed* the Applicant proposed for official control the ring-trial validated spread plate method EN 15784 as recently revised by CEN (equivalent to VDLUFA method 28.2.2). The Applicant did not recommend any method for the enumeration of the *feed additive* in *water*. However, in the frame of the stability studies supporting the dossier, the Applicant presented experimental results applying the VDLUFA method 28.2.2 for the enumeration of *Bacillus licheniformis* in *water* thus demonstrating its applicability also to this matrix. Based on the performance characteristics and the further experimental evidences presented within the dossier, the EURL recommends for official control the ring-trial validated EN 15784 method for the enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive*, *premixtures*, *compound feed* and *water*.

For the enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive*, *premixtures* and *compound feed* the Applicant proposed for official control a slightly modified method based on the ring-trial validated spread plate method EN 15787 dedicated for the analysis of *Lactobacillus spp.*. The method was formerly presented and positively evaluated by the EURL in the frame of a previous *Weizmannia faecalis* currently authorised by Commission Implementing Regulation (EU) 2020/1755. The Applicant did not recommend any method for the enumeration of the *feed additive* in *water*. However, in the frame of stability studies the Applicant presented experimental results applying the modified EN 15787 for the enumeration of *Weizmannia faecalis* DSM 32016 in *water* thus demonstrating its applicability also to this matrix. Based on the performance characteristics and the experimental data available, the EURL recommends for the official control the modified method based on ring-trial validated method EN 15787 for the enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive*, *premixtures*, *compound feed* and *water*.

For the identification of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 at a strain level, the EURL recommends for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) (CEN/TS 17697).

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Bacillus licheniformis DSM 33806, *Weizmannia faecalis* DSM 32016, zootechnical additives, gut flora stabilizers, piglets (suckling and weaned), pigs for fattening, sows and minor growing and reproductive porcine species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for a preparation containing *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilizers', according to Annex I of Regulation (EC) No 1831/2003 [1,2]. The authorisation is sought for the use of the *feed additive* for piglets (suckling and weaned), pigs for fattening, sows and physiologically related minor growing and reproductive porcine species [2].

According to the Applicant, the product is a preparation proposed to be marketed in two forms under the trade name TechnoCare[®] 50. A first formulation, containing calcium carbonate as carrier, is intended for *compound feed* while a second formulation, containing maltodextrin as carrier, is developed for the use in *water* and liquid feed. Both preparations contain as *active substances* viable spores of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 at a minimum amount of 2.4×10^{10} and 6.0×10^9 Colony Forming Unit (CFU) / g *feed additive*, respectively [3]. The *feed additive* is intended to be added to *compound feed* and *water* at a recommended dosage of 1.5×10^9 CFU / kg *feed* and 0.75×10^9 CFU / l *water*, respectively [4]. The Applicant stated that the microorganisms belong to non-genetically modified strains and are both deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) [5].

Note: The EURL, in former dossiers, has already evaluated analytical methods for the official control of various *Bacillus licheniformis* strains [6]. Furthermore, in the frame of a recent dossier presented by the present Applicant, the EURL has evaluated the analytical methods proposed for the official control of the authorised *feed additive* *Weizmannia faecalis* DSM 32016 (formerly indicated as *Bacillus coagulans* DSM 32016) [7,8].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with preparation of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive, premixtures and compound feed* the Applicant proposed for official control the ring-trial validated spread plate method EN 15784 as recently revised by CEN [9-11]. During the revision the method was adjusted to VDLUFA method 28.2.2 and completed with validation data from inter-laboratory studies using commercial feed products [12]. The revision resulted in the updated CEN method dedicated for the enumeration of *bacilli spp.* in feedingstuffs (additives, premixtures and compound feeds including mineral feeds) that contain bacilli as a single microorganism component or in a mixture with other microorganisms [11].

The sample (5 to 50 g) is suspended in 0.2 % sodium hydroxide solution containing Polysorbate 80 (Tween[®] 80) (tPBS). Decimal dilutions are prepared from the suspension using the above mentioned solution, spread plated on tryptone soya agar and incubated, aerobically at 37 °C for 16 to 24 h [11].

