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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**

Bacillus subtilis DSM 29784
(FAD-2016-0040; CRL/160023)



**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-2016-0040 - CRL/160023**

Name of Product: ***Alterion NE[®]***

Active Agent (s): **Bacillus Subtilis DSM 29784**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

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Date: **20/12/2016**

Report approved by: **Christoph von Holst**
Date: **20/12/2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis* DSM 29784 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for chickens for fattening.

According to the Applicant, the *feed additive* contains as active substance viable spores of non-genetically modified *Bacillus subtilis* DSM 29784. The *feed additive* is to be marketed as light brown to brown granular free flowing powder, containing a minimum *Bacillus subtilis* DSM 29784 content of 1×10^{10} colony forming units per gram of *feed additive* (CFU/g). The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* [5] at a minimum dose of 1×10^8 CFU /kg of complete *feedingstuffs*.

For the identification of *Bacillus subtilis* DSM 29784 the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification. This standard methodology for microbial identification is currently being evaluated by the CEN Technical Committee 327 to become a European Standard.

For the enumeration of *Bacillus subtilis* DSM 29784 in *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread plate method EN 15784 which was already evaluated by EURL in the frame of previous *bacilli* dossiers. Based on the performance characteristics available, the EURL recommends for official control the CEN method for the enumeration of *Bacillus subtilis* DSM 29784 in the three matrices.

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.

KEYWORDS

Bacillus subtilis DSM 29784, zootechnical additives, gut flora stabilisers, chickens for fattening.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis* DSM 29784 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, the authorisation is sought for the use of the *feed additive* for chickens for fattening [1][2].

According to the Applicant, the *feed additive* contains as active substance viable spores of non-genetically modified *Bacillus subtilis* DSM 29784 [2][3][4]. The strain is deposited at the Deutsche Sammlung von Mikro-organismen und Zellkulturen (DSMZ) [4].

The *feed additive* is to be marketed as light brown to brown granular free flowing powder, containing a minimum *Bacillus subtilis* DSM 29784 content of 1×10^{10} colony forming units per gram (CFU/g) of *feed additive* [3].

The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of 1×10^8 CFU /kg of complete *feedingstuffs* [2][5].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus subtilis* in the frame of several dossiers (e.g. FAD-2009-0023; FAD-2014-0037; or FAD-2015-0006) [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 subtilis* DSM 29784 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

For the identification of *Bacillus subtilis* DSM 29784, the Applicant used Pulsed Field Gel Electrophoresis (PFGE) of DNA digested with *Xba*I, *Spe*I or *Not*I enzymes and sequence analysis of 16S rDNA and *gyrB* genes [4][7]. The EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for

genetic identification [8]. This standard methodology for microbial identification is currently being evaluated by the CEN Technical Committee 327 to become a European Standard.

Qualitative and quantitative composition of impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (e.g. *Salmonella*, *Enterobacteriaceae*, *Escherichia coli*, yeast and moulds) by using appropriate ISO standards [9]. As for the determination of other undesirable substances in the *feed additive* (e.g. arsenic, cadmium, lead, mercury, mycotoxins and dioxins) several internationally recognised standard methods are available at the respective European Union Reference Laboratories [10].

Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs

For the enumeration of *Bacillus subtilis* DSM 29784 in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread plate method EN 15784 [11] that was already evaluated by the EURL in the frame of previous *bacilli* dossiers [6].

An initial suspension of the sample is prepared in a diluent using a suitable homogeniser. From this suspension, one new dilution is prepared and heat treated at 80 °C for 10 min. Decimal dilutions are then prepared, spread plated on tryptone soya agar (TSA), and incubated at 37 °C for 16 h to 24 h under aerobic conditions. Colonies of *Bacillus* species are counted and the number of colony forming units per g or kg is calculated [11].

The performance characteristics of the CEN method reported after logarithmic transformation are [11]:

- *repeatability* standard deviation (S_r) ranging from 0.07 to 0.09 \log_{10} CFU/g;
- *reproducibility* standard deviation (S_R) ranging from 0.32 to 0.35 \log_{10} CFU/g; and
- limit of quantification (LOQ) of 2×10^7 CFU/kg, well below the minimum dose of 1×10^8 CFU/kg of *feedingstuffs* proposed by the Applicant.

Based on the performance characteristics presented the EURL recommends for official control the ring-trial validated CEN method (EN 15784) for the enumeration of *Bacillus subtilis* DSM 29784 in the *feed additive*, *premixtures* and *feedingstuffs*.

Note: The EN 15784 method is not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash. For these matrices laboratories may consider using the ring-trial validated VDLUFA method 28.2.2 instead [12].

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE) for the identification of the bacterial strains. For the enumeration of *Bacillus subtilis* DSM 29784 in *feed additive*, *premixtures* and *feedingstuffs* the EURL recommends for official control the EN 15784 method.

Recommended text for the register entry (analytical method)

Identification of *Bacillus subtilis* (DSM 29784):

- Pulsed Field Gel Electrophoresis (PFGE)

Enumeration of *Bacillus subtilis* (DSM 29784) in the *feed additive*, *premixtures* and *feedingstuffs*:

- Spread plate method using tryptone soya agar (EN 15784)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Bacillus subtilis* DSM 29784 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 SANCO/G1: Forw. Appl. 1831-0035-2016
- [2] *Application, Proposal for Register Entry, Annex A
- [3] *Technical dossier, Section II– 2.1 Identity of the additive
- [4] *Technical dossier, Section II– 2.2 Characterisation of the active substance(s)/agent(s)
- [5] *Technical dossier, Section II– 2.5 Conditions of use of the additive
- [6] EURL Evaluation Reports:
<https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0023.pdf>
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2014-0037-bacillus_subtilis.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2015-0006-bacillus_subtilis.pdf
- [7] *Technical dossier, Section II– Annex II.2-3 & Annex II.2-4
- [8] 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)
- [9] *Technical dossier, Section II– 2.6 Methods of analysis and reference samples
- [10] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[11] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus spp.*

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

*Refers to Dossier no: FAD-2016-0040

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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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)