EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

JRC F.5/CvH/MGH/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 subtilis ABS747

(FAD-2019-0074; CRL/190049)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2019-0074 - CRL/190049**
Name of Product:	***Bacillus subtilis ABS747***
Active Agent (s):	**Bacillus subtilis ABS747**
Rapporteur Laboratory:	**Centre Wallon de Recherches Agronomiques (CRA-W), Gembloux, Belgium**
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Report checked by: Date:	**María José González de la Huebra** **21/04/2020**
Report approved by: Date:	**Christoph von Holst** **21/04/2020**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis* ABS747 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 chickens for fattening, turkeys for fattening, chickens reared for laying, turkeys reared for breeding and minor poultry species.

According to the Applicant, the *feed additive* contains as *active substance* viable spores of the non-genetically modified strain *Bacillus subtilis* ABS747. The *feed additive* is to be marketed as a dry preparation containing a minimum content of *active substance* of $1x10^{11}$ Colony Forming Unit (CFU)/g and to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $1x10^{5}$ CFU/g complete *feedingstuffs*.

For the identification of *Bacillus subtilis* ABS747, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains.

For the enumeration of *Bacillus subtilis* ABS747 in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted an in-house plate count method single-laboratory validated and further verified by another laboratory. However, the EURL is aware of a ring-trial validated spread plate CEN method EN 15784 developed to enumerate and differentiate spores of several *Bacillus* spp. Furthermore the Applicant confirmed, upon request of the EURL, the suitability of the CEN method EN 15784 for this product.

Based on the performance characteristics reported and the applicability statement provided by the Applicant, the EURL recommends instead the ring trial validated CEN method EN 15784 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), as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Bacillus subtilis ABS747, zootechnical additives, gut flora stabilisers, chickens for fattening, turkeys for fattening, chickens reared for laying, turkeys reared for breeding, minor poultry species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Bacillus subtilis* ABS747 under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1].

Authorisation is sought for the use of the *feed additive* for chickens for fattening, turkeys for fattening, chickens reared for laying, turkeys reared for breeding and minor poultry species [1,2].

According to the Applicant, the *feed additive* contains as *active substance* viable spores of the non-genetically modified strain *Bacillus subtilis* ABS747 [3]. The strain is deposited at the Agricultural Research Culture Collection (NRRL, Peoria, Il, USA) under the deposit number NRRL B-67257 [4].

The *feed additive* is to be marketed as a dry preparation containing a minimum content of *active substance* of $1x10^{11}$ Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $1x10^{5}$ CFU/g complete *feedingstuffs* [6].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus subtilis* in the frame of several dossiers (e.g. FADs 2017-0058; 2019-0009; 2019-0044) [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 subtilis* ABS747 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* ABS747 in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted an in-house plate count method, single-laboratory validated and further verified by another laboratory for the *feed additive* and *feedingstuffs* [8].

According to the Applicant, an initial suspension of the sample is prepared in 0.1% peptone water and heat-treated at 61 °C for 35 min (heat treatment is omitted for pelleted feeds). Decimal dilutions are prepared from this initial suspension, pour plated with Tryptic Soy Agar (TSA) and incubated aerobically at 32 °C for 18 h to 48 h.

The main performance characteristics reported by the Applicant in the frame of the validation and verification [9] studies are summarised in Table 1.

However, the EURL is aware of a ring-trial validated spread plate CEN method EN 15784 developed to enumerate and differentiate spores of several *Bacillus* spp in *feedingstuffs* that was already evaluated and recommended by the EURL in the frame of previous *Bacillus subtilis* dossiers [7].

According to the CEN method EN 15784, 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 new dilution is prepared with "Polysorbate 80" peptone salt solution and heat-treated at 80 °C for 10 minutes. Further 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 following performance characteristics were reported from the validation study after logarithmic transformation of the CFU values [10]:

- a *repeatability* standard deviation (S_r) ranging from 0.07 to 0.09 $\log_{10}$ CFU/g and
- a *reproducibility* standard deviation (S_R) ranging from 0.32 to 0.35 $\log_{10}$ CFU/g

In addition, the EURL calculated according to ISO 7218 a limit of quantification (LOQ) of $3x10^4$ CFU/g [11].

Furthermore, the Applicant confirmed, upon request of the EURL, the applicability of the EN 15784 to its product.

Based on the performance characteristics reported and the applicability statement from the Applicant, the EURL recommends for official control the ring trial validated CEN method

EN 15784 for the enumeration of *Bacillus subtilis* ABS747 in the *feed additive*, *premixtures* and *feedingstuffs*.

Table 1: Performance characteristics of the analytical method for the determination of *Bacillus subtilis* ABS747 in the *feed additive* (FA) and *feedingstuffs* (FS)

Matrix	Bacterial Counts (CFU/g)		S_r (log_{10} CFU/g)		S_{ip} (log_{10} CFU/g)	
	Val	Ver	Val	Ver	Val	Ver
FA	6.2×10^7 1.6×10^{10} 1.2×10^{11}	 9.6×10^{10} 1.0×10^{11}	0.02 0.05	 0.08 0.15	 0.03	 0.12
FS	5.2×10^7 6.4×10^9 7.1×10^9	 9.1×10^9 9.3×10^9	 0.03 0.05	0.06 0.05 	 0.08	 0.05

S_r: standard deviation for repeatability; S_{ip} standard deviation for intermediate precision; Val: Validation; Ver: verification

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].

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* ABS747, the Applicant developed a single nucleotide polymorphism (SNP) assay [3].

The EURL recommends instead for official control pulsed-field gel electrophoresis (PFGE), a generally recognised methodology for the 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 subtilis* ABS747, and the CEN method EN 15784 for the enumeration of *Bacillus subtilis* ABS747 in the *feed additive*, *premixtures* and *feedingstuffs*.

Note: The method EN 15784 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.

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 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* ABS747 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: F.A. 1831/0075-2019

[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.2.1.2.1

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

[6] *Technical dossier, Section II: 2.5 Conditions of use of the additive

[7] EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0044_syncra.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0009-galliprofit.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0058-baci_subtilis.pdf

[8] *Technical dossier, Section II: 2.6 Methods of analysis for the active substance

[9] *Technical dossier, Section II : Annex II 6.1.1, 6.1.2, 6.1.4 & 6.1.5.

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

[11] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations

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

[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-2019-0074

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the 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:

- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)