



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
European Union Reference Laboratory for Feed Additives

 Ref. Ares(2019)6899935 - 07/11/2019

JRC.F.5/CvH/ZE/AS/Ares

**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**

**Bacillus coagulans DSM 32016 (TechnoSpore®)
(FAD-2019-0024; CRL/190011)**



**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**

Dossier related to: **FAD-2019-0024 – CRL/190011**

Name of Product: ***Bacillus coagulans DSM 32016
(TechnoSpore®)***

Active Agent (s): ***Bacillus coagulans DSM 32016***

Rapporteur Laboratory: **Centre wallon de Recherches
agronomiques (CRA-W), Gembloux,
Belgium**

Report prepared by: **Véronique Ninane**

Report checked by: **Zigmas Ezerskis**
Date: **06/11/2019**

Report approved by: **Christoph von Holst**
Date: **07/11/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Bacillus coagulans* DSM 32016 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', 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), other growing *Suidae*, chickens for fattening, other poultry for fattening and ornamental birds.

According to the Applicant, the *feed additive* contains viable spores of the non-genetically modified strain *Bacillus coagulans* DSM 32016 as an active substance. The *feed additive* is to be marketed as a powder (under the trade name - *TechnoSpore*®) containing a minimum content of the active substance of 2×10^{10} Colony Forming Unit (CFU)/g *TechnoSpore*®. The *feed additive* is intended to be used in *feedingstuffs* and *premixtures* at a minimum dose of 1×10^9 CFU/kg complete *feedingstuffs*.

For the identification of *Bacillus coagulans* DSM 32016, the EURL recommends for the official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains.

For the enumeration of *Bacillus coagulans* DSM 32016 in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the slightly modified method based on ring-trial validated spread plate method EN 15787, where Tween® 80 is added at 1 % in buffer and diluent in the case of all matrices and where the final dilution is heated. Based on the performance characteristics and experimental data available, the EURL recommends the modified method EN 15787 for the official control for the enumeration of *Bacillus coagulans* DSM 32016 in the *feed additive*, *premixtures* and *feedingstuffs*.

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 coagulans DSM 32016, zootechnical additives, gut flora stabilisers, piglets, other growing *Suidae*, chickens for fattening, other poultry for fattening and ornamental birds

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new feed additive) for *Bacillus coagulans* DSM 32016 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the *feed additive* for piglets (suckling and weaned), other growing *Suidae*, chickens for fattening, other poultry for fattening and ornamental birds [2].

According to the Applicant, the *feed additive* contains viable spores of the non-genetically modified strain of *Bacillus coagulans* DSM 32016 as an active substance [3]. The strain is deposited at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under the deposit number DSM 32016 [4].

The *feed additive* is to be marketed as a powder (under the trade name - *TechnoSpore*®) containing a minimum content of the active substance of 2×10^{10} Colony Forming Unit (CFU)/g *TechnoSpore*® [5].

The *feed additive* is intended to be used in *feedingstuffs* and *premixtures* at a minimum dose of 1×10^9 CFU/kg complete *feedingstuffs* [1,2,6].

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 *Bacillus coagulans* DSM 32016 (*TechnoSpore*®) 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 and feedingstuffs (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of *Bacillus coagulans* DSM 32016 in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the slightly modified method [7] based on the ring-trial validated spread plate method EN 15787 [8] dedicated for the analysis of *Lactobacillus* spp.

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 [7]. The addition of Tween[®] 80 in initial suspension and dilutions are already prescribed by the method EN 15787 for the encapsulated products [8] 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* [9]. Therefore, the submitted protocol was considered as a minor modification of the EN 15787 method.

The Applicant performed in an external laboratory the verification study of the modified EN 15787 method for the enumeration of *Bacillus coagulans* DSM 32016 in the *feed additive* and *feedingstuffs* and the performance characteristics reported were the following [9]: a standard deviation for *repeatability* ranging from 0.02 to 0.06 log₁₀ CFU/g; a standard deviation for *intermediate* precision ranging from 0.08 to 0.09 log₁₀ CFU/g; and a limit of quantification (LOQ) of 3x10⁴ CFU/g. These performance characteristics are comparable with the ones obtained in the frame of the ring-trial validation of the method EN 15787 on MRS agar [8]. In addition, the Applicant demonstrated the suitability of the modified EN 15787 method for the enumeration of *Bacillus coagulans* DSM 32016 in the *feed additive* and *premixtures* in the frame of stability and homogeneity studies [10]. Finally, the Applicant provided the experimental evidence demonstrating that the modified and the original method EN 15787 developed for the analysis of *Lactobacillus* spp. is more suitable for the quantification of *Bacillus coagulans* DSM 32016 than the method EN 15784 developed for the analysis of *Bacillus* spp. [9].

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 *Bacillus coagulans* DSM 32016 in the *feed additive*, *premixtures* and *feedingstuffs*.

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 [11].

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 coagulans* DSM 32016, the Applicant applied a method of Whole Genome Sequencing (WGS) [3]. The WGS is a promising genetic typing technique for microbial identification at a strain level [12] though not yet standardised and not available in many control laboratories.

The EURL recommends instead for official control the pulsed-field gel electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains [13]. This methodology for bacterial identification of authorised additives at a strain level is currently being evaluated by the CEN Technical Committee 327 to become a European Standard.

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 the official control Pulsed Field Gel Electrophoresis (PFGE) for the identification of *Bacillus coagulans* DSM 32016 and the modified method based on ring-trial validated spread plate method EN 15787 for the enumeration of *Bacillus coagulans* DSM 32016 in the *feed additive, premixtures* and *feedingstuffs*.

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.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in the *feed additive, premixtures* and *feedingstuffs*: Spread plate method on MRS agar (based on EN 15787 method)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Bacillus coagulans* DSM 32016 (TechnoSpore®) 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] *Application, Reference SANTE/E5: FWD. APPL. 1831/0032-2019 & Annex I – submission number 1553704806652-2390
- [2] *Application, Proposal for Register Entry, Annex A
- [3] *Technical dossier, Section II: 2.2 Characterisation of the Active Substance
- [4] *Technical dossier, Section II: Annex II.10
- [5] *Technical dossier, Section II: 2.1. Identity of the additive
- [6] *Technical dossier, Section II: 2.5 Conditions of use of the additive
- [7] *Technical dossier, Section II: Annex II.37
- [8] EN 15787:2009 - Animal feeding stuffs - Isolation and enumeration of *Lactobacillus* spp.
- [9] *Technical dossier, Section II: Annex II.36
- [10] *Technical dossier, Section II: 2.4.1 Stability & 2.4.2 Homogeneity
- [11] VDLUFA method 28.2.4 – Determination of *Enterococcus faecium* and *Lactobacillus rhamnosus* (Method book III, 8 Suppl. 2012, VDLUFA, Darmstadt)
- [12] Jackson S.A., Schoeni J.L., Vegge C., Pane M., Stahl B., Bradley M., Goldman V.S., Burguière P., Atwater J.B., and Sanders M.E. (2019). Improving End-User Trust in the Quality of Commercial Probiotic Products. *Front. Microbiol.* Vol.10, Art. 739.
- [13] European Community Project SMT4-CT98-2235. "Methods for the Official Control of Probiotics Used as Feed Additives", Report 20873/1 EN (2002) ISBN 92-894-6250-7 (Vol. I) and Report 20873/3 EN (2002) ISBN 92-894-6252-3 (Vol. III)

*Refers to Dossier no: FAD-2019-0024

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Centre wallon de Recherches agronomiques (CRA-W), Gembloux, 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

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)