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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

Enviva® PRO 202 GT

(FAD-2015-0008; CRL/110006)

European Union Reference Laboratory
Feed Additives

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-2015-0008 - CRL/110006**
Name of Product:	**Enviva® PRO 202 GT**
Active Agent (s):	***Bacillus amyloliquefaciens* BS 15A-P4 (PTA-6507)** ***Bacillus amyloliquefaciens* LSSAO1 (NRRL B-50104)** ***Bacillus amyloliquefaciens BS 2084 (NRRL B-50013)***
Rapporteur Laboratory:	**Centre wallon de Recherches agronomiques (CRA-W), Gembloux, Belgium**
Report prepared by:	**Véronique Ninane**
Report checked by: Date:	**Stefano Bellorini and Zigmas Ezerskis (EURL-FA)** **06/08/2015**
Report approved by: Date:	**Christoph von Holst** **06/08/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Enviva® PRO 202 GT* 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, chickens reared for laying, minor poultry species for fattening and minor poultry species reared to point of lay.

According to the Applicant, the *feed additive* contains as active substances viable spores of the three non-genetically modified strains *Bacillus amyloliquefaciens* BS 15A-P4 (PTA-6507), *Bacillus amyloliquefaciens* LSSAO1 (NRRL B-50104) and *Bacillus amyloliquefaciens* BS 2084 (NRRL B-50013). The *feed additive* is to be marketed as granular dry product containing a minimum concentration of total active substances of $2.5x10^9$ Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of total active substances of $7.5x10^7$ CFU/kg complete *feedingstuffs*.

For the identification and characterization of *Bacillus amyloliquefaciens* BS 15A-P4 (PTA-6507), *Bacillus amyloliquefaciens* LSSAO1 (NRRL B-50104) and *Bacillus amyloliquefaciens* BS 2084 (NRRL B-50013), 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 all the *Bacillus amyloliquefaciens* strains (BS 15A-P4, LSSAO1 and BS 2084) 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 a previous *Bacillus amyloliquefaciens* dossier. Based on the performance characteristics available, the EURL recommends for official control this CEN method (EN 15784) for the enumeration of *Bacillus amyloliquefaciens* (BS 15A-P4, LSSAO1 and BS 2084) 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 amyloliquefaciens BS 15A-P4 (PTA-6507), *Bacillus amyloliquefaciens* LSSAO1 (NRRL B-50104) and *Bacillus amyloliquefaciens* BS 2084 (NRRL B-50013), technological additives, gut flora stabilisers, chickens for fattening chickens reared for laying, minor poultry species for fattening, minor poultry species reared to point of lay.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new feed additive) for *Enviva® PRO 202 GT* under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorization is sought for the use of the *feed additive* for chickens for fattening, chickens reared for laying, minor poultry species for fattening and minor poultry species reared to point of lay [1,2].

According to the Applicant, the *feed additive* contains as active substances viable spores of the three non-genetically modified strains *Bacillus amyloliquefaciens* BS 15A-P4, *Bacillus amyloliquefaciens* LSSAO1 and *Bacillus amyloliquefaciens* BS 2084 [2,3]. The strain *Bacillus amyloliquefaciens* BS 15A-P4 is deposited at the American Type Culture Collection with deposit number PTA-6507 while the two other strains (*Bacillus amyloliquefaciens* LSSAO1 and *Bacillus amyloliquefaciens* BS 2084) are deposited at the Agricultural Research Service Culture Collection of the United States Department of Agriculture with deposit numbers NRRL B-50104 and NRRL B-50013, respectively [3].

The *feed additive* is to be marketed as granular dry product containing a minimum concentration of total active substances of 2.5×10^9 Colony Forming Unit (CFU)/g [2,3].

The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of total active substances of 7.5×10^7 CFU/kg complete *feedingstuffs* [2,3].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus amyloliquefaciens* in the frame of dossier FAD 2009-0041[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 *Enviva® PRO 202 GT* 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 and characterization of Enviva® PRO 202 GT, the Applicant used sequence analysis of 16S rRNA, Pulsed Field Gel Electrophoresis (PFGE) of DNA digested with *ApaI* and *AscI* enzymes; and Single-Nucleotide Polymorphism (SNP) [3-5].

The EURL recommends for official control PFGE, a generally recognised standard methodology for genetic identification [6]. 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. coliforms, *Escherichia coli*, *Enterobacteriaceae*, *Listeria*, *Staphylococcus aureus* 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, mycotoxins), analytical methods for official control are available from the respective European Union Reference Laboratories [7].

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

The Applicant did not specify the concentrations of each individual strain of *Bacillus amyloliquefaciens* but provided instead overall concentrations of the three strains.

For the enumeration of the overall *Bacillus amyloliquefaciens* strains (BS 15A-P4, LSSAO1 and BS 2084) in *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread plate method EN 15784 [3,8].

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, one new dilution 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 (TSA) and incubated at 37 °C for 16-24 h aerobically. The following performance characteristics were reported after logarithmic transformation (CFU) [8]:

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

- a limit of quantification (LOQ) of $2x10^7$ CFU/kg of *feedingstuffs* [8], below the minimum dose of $7.5x10^7$ CFU/kg *feedingstuffs* proposed by the Applicant.

In addition, the Applicant provided experimental evidence of the applicability of the method to mineral feed in the frame of stability studies [3].

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 amyloliquefaciens* (BS 15A-P4, LSSAO1 and BS 2084) in *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.

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 European Standards. For the enumeration of *Bacillus amyloliquefaciens* (BS 15A-P4, LSSAO1 and BS 2084) 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: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration: Spread plate method following heat treatment - EN 15784

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Enviva® PRO 202 GT* 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 G1: F.A. 1831/0010-2015

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

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

[4] FAD 2009-0041 EURL report, ARES(2010)516717
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0041.pdf

[5] Technical dossier, Section II – Annex II.15.Conf

[6] 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)

[7] 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 Laboratory

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

*Refers to Dossier no: FAD-2015-0008

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

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)[1]
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ))

[1] Name and address according to Regulation (EC) No 885/2009: Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)