The following performance characteristics were reported from the ring-trial validation studies of non-transformed logarithmically CFU values of *bacilli spp.* ranging from 9.0×10^8 to 4.45×10^{14} CFU / kg *feed additives, premixtures and compound feed* (including a mineral feed): a relative standard deviation for *repeatability* (RSD_r) ranging from 9.1 to 19.6 %; and a relative standard deviation for *reproducibility* (RSD_R) ranging from 17.1 to 33.9 % [11].

Moreover, in the frame of the studies supporting the present dossier (i.e. batch to batch variation and stability studies), the Applicant presented experimental data applying the above-mentioned VDLUFA method 28.2.2 (equivalent to EN 15784) to the product for the

enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive*, *premixtures* and *compound feed* [3,13, 14].

The Applicant did not recommend any method for the enumeration of the *feed additive* in *water* [9]. However, in the frame of the above-mentioned stability studies, the Applicant presented experimental results applying the VDLUFA method 28.2.2 (equivalent to EN 15784) for the enumeration of *Bacillus licheniformis* in *water* thus demonstrating its applicability also to this matrix [15].

Based on the performance characteristics and the further experimental evidences presented within the dossier, the EURL recommends for official control the ring-trial validated

EN 15784 method for the enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive*, *premixtures*, *compound feed* and *water*.

For the enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive*, *premixtures* and *compound feed* the Applicant proposed for official control the slightly modified method based on the ring-trial validated spread plate method EN 15787 dedicated for the analysis of *Lactobacillus spp.* [9,16-18]. The method was formerly presented and positively evaluated by the EURL in the frame of a previous *Weizmannia faecalis* currently authorised by Commission Implementing Regulation (EU) 2020/1755. [7,8,18].

According to the modified EN 15787 method, the sample is suspended, further serially diluted in a buffer and a diluent each supplemented with 1 % Tween[®] 80. The appropriate dilutions are heated at 75 °C for 30 min and spread on MRS (de Man, Rogosa, Sharp) agar plates. The agar plates are incubated anaerobically at 37 °C for at least 72 hours [18]. The addition of Tween[®] 80 in initial suspension and dilutions are already prescribed by the method EN 15787 for the encapsulated products while the inclusion of the heating step was demonstrated as not having a significant impact on the enumeration *Bacillus coagulans* DSM 32016 in the feed additive [19]. Therefore, the submitted protocol was considered as a minor modification of the EN 15787 method.

In the frame of the above-mentioned formerly evaluated dossier, the Applicant verified via an external laboratory the modified EN 15787 method for the enumeration of *Weizmannia faecalis* in the *feed additive* and *compound feed* [19]. The performance characteristics reported were the following [19]: a standard deviation for repeatability (S_r) ranging from 0.02 to 0.06 log₁₀ CFU/g; a standard deviation for intermediate precision (S_{ip}) ranging from 0.08 to 0.09 log₁₀ CFU/g; and a limit of quantification (LOQ) of 3x10⁴ CFU/g. These performance characteristics were considered by the EURL comparable with the ones obtained in the frame of the ring-trial validation of the method EN 15787 on MRS agar [7,17].

In addition, in the present and in the former dossier, the Applicant demonstrated the suitability of the modified EN 15787 method for the enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive*, *premixtures* and *compound feed* in the frame of the batch to batch variation and stability studies [7,13,14].

The Applicant did not recommend any method for the enumeration of the *feed additive* in *water* [9]. However, in the frame of stability studies the Applicant presented experimental results applying the modified EN 15787 for the enumeration of *Weizmannia faecalis* DSM 32016 in *water* thus demonstrating its applicability also to this matrix [20].

Based on the performance characteristics and the experimental data available, the EURL recommends for the official control the modified method based on ring-trial validated method EN 15787 for the enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive*, *premixtures*, *compound feed* and *water*.

Note: The modified or original EN 15787 methods are not applicable to mineral feed composed mainly of minerals and containing at least 40 % crude ash. For these matrices laboratories may consider using instead the ring-trial validated VDLUFA method 28.2.4 [7,21].

