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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-2007-040 FAD-2006-0033
Name of Additive:	Calsporin® for chickens for fattening
Active Agent(s):	*Bacillus subtilis* C-3102 (DSM 15544)
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 Calsporin® under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Calsporin® as a gut flora stabiliser for chickens for fattening (broilers) is requested. Calsporin® consists of a minimum of 1 x 10^{10} of viable spores (c.f.u., colony-forming units) of *Bacillus subtilis* C-3102 (as active agent) per gram and calcium carbonate as carrier. The feed additive is intended to be mixed into complete feedingstuffs at a final concentration of 5 x 10^{8} to 1 x 10^{9} c.f.u./kg.

For the determination of the active agent in the *feed additive, premixtures and feedingstuffs,* a surface plate count method is proposed by the applicant to enumerate viable spores of *Bacillus subtilis* C-3102. Vegetative cells are inactivated by a heat treatment and not taken into account. The method is quantitative using trypticase soy agar (TSA) as medium. The method's performance characteristics for enumerating the active agent in the *feed additive, premixtures and feedingstuffs* revealed acceptable method's performance characteristics. However, for official controls a fully ring-trial validated, peer reviewed and published spread plate method for enumeration of bacilli spores including those of *B. subtilis,* is recommended [J. AOAC Int. 2003. 86, 568-575]. In the method a heat treatment of the initial sample suspension and tryptone soya agar is used. Methods performance characteristics for samples of premixtures and feedingstuffs were determined after logarithmic transformation of the measured colony forming units. A standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ and a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$ for premixtures were concluded. For feedingstuffs a s_r of 0.07 $\log_{10}$ and a s_R of 0.35 $\log_{10}$ were found. The limit of quantification (LOQ) for the method for feedingstuffs is around 1 x 10^{7} c.f.u./kg sample which is well below the minimum anticipated target level of application.

For identification of the active agent molecular methods suitable for the purpose of analysis were used by the applicant. For official controls pulsed-field gel electrophoresis (PFGE) is recommended.

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

KEYWORDS

Calsporin®, *Bacillus subtilis* C-3102, feed additive, zootechnical, gut flora stabiliser

1. BACKGROUND

Calsporin® 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. Calsporin® is provided in form of a powder containing at least 1.0 x 10^{10} cfu/g spores of *Bacillus subtilis* C-3102 as active agent. The strain is deposited at a culture collection, the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (DSMZ, Braunschweig, Germany) under accession number DSM 15544. The intended use of the current application is chickens for fattening (broilers), by mixing the feed additive into complete feedingstuffs at a final concentration of 5 x 10^{8} to 1 x 10^{9} c.f.u./kg [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 Calsporin® dossier (EFSA-Q-2007-040) 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 provided by the applicant to enumerate the active agent in the additive is a spread plate method to quantify spores of *Bacillus subtilis* C-3102. The method is based on ISO 4833 (2003), which is a horizontal method for enumeration of microorganisms, by counting the colonies growing in a solid medium after aerobic incubation at 30 °C. The method allows for enumeration and differentiation of bacterial spores capable of germinating. The results are reported as colony forming units (c.f.u.) per gram (g) sample. The samples are initially diluted and homogenised. A sample of the feed additive is suspended in a dilution buffer and blended at high speed followed by a thermal treatment. After refrigeration for 12-18 hours, decimal

dilution series are prepared. Duplicate plates containing trypticase soy agar (TSA) are inoculated with appropriate dilutions. Unless otherwise stated, each step is carried out at temperatures lower than 10 °C. The agar plates are incubated under aerobic conditions in plastic bags with ventilation holes at 37 °C for 20 - 24 hours. Only agar plates with maximum 60 colonies with typical *Bacillus subtilis* C-3102 morphology are counted. Means of two replicate plates are calculated. The proposed methods are in-house validated and suitable for the intended purpose [2, 3, 4]. However, parts of the procedures are not in accordance with standard procedures such as laid down by the International Organization for Standardization (ISO) or the European Committee for Standardization (CEN). For official controls in the frame of the authorisation a fully ring-trial validated method is recommended [5].

