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

Dossier related to: FAD-2009-0060  
CRL/ 090030

Name of additive: Cylactin LBC/Cernivet LBC

Active Agent (s): *Enterococcus faecium* NCIMB 10415

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## EXECUTIVE SUMMARY

In the current application authorisation under article 4(1) (new use) and 10(2) (re-evaluation of an authorised additive) is sought for the microbial feed additive *Enterococcus faecium* NCIMB 10415 under the category 'zootechnical additives', functional group 4(b), 'gut flora stabilisers' according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Cylactin LBC/Cernivet LBC* for calves, lambs and kids for rearing and fattening. The *feed additive* is presented as a white – yellow granulate in three formulations with different concentrations of *Enterococcus faecium* NCIMB 10415:

- Cylactin LBC ME10/Cernivet LBC ME10 ( $1 \times 10^{10}$  CFU/g);
- Cylactin LBC ME20 plus/Cernivet LBC ME20 plus ( $2 \times 10^{10}$  CFU/g); and
- Cylactin LBC G35/Cernivet LBC G35 ( $3.5 \times 10^{10}$  CFU/g).

It is intended to be mixed at a dose ranging from  $1 \times 10^9$  to  $6.6 \times 10^9$  CFU/kg of complete *feedingstuffs*.

For the enumeration of *Enterococcus faecium* NCIMB 10415 in *feed additives*, *premixtures* and *feedingstuffs* the CRL recommends a CEN spread plate method using Bile Esculin Azide Agar (EN 15788), instead of the single laboratory validated method submitted by the applicant. The performance characteristics of the EN 15788 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  $1 \times 10^5$  CFU/kg, well below the minimum dose proposed by the applicant ( $1 \times 10^9$  CFU/kg of *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

*Enterococcus faecium* NCIMB 10415, zootechnical additives, calves, lambs, kids for rearing and fattening, gut flora stabilisers.

## 1. BACKGROUND

*Enterococcus faecium* NCIMB 10415 is a feed additive for which authorisation under Article 4(1) (new use) and 10(2) (re-evaluation of an authorised additive) is sought under the category of 'zootechnical additives' functional group 4(b), 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003 [1]. The *feed additive* is already authorised for the use for calves (Regulation (EC) No 1288/2004), chickens and pigs for fattening (Regulation (EC) No 943/2005), sows (Regulation (EC) No 1200/2005), piglets (Regulation (EC) No 252/2006) and cats and dogs (Regulation (EC) No 102/2009) under the registration number E 1705. The strain is deposited in the 'National Collection of Industrial, Marine and Food Bacteria (NCIMB)' in Aberdeen, Scotland [2]. The *feed additive* is presented as a white – yellow granulate in three formulations with different concentrations of *Enterococcus faecium* NCIMB 10415:

- Cylactin LBC ME10/Cernivet LBC ME10 ( $1 \times 10^{10}$  CFU/g),
- Cylactin LBC ME20 plus/Cernivet LBC ME20 plus ( $2 \times 10^{10}$  CFU/g), and
- Cylactin LBC G35/Cernivet LBC G35 ( $3.5 \times 10^{10}$  CFU/g) [3].

It is intended to be mixed at a dose ranging from  $1 \times 10^9$  to  $6.6 \times 10^9$  CFU/kg of complete *feedingstuffs* for calves, lambs and kids for rearing and fattening [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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL 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 *Cylactin LBC/Cernivet LBC*, 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* NCIMB 10415 the applicant used molecular methods such as randomly amplified polymorphic DNA (RAPD) method and Pulsed Field Gel Electrophoresis (PFGE) [5,6]. These methods are suitable for

the purpose of analysis. The CRL recommends for official control, Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification [7].

*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*, *Pseudomonas aeruginosa* and total fungi) by using appropriate tests [8-10].

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 the active substance in feed additive, premixtures, feedingstuffs and water***

For enumeration of *Enterococcus faecium* NCIMB 10415 in *feed additive*, the applicant proposes a single laboratory validated pour plate/spread plate method [11]. For the manual procedure the sample is suspended and diluted in a buffer solution; the appropriate dilutions are then transferred into petri dishes and Columbia agar is added (pour plate method). When the agar is solidified, plates are incubated at 37°C for 48 hours before colony counting. For automatic plating procedure (IUL system), the appropriate dilutions are spread on Columbia agar plates followed by incubation for 24 hours at 37°C (spread plate method). After incubation, colonies are counted.

For *premixtures* and *feedingstuffs*, the applicant proposes another single laboratory validated spread plate method [12]. The sample is mixed and diluted with phosphate buffer. If copper is present, iminodiacetic acid (IDAA) is added to neutralize the copper. The appropriate dilutions are then spread on Slanetz and Bartley agar plates. The agar plates are incubated at 37°C for 24 hours before colony counting – manually or automatically.

The CRL recommends instead the internationally recognised ring trial validated spread plate method developed by 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  $1 \times 10^5$  CFU/kg [14], well is below the minimum dose proposed by the applicant ( $1 \times 10^9$  CFU/kg of *feedingstuffs*).

The CRL recommends, for official control, the CEN method EN 15788 for the enumeration of *Enterococcus faecium* NCIMB 10415 in *feed additives*, *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 CRL recommends the CEN method - EN 15788 - for the enumeration of the active agent *Enterococcus faecium* NCIMB 10415 in *feed additive*, *premixtures* and *feedingstuffs*.

For the identification of the bacterial strain *Enterococcus faecium* NCIMB 10415 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 in accordance with article 10 of Commission Regulation (EC) No 378/2005 is not considered necessary.

#### ***Recommended text for the register entry (analytical method)***

- Enumeration: Spread plate method using Bile Esculin Azide agar (EN 15788)
- 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 *Cylactin LBC/Cernivet LBC* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

## 6. REFERENCES

- [1] \*Application/Ref:SANCO/D/2:Forw.Appl.1831/0048-2009.
  - [2] \*Technical dossier, Section II/2.2. Characterisation of *E.Faecium* NCIMB 10415
  - [3] \*Technical dossier, Section II/2.1. Identity of the additive
  - [4] \*Application, Proposal for Register Entry, Annex A
  - [5] \*Technical dossier, Section II, Annex\_II\_26\_Coconcelli et al 1995
  - [6] \*Technical dossier, Section II, Annex\_II\_27\_Arini 2009a
  - [7] 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)
  - [8] \*Technical dossier, Section II, Annex\_II\_13\_Cerbios 2007d
  - [9] \*Technical dossier, Section II, Annex\_II\_82\_European Pharmacopoeia 2008d
  - [10] \*Technical dossier, Section II, Annex\_II\_83\_European Pharmacopoeia 2008e
  - [11] \*Technical dossier, Section II, Annex\_II\_75\_Cerbios 2007h
  - [12] \*Technical dossier, Section II, Annex\_II\_76\_Cerbios 2007i
  - [13] EN 15788 “Animal feeding stuffs - Isolation and enumeration of *Enterococcus (E. faecium)* spp.”
  - [14] ISO 7218:2007 'Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations'
- \*Refers to Dossier no: FAD-2009-0060

## 7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was The Danish Plant Directorate, Lyngby, Denmark. 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

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