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the identification of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 at strain level, the Applicant proposed Pulsed-Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains [9]. The PFGE method has been ring-trial validated and recently published as a CEN Technical Specification CEN/TS 17697 [22]. Additionally, in former reports for similar dossiers, the EURL recommended for official control DNA sequencing methods [6,7].

The EURL considers that all the above-mentioned methodologies are suitable for official control for the bacterial identification *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 at strain level.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control: i) the ring-trial validated spread plate CEN method (EN 15784) for the enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive, premixtures, compound feed* and *water*; ii) the modified method based on the ring-trial validated CEN method (EN 15787) for the enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive, premixtures, compound feed* and *water*; and iii) DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) of CEN Technical Specification (CEN/TS 17697) for the identification of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016.

Recommended text for the register entry (analytical method)

- Identification of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016: DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) (CEN/TS 17697)
- Enumeration of *Bacillus licheniformis* DSM 33806 in the *feed additive, premixtures, compound feed* and *water*: Spread plate method on tryptone soya agar (EN 15784)
- Enumeration of *Weizmannia faecalis* DSM 32016 in the *feed additive, premixtures, compound feed* and *water*: Spread plate method on MRS agar (based on EN 15787)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of preparation of *Bacillus licheniformis* DSM 33806 and *Weizmannia faecalis* DSM 32016 have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

- [1] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform – <https://webgate.ec.europa.eu/esfc/#/applications/48710>
<https://open.efsa.europa.eu/questions/EFSA-Q-2023-00667>
- [2] *Application – Annex 1
- [3] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition
- [4] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition
- [5] *Technical dossier, Section II: 2.2 Characterisation of the active substance(s)
- [6] EURL reports: https://joint-research-centre.ec.europa.eu/publications/feed-2021-0928_en

- https://joint-research-centre.ec.europa.eu/publications/fad-2021-0056_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0064_en
https://joint-research-centre.ec.europa.eu/publications/fad-2015-0016_en
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https://joint-research-centre.ec.europa.eu/publications/fad-2009-0023_en
https://joint-research-centre.ec.europa.eu/publications/fad-2010-0009_en
- [7] EURL reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0024_en
- [8] Commission Implementing Regulation (EU) 2020/1755 of 24 November 2020 concerning the authorisation of a preparation of *Bacillus coagulans* DSM 32016 as a feed additive for suckling and weaned Suidae piglets, poultry for fattening and ornamental birds (holder of authorisation Biochem Zusatzstoffe Handels- und Produktionsges. mbH) – OJ L 395, 25.11.2020
- [9] *Technical dossier, Section II: 2.6 Methods of analysis of the active substance
- [10] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [11] EN 15784:2021 – Animal feeding stuffs – Methods of sampling and analysis – Detection and enumeration of *Bacillus* spp. used as feed additives
- [12] VDLUFA method – Enumeration of *Bacillus licheniformis* and *Bacillus subtilis* (VDLUFA Methodenbuch Bd.III, 28.2.2)
- [13] *Technical dossier, Section II – Annex_II_4.a_Batch-to-Batch variation_Calcium carbonate_conf-2
- [14] *Technical dossier, Section II – Annex_II_4.b_Batch-to-Batch variation_Maltodextrin_conf
- [15] *Technical dossier, Section II - Annex_II_28_Stability water DSM33806_conf_Redacted
- [16] EN 15787:2009 – Animal feeding stuffs - Isolation and enumeration of *Lactobacillus* spp.
- [17] EN 15787:2021 – Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of *Lactobacillus* spp. used as feed additive
- [18] **Technical dossier, Section II – Annex II.37
- [19] **Technical dossier, Section II – Annex II.36
- [20] *Technical dossier, Section II - Annex_II_29_Stability water DSM32016_conf-1
- [21] VDLUFA method 28.2.4 – Determination of *Enterococcus faecium* and *Lactobacillus rhamnosus* (Method book III, 8 Suppl. 2012, VDLUFA, Darmstadt)
- [22] CEN/TS 17697:2023 – Animal feeding stuffs - Methods of sampling and analysis – PFGE typing of *Lactobacilli*, *Pediococci*, *Enterococci* and *Bacilli* in animal feeds

* Refers to Dossier n°: FEED-2023-18810

** Refers to Dossier n°: FAD-2019-0024

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)