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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2008-422 FAD-2008-0021 CRL/080008
Name of Additive:	Cylactin/Cernivet LBC ME 10 and 20
Active Agent(s):	*Enterococcus faecium* NCIMB 10415
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Cylactin/Cernivet LBC ME 10 and 20 under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Enterococcus faecium* NCIMB 10415. The additive is available in micro-encapsulated form and contains a minimum concentration of 1 x 10^{10} colony forming units (c.f.u.) per gram. Specifically, authorisation is sought to use Cylactin/Cernivet LBC ME 10 and 20 for chickens for fattening. The conditions of use are proposed with a recommended dosage of 0.3 to 2.8 x 10^{9} c.f.u./kg feed.

For the quantification of the active agent (*Enterococcus faecium* NCIMB 10415) of Cylactin/Cernivet LBC ME 10 and 20 in the *feed additive*, *premixtures* and *feedingstuffs*, an appropriate spread plate method was proposed by the applicant. The method was in-house validated and the method precision data were acceptable for the intended purpose.

For official controls regarding the quantitative determination of the colony forming units of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, a fully ring-trial validated spread plate enumeration method is recommended (J. Appl. Microbiol. 2002, 93, 781-786). The method's performance characteristics of the enumeration method are standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.12 – 0.20 $\log_{10}$ and 0.23 – 0.41 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The limits of quantification (LOQ) of this method are around 10^{4} colony forming units (c.f.u.) per gram (g) feed additive or premixture and around 10^{7} c.f.u./kg feedingstuff.

The identity of the bacterial strain, *Enterococcus faecium* NCIMB 10415, was analysed by microscopy, biochemistry and molecular methods such as randomly amplified polymorphic DNA (RAPD) methodology. Pulsed-field gel electrophoresis (PFGE) is recognised as a standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

KEYWORDS

Cylactin/Cernivet LBC ME 10 and 20, *Enterococcus faecium*, zootechnical additive

1. BACKGROUND

Cylactin/Cernivet LBC ME 10 and 20 is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Cylactin/Cernivet LBC ME 10 and 20 is provided in form of micro-encapsulated granules supplemented with a strain of *Enterococcus faecium.* The strain is deposited at the National Collection of Industrial, Marine and Food Bacteria (NCIMB), Aberdeen, Scotland under the deposit number NCIMB 10415. Cylactin/Cernivet LBC ME 10 and 20 contains at least 1 x 10^{10} c.f.u. of *Enterococcus faecium* NCIMB 10415 per gram in the feed additive [1]. The intended use of the current application (EFSA-Q-2008-422) is for chickens for fattening. The proposed conditions of use are for chickens for fattening at a recommended dosage of 0.3 to 2.8 x 10^{9} c.f.u./kg complete feedingstuffs [1].

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 authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application. For this particular dossier, the methods of analysis submitted in connection with the Cylactin/Cernivet LBC ME 10 and 20 dossier (EFSA-Q-2008-422) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5 Control methods), in the following referred to as 'the Guidelines'.

Description of some of the methods listed under item 2.5.1. of the Guidelines

Qualitative and quantitative composition of the additive

The method for quantifying the active agent as proposed by the applicant represents a spread plate method using an appropriate agar for enumeration. The method is suitable for Cylactin/Cernivet LBC ME 10 and 20 according to the applicant and was in-house validated [2, 3]. A sample is suspended in a dilution buffer and decimally diluted. It is recommended to incubate the initial suspension of Cylactin/Cernivet LBC ME 10 and 20 for 30 min at room temperature, followed by homogenization prior to perform decimal dilutions. Appropriate

dilutions were transferred to agar plates. The agar plates are incubated at 37 °C for 48 h [2]. Within-laboratory repeatability and reproducibility, accuracy and linearity of the method were determined [3]. The reported precision data are acceptable and within the range of data reported in a published interlaboratory ring-trial validation study that used bile esculin azid (BEA) agar to selectively enumerate enterococci [4]. The published ring-trial validated method is recommended for official controls to determine the colony forming units in the frame of the authorisation.

