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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 amyloliquefaciens DSM 25840
(FAD-2016-0069; CRL/160048)

**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-2016-0069 - CRL/160048**

Name of Product / Feed Additive: ***Bacillus amyloliquefaciens DSM 25840***

Active Agent (s): **Bacillus amyloliquefaciens DSM 25840**

Rapporteur Laboratory: **Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, Italy**

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Date: **(EURL-FA)
11/04/2017**

Report approved by: **Christoph von Holst**
Date: **11/04/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Bacillus amyloliquefaciens* DSM 25840 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for weaned piglets and other weaned minor porcine species.

According to the Applicant, the *feed additive* contains as active substance viable spores of the non-genetically modified strain *Bacillus amyloliquefaciens* DSM 25840. The *feed additive* is to be marketed as a dry powder containing a minimum *Bacillus amyloliquefaciens* DSM 25840 content of 1.3×10^{10} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used in *drinking water* at a minimum dose of 1.7×10^8 CFU/l, included through *premixtures* or added directly into *feedingstuffs* at a minimum dose of 5×10^8 CFU/kg complete *feedingstuffs*.

For the identification of *Bacillus amyloliquefaciens* DSM 25840, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strain.

For the enumeration of *Bacillus amyloliquefaciens* DSM 25840 in the *feed additive*, in *premixtures*, *feedingstuffs* and *water* the Applicant submitted the ring-trial validated spread plate CEN method 15784. Based on the performance characteristics available, the EURL recommends this method for official control.

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 amyloliquefaciens DSM 25840, zootechnical additives, gut flora stabilizers, weaned piglets, weaned minor porcine species.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new feed additive to be authorised or new use) for *Bacillus amyloliquefaciens* DSM 25840 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilizers', according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of the *feed additive* for weaned piglets and other weaned minor porcine species. [1,2].

According to the Applicant, the *feed additive* contains as active substance viable cells of the non-genetically modified strain *Bacillus amyloliquefaciens* DSM 25840. The strain is deposited at Leibniz-Institut DSMZ-Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) [2,3].

The *feed additive* is to be marketed as a powder containing a minimum *Bacillus amyloliquefaciens* DSM 25840 content of 1.3×10^{10} Colony Forming Unit (CFU)/g [4,5].

The *feed additive* is intended to be used in *drinking water* at a minimum dose of 1.7×10^8 CFU/l, included through *premixtures* or added directly into *feedingstuffs* at a minimum dose of 5×10^8 CFU/kg complete *feedingstuffs* [6].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus spp.* in the frame of several dossiers [7].

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 amyloliquefaciens* DSM 25840 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 amyloliquefaciens* DSM 25840, the Applicant applied multi locus sequence analysis and Pulsed Field Gel Electrophoresis (PFGE) [8].

The EURL recommends for official control PFGE, a generally recognised methodology for genetic identification [3,9]. This 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. coliforms, yeast and moulds, *Escherichia coli*, *Enterobacteriaceae* and *Salmonella*) using the methods described in the technical dossier [3]. As for the determination of other undesirable substances in the *feed additive* (e.g. arsenic, cadmium, lead, mercury, Aflatoxin B1), analytical methods for official control are available from the respective European Union Reference Laboratories [10].

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

For the enumeration of *Bacillus amyloliquefaciens* DSM 25840 in *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant submitted the spread plate method based on EN 15784:2009 [11,12].

Samples (20 g) of the *feed additive* (or 50 g of *premixtures* or *feedingstuffs*) are suspended in a phosphate buffered saline (or in 0.2 % sodium hydroxide solution for *premixtures* or *feedingstuffs*). From this, one suspension is prepared and heat-treated at 80 °C for 10 minutes. Decimal dilutions are prepared from the heat treated suspension, spread plated on tryptone soya agar and incubated at 37 °C for 16-24 h aerobically. The reported [12] performance characteristics of the EN method are:

- a standard deviation for *repeatability* (S_r) ranging from 0.07 to 0.09 log₁₀ CFU/g;
- a standard deviation for *reproducibility* (S_R) ranging from 0.32 to 0.35 log₁₀ CFU/g; and
- a *limit of quantification* (LOQ) of 2x10⁴ CFU/g.

In the frame of the stability studies, the Applicant provided additional experimental evidence demonstrating the applicability of the CEN method for the enumeration of *Bacillus subtilis* DSM 25841 in *water* [13].

Based on the performance characteristics presented and on experimental data provided, the EURL recommends for official control the ring-trial validated EN 15784 method for the enumeration of *Bacillus amyloliquefaciens* DSM 25840 in *feed additive, premixtures, feedingstuffs* and *water*.

Note: The EN 15784 method is not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash.

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 *Bacillus amyloliquefaciens* DSM 25840 and the ring-trial validated spread plate method EN 15784 for enumeration of this strain in the *feed additive, premixtures, feedingstuffs* and *water*.

Note: The EN 15784 method is not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in *feed additive, premixtures, feedingstuffs* and *water*: Spread plate method on 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 amyloliquefaciens* DSM 25840 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: Forw. Appl. 1831/0050-2016
- [2] Application, Proposal for Register Entry – Annex A
- [3] *Technical dossier, Section II: 2.2. Characterisation of the Active substance(s) / agent(s)
- [4] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition
- [5] *Technical dossier, Section II: 2.1.5. Physical state of each form of the product

- [6] *Technical dossier, Section II: 2.5 Conditions of use of the additive
- [7] Examples of EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0040_alterionne.pdf
<https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2014-0038-probion-forfe.pdf>
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2014-0037-bacillus_subtilis.pdf
<https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2015-0006.pdf>
- [8] *Technical dossier, Annex II: 2.1.2b - ID Certificate, genetic stability and absence of plasmids / 2.1.2c – PFGE Method
- [9] 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)
- [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] *Technical dossier, Section II: 2.6 Methods of analysis
- [12] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [13] *Technical dossier, Section II: 2.4.1 Stability
- *Refers to Dossier no: FAD-2016-0069

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the "Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, Italy". 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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- Centre wallon de Recherches agronomiques (CRA-W), Gembloux (BE)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- 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)