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**CRL Evaluation Report on the Analytical Methods  
submitted in connection with the Application for the  
Authorisation of a Feed Additive  
according to Regulation (EC) No 1831/2003**

**Dossier related to:** FAD-2009-0041  
CRL/090007

**Product Name:** Ecobiol®

**Active Substance(s):** *Bacillus amyloliquefaciens* CECT 5940

**Rapporteur Laboratory:** Community Reference Laboratory for Feed Additives (CRL-FA)

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**Date:** 16/082010

## EXECUTIVE SUMMARY

In the current application, modification of the authorisation of the microbial feed additive *Bacillus amyloliquefaciens* CECT 5940 is sought consisting in the extension of the shelf-life of the product to two years and the compatibility with several coccidiostats. The feed additive is already authorised for chickens for fattening under the category 'zootechnical additives', functional group 'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003. The target level of *Bacillus amyloliquefaciens* CECT 5940 in complete feedingstuffs is  $1 \times 10^9$  colony forming units (CFU)/kg.

For enumeration of *Bacillus amyloliquefaciens* CECT 5940 in *feed additive, premixtures* and *feedingstuffs* the applicant submitted a spread plate method based on the ring-trial validated CEN method (EN 15784). The performance characteristics of the EN 15784 method reported after logarithmic transformation of measured values (CFU) are:

- 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 0.32 to 0.35  $\log_{10}$  CFU/g; and
- a limit of detection (LOD) of  $10^5$  CFU/kg of *feedingstuffs*.

Based on these acceptable performance characteristics, the CRL recommends for official control the spread plate CEN method (EN 15784) for the determination of *Bacillus amyloliquefaciens* CECT 5940 in the *feed additive, premixtures* and *feedingstuffs*.

Molecular methods were used by the applicant for identification of the active agent. The CRL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

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* CECT 5940, Ecobiol<sup>®</sup>, Ecobiol<sup>®</sup> plus, zootechnical additives, poultry, chicken for fattening, gut flora stabilisers.

## 1. BACKGROUND

*Bacillus amyloliquefaciens* CECT 5940 is a feed additive with the identification number 4b1822, for which modification of an existing authorisation is sought under Article 13(1) of Regulation (EC) No 1831/2003 [1]. The sought modification of the authorisation consists in the extension of the shelf-life of the product to two years and the compatibility with several coccidiostats [2]. The feed additive is already authorised for chickens for fattening under the category of 'zootechnical additives' functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003 [1]. The target level of the feed additive in complete feedingstuffs is  $1 \times 10^9$  colony forming units (CFU)/kg. The strain *Bacillus amyloliquefaciens* CECT 5940 originally isolated from the soil is deposited in the Spanish Type Culture Collection (CECT) of the University of Valencia [3]. Authorisation is sought for use of *Bacillus amyloliquefaciens* CECT 5940 for poultry and chicken for fattening [2, 4].

## 2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorizations of *feed additives*, as last amended by Regulation (EC) No 885/2009, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application, or for each group of applications. For this particular dossier, the methods of analysis submitted in connection with the *Bacillus amyloliquefaciens* CECT 5940 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*

#### *Qualitative and quantitative composition of the additive*

For identification and characterization of the strain *Bacillus amyloliquefaciens* CECT 5940 the applicant used PCR amplification of 16S rRNA [5,6]. This method is suitable for the purpose of analysis. However, the CRL recommends for official controls Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [7].

### *Qualitative and quantitative composition of any impurities in the additive*

The applicant analysed the *feed additive* for microbial contaminants (such as Enterobacteria, *Escherichia coli*, *Salmonella*) by using appropriate EN ISO tests [8]. For undesirable substances (i.e. arsenic, cadmium, mercury, lead, selenium, copper, zinc, chrome, aflatoxins) internationally recognised standard methods are available at the respective Community Reference Laboratories, in accordance with Commission Regulation (EC) No 776/2006.

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

For the enumeration of spores of *Bacillus amyloliquefaciens* CECT 5940 in *feed additive*, *premixtures* and *feedingstuffs*, the applicant submitted a spread plate method [9] based on the ring-trial validated CEN method (EN 15784).

This method applies a heat treatment of the initial sample suspension at 80°C for 10 min to reduce the vegetative background flora. Subsequently, appropriate dilutions are spread on non-selective tryptone soya agar (TSA) and the plates are incubated at 37°C for 16 – 24 h.

The performance characteristics of the EN 15784 method reported after logarithmic transformation of measured values (CFU) are [10]:

- 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 0.32 to 0.35  $\log_{10}$  CFU/g; and
- a limit of detection (LOD) of  $10^5$  CFU/kg of *feedingstuffs* [11].

Based on these acceptable performance characteristics, the CRL recommends for official control the dedicated CEN method EN 15784 for the determination of *Bacillus amyloliquefaciens* CECT 5940 in the *feed additive*, *premixtures* and *feedingstuffs*.

## **4. CONCLUSIONS AND RECOMMENDATIONS**

In the frame of this authorisation the CRL recommends the spread plate method EN 15784 for the enumeration of the active agent *Bacillus amyloliquefaciens* CECT 5940 in the *feed additive* *premixture* and *feedingstuffs*.

For the analysis of the identity of the bacterial strain *Bacillus amyloliquefaciens* CECT 5940, the CRL recommends Pulsed Field Gel Electrophoresis (PFGE) 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.

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***Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)***

- Enumeration: Spread plate method using tryptone soya agar (EN 15784)
- Identification: Pulsed Field Gel Electrophoresis (PFGE)

## **5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL**

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive *Bacillus amyloliquefaciens* CECT 5940, for chickens to slaughter age, have been sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

## **6. REFERENCES**

- [1] \*Application/Ref: SANCO/D/2: Forw.Appl.1831/031-2009
- [2] \*Application, Annex A, Proposal for register entry
- [3] \*Technical Dossier, Section II, Annex II\_2
- [4] \*Technical Dossier, Section II 2.5.1
- [5] \*Technical Dossier, Section II 2.2.1
- [6] \*Technical Dossier, Section II, Annex II.9
- [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)
- [8] \*Technical Dossier, Section II 2.1.4
- [9] \*Technical Dossier, Section II 2.6.1
- [10] EN 15784: "Animal feeding stuffs – Isolation and enumeration of presumptive *Bacillus* spp."
- [11] ISO 7218:2007 "Microbiology of food and animal feeding stuffs -- General requirements and guidance for microbiological examinations"

\*Refers to Dossier no: FAD-2009-0041

## **7. RAPPORTEUR LABORATORY**

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, 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.

## **8. Acknowledgements**

The following National Reference Laboratories contributed to this report:

- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT).
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ).