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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-2008-287
FAD-2008-0009

Name of Additive: Toyocerin®

Active Agent(s): *Bacillus cereus* var. *toyoi*
NCIMB 40112/CNCM I-1012

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

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Date: 17/10/2008

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Toyocerin® under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Toyocerin® for rabbit breeding does from service until weaning is requested. Toyocerin® contains a minimum of 1×10^{10} colony forming units (c.f.u.) of viable spores of *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012 per gram (g). The feed additive is intended to be mixed into complete feedingstuffs at a final concentration of 0.2 to 1×10^9 c.f.u./kg.

For the quantification of the active agent (*Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012) of Toyocerin® in the *feed additive*, *premixtures* and *feedingstuffs* appropriate enumeration methods were proposed by the applicant. Analysis data confirmed an appropriate method performance in a second laboratory.

For official controls regarding the quantitative determination of colony forming units of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, another surface plate count enumeration method is recommended which includes a heat-treatment and uses subsequently a non-selective agar. This method has been fully ring-trial validated (J.AOAC Int. 2003, 86, 568-575). The method's performance characteristics revealed standard deviations for repeatability (s_r) and reproducibility (s_R) of around $0.07 - 0.09 \log_{10}$ and $0.35 - 0.32 \log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g premixture or feedingstuff, respectively. The limits of quantification (LOQ) of this method are around 10^4 c.f.u./g feed additive or premixture and around 10^7 c.f.u./kg feedingstuff which is well below the minimum anticipated target level of application.

The identity of the bacterial strain, *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012, was analysed by a range of techniques including biochemistry, phage-typing, molecular methods and pyrolysis mass spectrometry. Pulsed field gel electrophoresis (PFGE) is a generally recognised standard methodology for microbial identification and is therefore 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

Toyocerin®, feed additive, *Bacillus cereus* var. *toyoi*, zootechnical additive, rabbit breeding does

1. BACKGROUND

Toyocerin[®] is a feed additive for which authorisation is sought under the category 'zotechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Toyocerin[®] is provided in form of an odourless, white to greyish-brown dry powder supplemented with *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012 (EC No.: E-1701) containing at least 1×10^{10} c.f.u./g of viable spores of the active agent [1, 2]. The strain has been deposited at culture collections such as the National Collections of Industrial, Food and Marine Bacteria in Aberdeen (UK) [3]. The intended use of the current application (EFSA-Q-2008-287) is for rabbit breeding does from service until weaning. The proposed conditions of use are a recommended dosage of 0.2 to 1×10^9 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 Toyocerin[®] dossier (EFSA-Q-2008-287) 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 in the *feed additive* as proposed by the applicant represents a pour plate method using a nutrient agar. The method was in-house validated by an external laboratory [4]. The method is suitable for the intended purpose. However, a fully ring-trial validated method is recommended for official controls in the frame of the authorisation [5].

The genetic identity of the strain is examined by a range of techniques including biochemistry, phage-typing and molecular methods such as nucleotide sequencing, rRNA restriction analysis, plasmid profiling and pyrolysis mass spectrometry [6]. Pulsed field gel electrophoresis is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

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 *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012 in *premixtures* and *feedingstuffs*, the applicant proposes a similar in-house validated method as mentioned above for the additive [4, 7]. The method is suitable for the intended purpose.

For official controls the same fully ring-trial validated method as cited above is recommended [5]. The ring-trial validated method includes a heat-treatment to inactivate vegetative background flora at 80°C for 10 min of the first decimal dilution of the initial suspension. Subsequently, appropriate dilutions are spread on non-selective tryptone soya agar and plates are incubated at 37 °C for 16 – 24 h. Performance characteristics of this method obtained in the collaborative study were expressed in terms of standard deviations for repeatability (s_r) and reproducibility (s_R). Analysis results of samples of feedingstuffs with a mean concentration of 5.95 \log_{10} c.f.u./g had a repeatability standard deviation s_r of 0.07 \log_{10} and a reproducibility standard deviation s_R of 0.35 \log_{10} calculated on the base 10 logarithms of the measured c.f.u./g feedingstuff. Samples with a concentration of 9.53 \log_{10} c.f.u./g revealed a s_r of 0.09 \log_{10} and a s_R of 0.32 \log_{10} calculated on the base 10 logarithms of the measured c.f.u./g premixture. The limits of quantification (LOQ) of this method are 100 c.f.u./g feed additive or premixture and 10^7 c.f.u./kg feedingstuff which is well below the minimum anticipated target level of application.

The applicant used a range of techniques to identify the *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012 strain used as active agent as described above [6]. Pulsed field gel electrophoresis is considered as a suitable method for official controls.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant uses appropriate conventional methods to enumerate the active agent. A ring-trial validated method using a heat treatment and a non-selective agar is recommended for official controls in the frame of the authorisation [5]. As pulsed field gel electrophoresis 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)

Quantification: Spread plate method using tryptone soya agar and a heat treatment

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 Toyocerin® for rabbit breeding does 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, Summary for publication
- [2] Technical dossier, section II, 2.2.2. Biological origin
- [3] Technical dossier, section II, Annex 15 Official deposits
- [4] Technical dossier, section II, Annexes 91, 93
- [5] Leuschner R.G.K., Bew J., Cruz A. 2003. Enumeration of probiotic bacilli spores in animal feed: Interlaboratory study. J. AOAC Int. 86(3), 568-575
- [6] Technical dossier, section II, Annexes 16-23
- [7] Technical dossier, section II, Annexes 92, 93

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

The following National Reference Laboratory contributed to this report:

- National Veterinary Research Institute, Pulawy, Poland