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

Dossier related to: **FAD-2010-0029
EURL/ 110004**

Name of product: **Fecibiol/Fecibiol Plus**

Active Agent (s): ***Enterococcus faecium* CECT 4515
Bacillus amyloliquefaciens CECT 5940**

Rapporteur Laboratory: **Skúšobné laboratórium –
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EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1), for feed additive containing *Enterococcus faecium* CECT 4515 and *Bacillus amyloliquefaciens* CECT 5940, under the category/functional group 4(b) "zootechnical additives/gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The Applicant intends to market the product in two formulations: (i) *Fecibiol* and (ii) *Fecibiol plus* (10 x concentrated), with excipients being either calcium carbonate and sepiolite diluents (for administration via feed) or whey powder (for administration via water). *Fecibiol* and *Fecibiol Plus* are preparations containing *Enterococcus faecium* CECT 4515 and *Bacillus amyloliquefaciens* CECT 5940 (in a 1:1 ratio), with a minimum concentration of 5×10^8 and 5×10^9 CFU/g of each microorganism, respectively. Specifically, authorisation is sought for the use of *Fecibiol* and *Fecibiol Plus* for pets and fur animals. The product is intended to be mixed to complete *feedingstuffs* at a concentration of 5×10^8 CFU/kg of each microorganism. For the use in *water*, the Applicant recommends to reduce concentration by a factor of 2.5.

For the enumeration of *Enterococcus faecium* CECT 4515 in *feed additive*, *feedingstuffs* and *water* the Applicant proposed a single laboratory validated and further verified pour plate method using Slanetz and Bartley agar. The EURL identified instead the internationally recognised ring trial validated spread plate method issued by the European Committee for Standardization (CEN) for the enumeration of *Enterococcus* spp (EN 15788). The performance characteristics of the CEN method reported after logarithmic transformation (CFU) are:

- a repeatability standard deviation (s_r) ranging from 0.12 to 0.2 \log_{10} CFU/g,
- a reproducibility standard deviation (s_R) ranging from 0.23 to 0.41 \log_{10} CFU/g; and
- a limit of detection (LOD) of 1×10^5 CFU/kg *feedingstuffs*.

Based on these performance characteristics, the EURL recommends, for official control, the CEN method EN 15788 for the enumeration of *Enterococcus faecium* CECT 4515 in *feed additive*, *feedingstuffs* and *water*.

For the enumeration of spores of *Bacillus amyloliquefaciens* CECT 5940 in *feed additive*, *feedingstuffs* and *water*, the Applicant proposed a method, similar to the ring-trial validated spread plate method, issued by CEN for the enumeration of *Bacillus* spp (EN 15784). The performance characteristics of the CEN method reported after logarithmic transformation are:

- s_r ranging from 0.07 to 0.09 \log_{10} CFU/g;
- s_R ranging from 0.32 to 0.35 \log_{10} CFU/g; and

- an LOD of 1×10^5 CFU/kg of *feedingstuffs*.

Based on these performance characteristics, the EURL recommends, for official control, the CEN method EN 15784 for the enumeration of *Bacillus amyloliquefaciens* CECT 5940 in *feed additive, feedingstuffs* and *water*.

Molecular methods were used by the Applicant for identification of the active agents. The EURL 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

Enterococcus faecium CECT 4515, *Bacillus amyloliquefaciens* CECT 5940, zootechnical additives, gut flora stabilisers, pets, fur animals.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use), for feed additives containing *Enterococcus faecium* CECT 4515 and *Bacillus amyloliquefaciens* CECT 5940, under the category/functional group 4(b) "zootechnical additives/gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. *Enterococcus faecium* CECT 4515 is already authorised for the use for weaned piglets (Commission Regulation (EC) No 2036/2005) and chickens for fattening (Commission Regulation (EU) No 887/2011); while *Bacillus amyloliquefaciens* CECT 5940 is already authorised for the use for chickens for fattening (Commission Regulation (EC) No 1292/2008). Both strains are deposited in the 'Colección Española de Cultivos Tipo (CECT)' at the University of Valencia, Spain [2, 3].

The Applicant intends to market the product in two formulations: (i) *Fecibiol* and (ii) *Fecibiol plus* (10 x concentrated), with excipients being either calcium carbonate and sepiolite diluents (for administration via feed) or whey powder (for administration via water). *Fecibiol* and *Fecibiol Plus* are preparations containing *Enterococcus faecium* CECT 4515 and *Bacillus amyloliquefaciens* CECT 5940 (in a 1:1 ratio), with a minimum concentration of 5×10^8 and 5×10^9 CFU/g of each microorganism, respectively [4, 5]. Specifically, authorisation is sought for the use of *Fecibiol* and *Fecibiol Plus* for pets and fur animals. The product is intended to

be mixed to complete *feedingstuffs* at a concentration of 5×10^8 CFU/kg of each microorganism [4]. For the use in *water*, the Applicant recommends to reduce concentration by a factor of 2.5 [5].

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 (EFSA) for each application or group of applications. For this dossier, the methods of analysis submitted in connection with *Fecibiol/Fecibiol Plus*, 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 impurities in the additive

For identification and characterisation of the strain *Enterococcus faecium* CECT 4515 the Applicant used molecular methods such as Polymerase Chain Reaction (PCR) amplifications of 16S rRNA, 23S rRNA genes and intergenic regions between 16S and 23S rRNA genes, as well as Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Pulsed Field Gel Electrophoresis (PFGE) [6-8].

