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Feed Additives

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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 subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856

(FEED-2023-14050; CRL/220023)

European Union Reference Laboratory
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Dossier related to:	**FEED-2023-14050 - CRL/220023**
Name of Product:	***Preparation of Bacillus subtilis DSM 33862 and Lentilactobacillus buchneri DSM 12856***
Active Agent (s):	**Bacillus subtilis DSM 33862 and Lentilactobacillus buchneri DSM 12856**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**María José González de la Huebra 26/02/2024**
Report approved by: Date:	**Ursula Vincent 27/02/2024**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for a preparation of *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 under the category / functional group 1(k) 'technological additives' / 'silage additives', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all animal species.

According to the Applicant, the *feed additive* contains *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 as an *active substance* at a minimum amount of 7.20×10^{10} and 2.88×10^{11} Colony Forming Unit (CFU) / g *feed additive*, respectively.

The *feed additive* is intended to be added to the forage, used for ensiling process at a recommended dosage of 1×10^5 CFU / g fresh *silage*.

For the identification of *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 at a strain level, the EURL recommends for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) (CEN/TS 17697).

For the enumeration of *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 in the *feed additive*, the EURL recommends for official control the ring-trial validated CEN methods (EN 15784 and EN 15787), respectively.

As the unambiguous determination of *Bacillus subtilis* DSM 33862 or *Lentilactobacillus buchneri* DSM 12856 added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend the two above mentioned CEN methods, or any other methods for official control to enumerate *Bacillus subtilis* DSM 33862 or *Lentilactobacillus buchneri* DSM 12856 in *silage*.

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 subtilis DSM 33862, *Lentilactobacillus buchneri* DSM 12856, technological additives, silage additives, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for a preparation of *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 under the category / functional group 1(k) 'technological additives' / 'silage additives',

according to Annex I of Regulation (EC) No 1831/2003 [1,2]. The authorisation is sought for the use of the *feed additive* for all animal species [2].

According to the Applicant, the *feed additive* contains *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 as *active substances* at a minimum amount of 7.20×10^{10} and 2.88×10^{11} Colony Forming Unit (CFU) / g *feed additive*, respectively [3].

The Applicant stated that the *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 are a non-genetically modified strains [4]. The microorganisms are deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) [4].

The *feed additive* is intended to be added to the forage, used for ensiling process at a recommended dosage of 1×10^5 CFU / g fresh *silage* [5].

Note: The EURL has previously evaluated the analytical methods for the determination of another *Bacillus subtilis* and *Lentilactobacillus buchneri* strains in the frame of a recent dossiers [6].

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 subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 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 subtilis* DSM 33862 in the *feed additive* and *silage* the Applicant proposed for official control [7] the ring-trial validated spread plate CEN method EN 15784:2009 [8], which was recently revised by CEN. During the revision of the CEN method it was adjusted to VDLUFA method 28.2.2 [9] and completed with validation data from inter-laboratory studies using commercial feed products. 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 [10].

Following the protocol of the updated CEN method, 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 [10].

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 x 10^8 to 4.45 x 10^{14} CFU / kg *feed additives*, *premixtures* and *compound feed* (including a mineral feed) [10]: a relative standard deviation for *repeatability* ranging from 9.1 to 19.6 %; and a relative standard deviation for *reproducibility* (RSD_R) ranging from 17.1 to 33.9 %.

The Applicant demonstrated the fitness-for-purpose of the EN 15784 method by providing experimental data obtained for the *feed additive* in the frame of the batch-to-batch variation [11] and the stability studies [12].

Based on the performance characteristics, the EURL recommends for official control the ring-trial validated EN 15784 method for the enumeration of *Bacillus subtilis* DSM 33862 in the *feed additive.*

As the unambiguous determination of *Bacillus subtilis* DSM 33862 added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend the EN 15784 method or any other method for official control to enumerate *Bacillus subtilis* DSM 33862 in *silage*.

For the enumeration of *Lentilactobacillus buchneri* DSM 12856 in the *feed additive* and *silage* the Applicant proposed for official control [7] the ring-trial validated EN 15787:2009 method [13]. However, the EURL is aware that this method has been revised by CEN resulting in updated method dedicated for the enumeration of *lactobacilli spp*. in feedingstuffs (feed additives, premixtures and compound feeds excluding mineral feeds) that contain lactobacilli as a single microorganism component or in a mixture with other microorganisms [14].

