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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-2006-062 FAD-2006-0002
Name of Additive:	TOYOCERIN® (sows)
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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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. The active agent in the additive are viable spores of the microorganism Bacillus cereus var. *toyoi* NCIMB 40112/CNCM I-1012. The additive is an odourless, white greyish-brown dry powder containing a minimum concentration of 1 x 10^{10} colony forming units (c.f.u.) per gram additive. Specifically, authorisation is sought to use Toyocerin® for sows from service until weaning. The conditions of use are proposed with a recommended dosage of 0.5 to 2 x 10^{9} c.f.u./kg complete feedingstuffs.

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 using a selective agar were proposed by the applicant. Analysis data confirmed appropriate method performance in a second laboratory. It is however recommended to use a larger amount of feed sample for analysis than the proposed 2 g, such as for example 50 g to take account of potential sample heterogeneity.

For official controls regarding the quantitative determination of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, another surface plate count enumeration method is recommended which has been fully ring-trial validated (J.AOAC Int. 2003, 86, 568-575). The method performance characteristics include a relative standard deviation for repeatability (RSD_r) of around 1 % and a relative standard deviation for reproducibility (RSD_R) of around 6 %. The limit of quantification (LOQ) for the method is around 2 to 3 x 10^{6} c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

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 considered suitable for official controls in the frame of the authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

Toyocerin®, feed additive, *Bacillus cereus*, zootechnical additive, sows

1. BACKGROUND

Toyocerin® 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. Toyocerin® is provided in form of an odourless, white to greyish-brown dry powder supplemented with a strain of Bacillus cereus var. *toyoi* NCIMB 40112/CNCM I-1012 (EC No.: E-1701) containing at least 1 x 10^{10} c.f.u./g of the active agent [1]. The intended use of the current application (EFSA-Q-2006-062) is for sows from service until weaning. The proposed conditions of use are a recommended dosage of 0.5 to 2 x 10^{9} c.f.u./kg complete feedingstuffs [1, 2].

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 authorizations 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-2006-037) 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 [3]. 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 [4].

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 [5]. 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.

Qualitative and quantitative composition of any impurities in the additive

The applicant provided methods for the analysis of microbiological contaminants such as *Escherichia coli*, coliforms, *Salmonella* species, *Staphylococcus aureus* in the feed additive some of which represented AOAC official methods [6]. An analysis of aflatoxins B_1, B_2, G_1 and G_2 was carried out using High Performance Liquid Chromatography (HPLC) analysis. Arsenic and heavy metals (cadmium, mercury, lead) were further analysed in the feed additive. Method descriptions are submitted [6]. The methods are suitable for the intended purpose, however for official controls the provided information lacks some details. Therefore, standardized methods such as ISO/CEN standards where available are recommended for official controls in line with current EU legislation

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 [7]. This method is suitable for the intended purpose.

For official controls the same fully ring-trial validated method as cited above is recommended [4]. The ring-trial validated method includes a heat-treatment of the initial sample suspension at 80°C for 10 min to inactivate vegetative background flora. Subsequently, appropriate dilutions are spread on tryptone soya agar and plates were incubated at 37 °C for 16 – 24 h. This method revealed relative standard deviations for repeatability (RSD_r) between 1.0 – 1.1 % and for reproducibility (RSD_R) between 3.4 – 5.8 %.

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 [5]. 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 [4]. 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)

Analytical method suitable for official controls: Enumeration spread plate method using tryptone soya agar with pre-heat treatment of feed samples.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive Toyocerin® for sows have been sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Summary for publication

[2] Proposal of Register entry

[3] Technical dossier, section II, Annexes 90, 92

[4] Leuschner R.G.K., Bew J., Domig K., Kneifel W. 2002. Enumeration of probiotic bacilli spores in animal feed: Interlaboratory study. J. AOAC Int. 86(3), 568-575

[5] Technical dossier, section II, Annexes 16-23

[6] Technical dossier, section II, Annexes 67, 68

[7] Technical dossier, section II, Annexes 91a, 91, 92

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive Authorisation (CRL-FAA), Geel, Belgium

8. ACKNOWLEDGEMENTS

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- Thüringer Landesanstalt für Landwirtschaft, Jena, Germany
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