For identification of the strain *Bacillus subtilis* C-3102 molecular methods such as 16S rDNA gene sequence analysis, ribotyping and polymerase chain reaction (PCR) have been used. The methods are suitable for the purpose of analysis [6]. Pulsed field gel electrophoresis (PFGE) is recommended for official controls in the frame of the authorisation.

Qualitative and quantitative composition of any impurities in the additive

The applicant analyses the feed additive for microbial contaminants such as *Enterobacteriaceae*, *Salmonella* species, *Staphylococcus aureus*, yeasts and moulds by using appropriate standard methods from the ISO. Heavy metals including lead, arsenic, mercury, cadmium and other impurities such dioxins, aflatoxins, zearalenon, ochratoxin, deoxynivalenol are further monitored. The applicant uses Official Community and ISO methods or commercially available kits [7]. The methods are considered suitable for the proposed application. Internationally recognised standardised 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 spores of *Bacillus subtilis* C-3102 (Calsporin®) in *premixtures* and *feedingstuffs* the same plate count method as for feed additives is proposed by the applicant [2]. The obtained performance characteristics of the method for both matrices are considered acceptable [8].

For official controls a fully ring-trial validated, peer reviewed and published method is recommended by the CRL [5]. This method for the enumeration of *B. subtilis* spores in premixtures and feedingstuffs applies similar principles as used in the method proposed by the applicant i.e. a heat treatment to reduce the vegetative background flora in combination

with a non-selective tryptone soya agar. The recommended method was validated using samples containing *B. subtilis* spores as active agents used as feed additives and is expected to perform adequately for the enumeration of spores of *B. subtilis* C-3102. In the method a heat treatment of the initial sample suspension is applied and tryptone soya agar is used. Methods performance characteristics for samples of premixtures (mean: 9.53 $\log_{10}$ c.f.u./g) and feedingstuffs (mean: 5.95 $\log_{10}$ c.f.u./g) were determined after logarithmic transformation of the measured colony forming units. A standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ and a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$ for premixtures were concluded. For feedingstuffs a s_r of 0.07 $\log_{10}$ and a s_R of 0.35 $\log_{10}$ were found. The limit of quantification (LOQ) for the method for feedingstuffs is around 1 x 10^7 c.f.u./kg sample which is well below the minimum anticipated target level of application.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant provided a method for the enumeration of the active agent *Bacillus subtilis* C-3102 in the additive, premixtures and feedingstuffs. Validation data for the proposed enumeration method are described in the dossier for Calsporin® and are in general considered appropriate. For official controls a fully ring-trial validated method for the enumeration of *B. subtilis* spores in premixtures and feedingstuffs which includes a heat treatment to inactivate any vegetative cells is recommended.

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 tryptone soya agar with pre-heat treatment of feed samples.

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 Calsporin® 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] ISO 4833:2003 Microbiology of food and animal feedingstuffs: Horizontal method for the enumeration of microorganisms – colony count technique at 30 ºC. (http://www.iso.org)

[3] Technical dossier. Annex II. 2.5.1.1. 'Calpis Co. Ltd. In-house analytical methods for *Bacillus subtilis* C-3102 in Calsporin®, premix, feed and faeces'

[4] Technical dossier. Annex II. 2.5.2.2. 'Method validation'

[5] Leuschner, R.G.K., Bew, J., & Cruz, A. (2003). Enumeration of probiotic bacilli spores in animal feed: Interlaboratory study. J. AOAC Int. 86, 568-575

[6] Technical dossier. Updated section II text (identity). 2.2.2. Biological origin

[7] Technical dossier. Updated section II text (identity). 2.5.1. General methods

[8] Technical dossier. Updated section II text (identity). 2.5.2. Description of the qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium.

8. ACKNOWLEDGEMENTS

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- National Reference Laboratory RO Praha, Praha, Czech Republic
- Agricultural Research Centre, Saku, Harjumaa, Estonia
- Laboratoire de Rennes, Rennes, France
- National Feed Laboratory, Lublin, Poland
- University of Ljubljana, Ljubljana, Slovenia