



JRC.D.5/FSQ/CvH/DM/ag/ARES(2012)858055

**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-2010-0363 - CRL/100345**

Name of Feed Additive: **Fecinor/Fecinor Plus**

Active Substance(s): ***Enterococcus faecium* CECT 4515 (E 1713)**

Rapporteur Laboratory: **European Union Reference Laboratory
for Feed Additives (EURL-FA)
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Date: **13/07/2012**

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Date: **13/07/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 10(2) for *Enterococcus faecium* CECT 4515, 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 *feed additive* will be marketed in two powder forms: *Fecinor* and *Fecinor Plus*, containing a minimum concentration of 1×10^9 and 1×10^{10} CFU/g of *Enterococcus faecium* CECT 4515 on a sepiolite carrier. Specifically, authorisation is sought for the use of the *feed additive* for weaned piglets and the *feed additive* is intended to be mixed at a concentration of 1×10^9 CFU/kg of complete *feedingstuffs*.

For the enumeration of *Enterococcus faecium* CECT 4515 in the *feed additive* and *feedingstuffs* 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 CEN method (EN 15788), using Bile Esculin Azide Agar, for the enumeration of *Enterococcus* spp. 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 the performance characteristics presented, the EURL recommends for official control, the CEN method (EN 15788) for the enumeration of *Enterococcus faecium* CECT 4515 in *feed additive* and *feedingstuffs*.

Molecular methods were used by the Applicant to identify the active agent in the *feed additive*. The EURL recommends instead 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, E 1713, zootechnical additives, gut flora stabilisers, weaned piglets.

1. BACKGROUND

In the current application authorisation is sought under Article 10(2) (re-evaluation of already authorised additives) for *Enterococcus faecium* CECT 4515, 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]. The *feed additive* is already authorised under Commission Regulation (EC) No 2036/2005 for the use for weaned piglets. The strain is deposited in 'Colección Española de Cultivos Tipo (CECT)' at the University of Valencia, Spain [2].

The *feed additive* will be marketed in two powder forms: *Fecinor* and *Fecinor Plus*, containing a minimum concentration of 1×10^9 and 1×10^{10} CFU/g of *Enterococcus faecium* CECT 4515 on a sepiolite carrier [2].

Specifically, authorisation is sought for the use of the *feed additive* for weaned piglets and the *feed additive* is intended to be mixed at a concentration of 1×10^9 CFU/kg of complete *feedingstuffs* [3].

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 European Union Reference Laboratory concerning applications for authorisations of feed additives, as last amended by Regulation (EC) No 885/2009, the EURL 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 *Fecinor/Fecinor 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 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 region between 16S and 23S rRNA genes, as well as Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Pulsed Field Gel Electrophoresis (PFGE) [4-6]. These methods are suitable for the purpose of analysis. However, the EURL recommends instead for official control Pulsed Field Gel

Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [7].

Qualitative and quantitative composition of impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (such as coliforms, *Escherichia coli*, *Salmonella* spp., yeasts and moulds) by using appropriate AOAC and FDA 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 European Union Reference Laboratory, in accordance with Commission Regulation (EC) No 776/2006.

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

For the enumeration of *Enterococcus faecium* CECT 4515 in the *feed additive* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified pour plate method using Slanetz and Bartley agar [9-14]. 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) [15]. 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* [16].

Based on the performance characteristics presented, the EURL recommends for official control, the CEN method (EN 15788) for the enumeration of *Enterococcus faecium* CECT 4515 in *feed additive* 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 the CEN method (EN 15788) for the enumeration of *Enterococcus faecium* CECT 4515 in the *feed additive* and *feedingstuffs* and Pulsed Field Gel Electrophoresis (PFGE) for the identification of the strain.

Recommended text for the register entry (analytical method)

Enumeration of *Enterococcus faecium* CECT 4515 in the *feed additive* and *feedingstuffs*:

- Pour plate method using Bile Esculin Azide Agar (EN 15788)

Identification of *Enterococcus faecium* CECT 4515:

- Pulsed Field Gel Electrophoresis (PFGE)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of *Fecinor/Fecinor 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/Ref: SANCO/G1: Forw.Appl.1831/0030-2012
- [2] *Technical Dossier, Section II, Annex_II_04
- [3] *Application, Proposal for Register Entry, Annex A
- [4] *Technical dossier, Section II, Annex_II_11
- [5] *Technical dossier, Section II, Annex_II_12
- [6] *Technical dossier, Section II, Annex_II_13
- [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.2.6.3. Methods of the analysis relating to the identity and characterisation of the additive
- [9] *Technical dossier, Section II, Annex_II_33
- [10] *Technical dossier, Section II, Annex_II_34_1
- [11] *Technical dossier, Section II, Annex_II_34_2
- [12] *Technical dossier, Section II, Annex_II_34_3
- [13] *Technical dossier, Section II, Annex_II_34_4
- [14] *Technical dossier, Section II, Annex_II_34_5
- [15] EN 15788 'Animal feeding stuffs - Isolation and enumeration of *Enterococcus* (*E. faecium*) spp'

[16] ISO 7218:1996 - Microbiology of food and animal feedingstuffs – General rules for microbiological examinations

*Refers to Dossier No: FAD-2010-0363

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union 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, as last amended by Regulation (EC) No 885/2009.

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)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Państwowy Instytut Weterynaryjny, Puławy (PL)