The genetic identity of the strain is examined by a combination of techniques. The production strain was examined by microscopy, biochemically and by molecular methods such as randomly amplified polymorphic DNA (RAPD) methodology [6]. For official controls pulsed-field gel electrophoresis (PFGE) is recommended in the frame of the authorization.

Description of qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (cf. requirements of Guidelines section 2.5.2)

For the enumeration of the active agents *Enterococcus faecium* NCIMB 10415 in *premixtures* and *feedingstuffs*, the applicant proposes the same spread plate method as described above [5].

The fully ring-trial validated method [4] as described above is recommended for official controls for quantification of the active substance in premixtures and feedingsstuffs. The validated spread plate method uses bile esculine azide (BEA) agar. A sample is suspended in a dilution buffer and decimally diluted followed by a transfer of appropriate dilutions to agar plates. Incubation of BEA plates was carried out at 37 ºC for 24 hours. The enumeration of enterococci on BEA agar showed standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.12 – 0.20 $\log_{10}$ and 0.23 – 0.41 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The statistical analysis was carried out on $\log_{10}$ transformed raw data of the enumeration results. BEA agar was selective for enterococci in the presence of other probiotic micro-organisms such as pediococci, lactobacilli and yeast. The results of the collaborative study were published [4]. The limits of quantification (LOQ) of this method are around 10^4 colony forming units (c.f.u) per gram (g) feed additive or premixture and around 10^7 c.f.u./kg feedingstuff. This method is recommended by the CRL for official controls in the frame of the authorisation.

The applicant made some recommendations regarding the preparation of an initial suspension of a sample when trace elements, in particular copper, are present in premixtures or mineral feeds which may affect the viable counts [5]. It is recommended to examine the potential presence of copper using for example a stick test format such as Quantofix Copper. In case of presence of copper it is recommended to add iminodiacetic acid (IDAA) whereby 350 mg of IDAA were found to neutralise 166 mg copper and the pH has to be verified and possibly to be adjusted with 20 % NaOH.

The applicant has used a range of techniques to identify the *Enterococcus faecium* strain used as active agent as described above [6]. Pulsed-field gel electrophoresis (PFGE) is considered as a suitable method for official controls [7].

4. CONCLUSIONS AND RECOMMENDATIONS

A ring-trial validated spread plate method using BEA agar to enumerate the active agent is recommended for official controls in the frame of the authorisation [4].

For the analysis of the identity of the bacterial strain, *Enterococcus faecium* NCIMB 10415, the applicant uses also a range of techniques which are appropriate to identify the strain. As pulsed-field gel electrophoresis (PFGE) is already widely used by reference laboratories to identify bacterial isolates it is recommended as a suitable methodology in this context for official controls.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

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

Enumeration: Spread plate method using bile esculin azid agar.

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 Cylactin/Cernivet LBC ME 10 and 20 for chickens for fattening 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] Proposal of Register entry, Annex III

[2] Dossier Section II. Appendix II 67 'Quantitative determination of *Enterococcus faecium* in pure product'

[3] Dossier Section II. Appendix II 69 'Method validation'

[4] Leuschner R.G.K., Bew J., Domig K.J., Kneifel W. 2002. A collaborative study of a method for the enumeration of probiotic enterococci in animal feed. J. Appl. Microbiol. 93, 781-786

[5] Dossier Section II. Appendix II 68 'Quanititative determination of *Enterococcus faecium* in premixtures and feedingstuffs'

[6] Dossier Section II. 2.2.1. Strain identity

[7] Domig K.J., Mayer H.K., Kneifel W. 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. Int. J. Food Microbiol. 88, 165 - 188

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium. The initial evaluation report was made available for commenting to the consortium of National Reference Laboratories.

8. ACKNOWLEDGEMENTS

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- National Veterinary Research Institute, Pulawy, Poland