Following the updated method's protocol, the sample (5 to 50 g) is suspended in phosphate buffered saline containing Polysorbate 80 (Tween® 80) (tPBS). For serial dilutions, the peptone salt solution (PSS) is used. The appropriate dilutions are then mixed on Petri plates using spread plate (or pour plate) methods with MRS (de Man, Rogosa, Sharp) agar. Alternatively, the MRS agar can be acidified or include triphenyl tetrazolium chloride (TTC). However, for routine purposes the non-modified MRS agar is an appropriate medium. The agar plates are incubated anaerobically at 37 °C for 48 to 72 h [14].

The following performance characteristics, expressed in terms of precision, are reported in the frame of the ring-trial validation studies after logarithmic transformation of the CFU values ranging from 7.40 to 8.03 $\log_{10}$ CFU/g: a standard deviation for *repeatability* (S_r) ranging from 0.10 to 0.26 $\log_{10}$ CFU/g and a standard deviation for *reproducibility* (S_R) ranging from 0.18 to 0.39 $\log_{10}$ CFU/g [14].

In addition, the Applicant demonstrated the fitness-for-purpose of the EN 15787 method by providing experimental data obtained by using an equivalent pour plate in-house method [15,16] for the *feed additive* in the frame of the batch-to-batch variation [11] and the stability studies [12].

Based on the performance characteristics, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lentilactobacillus buchneri* DSM 12856 in the *feed additive.*

As the unambiguous determination of *Lentilactobacillus buchneri* DSM 12856 added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend the EN 15787 or any other method for official control to enumerate *Lentilactobacillus buchneri* DSM 12856 in *silage*.

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 subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 at strain level, the Applicant applied and DNA sequencing methods such a comparative 16 S rDNA gene sequencing and Whole Genome Sequencing [4].

In former reports for similar dossiers, the EURL recommended for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains. The PFGE method has been ring-trial validated and recently published as a CEN Technical Specification CEN/TS 17697 [17].

The EURL considers that all the above-mentioned methodologies are suitable for official control for the bacterial identification *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 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) DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) of CEN Technical Specification (CEN/TS 17697) for the identification of *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856; ii) the ring-trial validated spread plate CEN method (EN 15784) for the enumeration of *Bacillus subtilis* DSM 33862 in the *feed additive*; and iii) the ring-trial validated spread plate (or pour plate) CEN method (EN 15787) for the enumeration of *Lentilactobacillus buchneri* DSM 12856 in the *feed additive.*

As the unambiguous determination of *Bacillus subtilis* DSM 33862 or *Lentilactobacillus buchneri* DSM 12856 added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend the two above mentioned CEN methods, or any other methods for official control to enumerate *Bacillus subtilis* DSM 33862 or *Lentilactobacillus buchneri* DSM 12856 in *silage.*

Recommended text for the register entry (analytical method)

- Identification of *Bacillus subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856: DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) (CEN/TS 17697)
- Enumeration of *Bacillus subtilis* DSM 33862 in the *feed additive*: Spread plate method on tryptone soya agar (EN 15784)
- Enumeration of *Lentilactobacillus buchneri* DSM 12856 in the *feed additive*: Spread plate (or pour plate) method on MRS agar (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 subtilis* DSM 33862 and *Lentilactobacillus buchneri* DSM 12856 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/42370 https://open.efsa.europa.eu/questions/EFSA-Q-2023-00631

[2] *Application – Annex 1

[3] *Technical dossier, Section II : 2.1.3. Qualitative and quantitative composition

[4] *Technical dossier, Section II : 2.2.1. Identification

[5] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition

[6] EURL reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2020-0058_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0090_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0086_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0074_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0044_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0009_en
https://joint-research-centre.ec.europa.eu/publications/feed-2021-0246_en

[7] *Technical dossier, Section II: 2.6.1. Methods of analysis for the active substance

[8] EN 15784:2009 – Animal feeding stuffs – Isolation and enumeration of presumptive Bacillus spp.

[9] VDLUFA method – Enumeration of Bacillus licheniformis and Bacillus subtilis (VDLUFA Methodenbuch Bd.III, 28.2.2)

[10] EN 15784:2021 – Animal feeding stuffs: Methods of sampling and analysis – Isolation and enumeration of Bacillus spp. used as feed additive

[11] *Technical dossier, Section II – Annex_II_2

[12] *Technical dossier, Section II – Annex_II_18

[13] EN 15787:2009 – Animal feeding stuffs - Isolation and enumeration of Lactobacillus spp.

[14] EN 15787:2021 – Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Lactobacillus spp. used as feed additive

[15] *Technical dossier, Section II – Annex_II_23

[16] *Technical dossier, Section II – Annex_II_24

[17] 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 no: FEED-2023-14050

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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- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
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