For identification and characterisation of the strain *Bacillus amyloliquefaciens* CECT 5940 the Applicant used PCR amplification of 16S rRNA [9].

All these methods are suitable for the purpose of analysis. However, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [10].

Qualitative and quantitative composition of any impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (such as *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens*, coliforms, molds and yeasts and Enterococci) using appropriate AOAC and FDA tests [5].

For undesirable substances (i.e. arsenic, cadmium, mercury, lead, selenium, copper, zinc, chrome, aflatoxins) internationally recognised standard methods are available at the respective European Union Reference Laboratories, in accordance with Commission Regulation (EC) No 776/2006.

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

For the enumeration of *Enterococcus faecium* CECT 4515 in *feed additive, feedingstuffs and water* the Applicant proposed a single laboratory validated and further verified pour plate method using Slanetz and Bartley agar [11, 12]. The sample is suspended and diluted in a saline solution with added Tween-80; appropriate dilutions are then transferred into petri dishes and Slanetz and Bartley agar is added. When the agar is solidified, plates are incubated at 37 °C for 48 hours before colony counting.

The EURL identified instead the internationally recognised ring trial validated spread plate method issued by the European Committee for Standardization (CEN) for the enumeration of *Enterococcus* spp (EN 15788) [13]. The sample is suspended in phosphate buffered saline (PBS) and diluted in a peptone salt solution, the appropriate dilutions are then spread on Bile Esculin Azide Agar. The agar plates are incubated at 37 °C for 24 hours before colony counting. The performance characteristics of the CEN method reported after logarithmic transformation (CFU) are:

- a repeatability standard deviation (s_r) ranging from 0.12 to 0.2 \log_{10} CFU/g,
- a reproducibility standard deviation (s_R) ranging from 0.23 to 0.41 \log_{10} CFU/g; and
- a limit of detection (LOD) of 1×10^5 CFU/kg *feedingstuffs* [14].

Based on these performance characteristics, the EURL recommends, for official control, the CEN method EN 15788 for the enumeration of *Enterococcus faecium* CECT 4515 in *feed additive, feedingstuffs and water*.

For the enumeration of spores of *Bacillus amyloliquefaciens* CECT 5940 in *feed additive, feedingstuffs and water*, the Applicant proposed a method [15], similar to the ring-trial validated spread plate method, issued by CEN for the enumeration of *Bacillus* spp (EN 15784) [16]. According to the CEN method, 20 g of additive are suspended in PBS. Decimal dilutions are prepared in peptone salt diluent and subjected to a heat treatment at 80 °C for 10 minutes. Subsequently, appropriate dilutions are spread on tryptone soya agar (TSA) and

plates are incubated at 37 °C for 16-24 hours. The performance characteristics of the CEN method reported after logarithmic transformation are [16]:

- s_r ranging from 0.07 to 0.09 \log_{10} CFU/g;
- s_R ranging from 0.32 to 0.35 \log_{10} CFU/g; and
- an LOD of 1×10^5 CFU/kg of *feedingstuffs* [14].

Based on these performance characteristics, the EURL recommends, for official control, the CEN method EN 15784 for the enumeration of *Bacillus amyloliquefaciens* CECT 5940 in *feed additive, feedingstuffs* and *water*.

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 the following CEN methods: EN 15788 and EN 15784 for the enumeration of *Enterococcus faecium* CECT 4515 and *Bacillus amyloliquefaciens* CECT 5940 in *feed additive, feedingstuffs* and *water*.

For the identification of the microbial strains *Enterococcus faecium* CECT 4515 and *Bacillus amyloliquefaciens* CECT 5940 the EURL recommends Pulsed Field Gel Electrophoresis (PFGE) for official control.

Recommended text for the register entry (analytical method)

- Enumeration in the *feed additive, feedingstuffs* and *water*:
 - for *Enterococcus faecium* CECT 4515:
Spread plate method using Bile EsculineAzide Agar (EN 15788)
 - for *Bacillus amyloliquefaciens* CECT 5940:
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, reference samples of *Fecibiol/Fecibiol Plus* 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/D/2: Forw. Appl. 1831/0028-2011
- [2] *Technical dossier, Section II, Annex_II_09
- [3] *Technical dossier, Section II, Annex_II_10
- [4] *Application, Proposal for Register Entry – Annex A
- [5] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis
- [6] *Technical dossier, Section II, Annex_II_20
- [7] *Technical dossier, Section II, Annex_II_21
- [8] *Technical dossier, Section II, Annex_II_22
- [9] *Technical dossier, Section II, Annex_II_19
- [10] European Community Project SMT4-CT98-2235.'Methods for the Official Control of Probiotics Used as Feed Additives, Volume 1. 2002. Report 20873-1. Office for official Publications of the European Communities. ISBN 92-894-6250-7 (Vol. I)
- [11] *Technical dossier, Section II, Annex_II_46
- [12] *Technical dossier, Section II, Annex_II_47
- [13] EN 15788 'Animal feeding stuffs - Isolation and enumeration of *Enterococcus (E. faecium)* spp'
- [14] ISO 7218 'Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations'
- [15] *Technical dossier, Section II, Annex_II_48
- [16] EN 15784: "Animal feeding stuffs – Isolation and enumeration of presumptive *Bacillus* spp."

*Refers to Dossier no: FAD-2011-0029

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky (UKSUP), Bratislava, Slovakia. 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:

- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)