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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 28343

(FAD-2015-0006; CRL/150000)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2015-0006 - CRL/150000**
Name of Feed Additive:	***Bacillus subtilis DSM 28343***
Active Agent (s):	**Bacillus subtilis DSM 28343**
Rapporteur Laboratory:	**Centre wallon de Recherches agronomiques (CRA-W), Gembloux, Belgium**
Report prepared by:	**Véronique Ninane**
Report checked by: Date:	**Rebeca Fernandez Orozco & Zigmas Ezerskis (EURL-FA)** **29/08/2015**
Report approved by: Date:	**Christoph von Holst** **30/08/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis DSM 28343* 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 the viable spores of non-genetically modified *Bacillus subtilis DSM 28343*. The *feed additive* is to be marketed as cream-coloured free-flowing granules, containing a minimum *Bacillus subtilis DSM 28343* concentration of $1x10^{10}$ Colony Forming Units (CFU)/g. The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $1x10^{9}$ CFU /kg complete *feedingstuffs*.

For the identification of *Bacillus subtilis DSM 28343* 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 28343* 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 (EN 15784) for the enumeration of *Bacillus subtilis DSM 28343* 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) is not considered necessary.

KEYWORDS

Bacillus subtilis DSM 28343, zootechnical additives, gut flora stabilisers, chickens for fattening.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new use of a feed additive already authorised) for *Bacillus subtilis DSM 28343* 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 the viable spores of non-genetically modified *Bacillus subtilis DSM 28343* [2,3]. The strain is deposited at the Deutsche Sammlung von Mikroorganismen und Zelkulturen (DSMZ) [3].

The *feed additive* is to be marketed as cream-coloured free-flowing granules, containing, a minimum *Bacillus subtilis DSM 28343* concentration of $1x10^{10}$ Colony Forming Units (CFU) /g [3].

The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* [3] at a minimum dose of $1x10^{9}$ CFU /kg complete *feedingstuffs* [2].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus subtilis* in the frame of several dossiers e.g. FADs 2009-0023; 2009-0007; 2009-0013 [4].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 28343* 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 28343*, the Applicant used Pulsed Field Gel Electrophoresis (PFGE) of DNA digested with *Apa*I, *Asc*I, *Not*I, *Sma*I and *Spe*I enzymes [5]; and sequence analysis of 16S rRNA and gyrB genes [6]. The EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification [7]. 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*, *Enterobacteria*, yeast and moulds) by using appropriate EN ISO standards [3]. 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 [8].

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 28343* in *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread plate method EN 15784 [9] that was already evaluated by EURL in the frame of previous *bacilli* dossiers [4].

Twenty grams of the *feed additive* (or 50g of *premixtures* or *feedingstuffs*) are suspended in a phosphate buffered saline (PBS) (or in 0.2% sodium hydroxide solution for premixtures or feedingstuffs). From this suspension, one new dilution is prepared, heat treated at 80 ^{o}C for 10 minutes, and cooled at room temperature. Decimal dilutions are then prepared, spread plated on tryptone soya agar (TSA) and incubated at 37 °C for 16-24 h aerobically.

The performance characteristics of the CEN method reported after logarithmic transformation are [9]:

- *repeatability* standard deviation (S_r) ranging from 0.07 to 0.09 $\log_{10}$ CFU/g;
- *reproducibility* standard deviation (S_R) ranging from of 0.32 to 0.35 $\log_{10}$ CFU/g; and
- limit of quantification (LOQ) of $2x10^7$ CFU/kg, well below the minimum dose of $1x10^9$ CFU/kg *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 28343* 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 [10].

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; this method is currently evaluated by the CEN technical Committee 327 to become a European Standard. For the enumeration of *Bacillus subtilis* DSM 28343 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 28343*):

- Pulsed Field Gel Electrophoresis (PFGE)

Enumeration of *Bacillus subtilis* (*DSM 28343*) 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 28343* 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. 1831/0009-2015

[2] *Application, Proposal for Register Entry, Annex A

[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; methods of analysis

[4] EURL Evaluation Reports: https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0023.pdf

https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0007.pdf
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0013.pdf

[5] *Technical dossier, Section II – Annex II.2-2

[6] *Technical dossier, Section II – Annex II.2-3

[7] 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)

[8] 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

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

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

* Refers to Dossier no: FAD-2015-0006

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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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