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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-2005-150
Name of Additive:	Calsporin®
Active Agent(s):	*Bacillus subtilis* C-3102
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the probiotic, Calsporin®, under the category zootechnical additives, group 4, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Calsporin® as a gut flora stabiliser for chickens for fattening (broilers), to improve growth and feed efficiency, is requested. Calsporin® consists of viable spores of *Bacillus subtilis* C-3102 (as active agent) and calcium carbonate (as carrier). The appearance of the additive is a dry and granular powder. The feed additive is intended to be mixed into compound feedingstuffs at a final concentration of 1 x 10^9 c.f.u./kg (c.f.u. = colony-forming units).

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 and uses trypticase soy agar (TSA) as medium.

The plate count method's performance characteristics for enumerating the active agent in the *feed additive* include a relative standard deviation of repeatability (RSD_r) and within-laboratory relative standard deviation reproducibility (RSD_R) values in the 8 – 10 % range. The limit of quantification (LOQ) for the method is 2.5 x10^3 c.f.u./kg. The performance characteristics are considered acceptable.

For the enumeration of the active agent in *premixtures and feedingstuffs* the method's performance characteristics include RSD_r and within-laboratory RSD_R values in the 10 – 12 % range. The LOQ of the method is 2.5 x 10^4 c.f.u./kg.

The performance characteristics obtained for *premixtures and feedingstuffs* are considered acceptable. However, for official control purposes a fully ring-trial validated, peer reviewed and published method for enumeration of bacilli spores including those of *B. subtilis*, is recommended [J.AOAC Int. 2003. 86, 568-575]. Methods performance characteristics for the AOAC method using samples of premixtures and feedingstuffs include a RSD_r and a between-laboratory RSD_R of around 1 % and 6 %, respectively.

For identification of the active agent a standardised, automated molecular method based on restriction analysis of DNA is proposed and appears suitable for the purpose of analysis.

Further testing or validation is not considered necessary, as appropriate methods for official control are available.

KEYWORDS

Calsporin®, *Bacillus subtilis* C-3102, zootechnical additive group 4, feed additive

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1. BACKGROUND

Calsporin® contains spores of *Bacillus subtilis* C-3102 as active agent, originally isolated from soil. The strain has been deposited in an international culture collection (DSMZ, Germany) under accession number DSM 15544. The intended use of the current application is to enhance the growth and feed efficiency of chickens (broilers), by mixing the feed additive into compound feedingstuffs in a final concentration of 1×10^9 c.f.u./kg.

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 for each application. For this particular dossier, the suitability of the control methods submitted in connection with Calsporin®, EFSA- Q – 2005 -150 was evaluated.

3. EVALUATION

The numbering system under this point refers to the report of the Scientific Committee on Animal Nutrition on the revision of the guidelines for the assessment of additives in animal nutrition, adopted on 22 October 1999 (Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms).

Description of the methods used for the determination of the criteria listed under item 2.5.*1*

Qualitative and quantitative composition of the additive

The method provided by the applicant to enumerate the active agent in the additive is a pour plate method to quantify spores of *Bacillus subtilis* C-3102. The method is based on ISO 4833 [1], 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 feed additive is weighed, suspended in a dilution buffer and blended at high speed. Thermal treatment follows. 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 plate count method's performance characteristics for enumerating the active agent in the feed additive include RSD_r and within-laboratory RSD_R values of 8 – 10 %. The LOQ of the method is 2.5×10^3 c.f.u./kg. These performance characteristics are considered acceptable.

For identification of the strain *Bacillus subtilis* C-3102 a standardised and automated molecular method based on restriction analysis of DNA is proposed and appears suitable for the purpose of analysis.
(*Cf*. the requirements listed in point 2.1.3 of the Guidelines.)

Antibiotic production and antibiotic resistance of the active agent

The possible production of antibiotics by *Bacillus subtilis* C-3102 was tested according to an original FAO (Food and Agriculture Organization of the United Nations) protocol using six different test strains. The methodology is considered appropriate.
(*Cf*. the requirements listed in point 2.2.6 of the Guidelines).

Stability of the additive

The applied analytical methods for determining stability of the active agent in Calsporin®, in premixtures and in feedingstuffs are similar to those described above and are considered suitable.

Results of genetic stability testing are provided and are considered appropriate.
(*Cf.* the requirements listed in point 2.3.1 of the Guidelines).

Description of qualitative and quantitative analytical methods for routine control of the active agent (*Bacillus subtilis* C-3102***) in premixtures and feedingstuffs***.

For the enumeration of spores of *Bacillus subtilis* C-3102 (Calsporin®) in premixtures and feedingstuffs the same plate count method [1] as for feed additives is proposed by the applicant.

Although the applicant does not seek authorisation for the use of Calsporin® in *premixtures*, method performance characteristics for this matrix are also provided, and include RSD_r and within-laboratory RSD_R values of 8 - 9 %. The LOQ is 2.5 x 10^3 c.f.u./kg. In *feedingstuffs* the method's performance characteristics include RSD_r and within-laboratory RSD_R values of 10 – 12 %. The LOQ for the method is 2.5 x 10^4 c.f.u./kg. The obtained performance characteristics of the method for both matrices are considered acceptable.

However, an exceptionally high inter-laboratory variation (30 – 40 %) was observed, probably because only two laboratories participated in the validation study and some modifications were applied. This raises some concerns about the transferability between laboratories of the method. Therefore, other well established methods for the enumeration of spore-containing probiotic feed additives are considered more suitable [2, 3].

For official control purposes a fully ring-trial validated, peer reviewed and published method is recommended by the CRL [3]. 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. Methods performance characteristics for this method using samples of premixture and feed include RSD_r and between-laboratory RSD_R of around 1 % and 6 %, respectively [3].
(*Cf.* the requirements listed in point 2.5.2 of the Guidelines).

CHECKLIST FOR THE METHODS PROPOSED BY THE APPLICANT

		Y	N	Comments
1.1	Is/Are the method(s) mentioned on Premixtures accompanied by information on:			
	- Sampling Method used		**X**	
	- Percentage Recovery	**X**		
	- Specificity	**X**		
	- Accuracy	**X**		
	- Precision	**X**		
	- Limit of quantification	**X**		
	- Validation procedure used	**X**		
1.2	Is/Are the method(s) mentioned on Feedingstuffs accompanied by information on:			
	- Sampling Method used		**X**	
	- Percentage Recovery	**X**		
	- Specificity	**X**		
	- Accuracy	**X**		
	- Precision	**X**		
	- Limit of quantification	**X**		
	- Validation procedure used	**X**		

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant provided a method, used in-house, for the enumeration of Calsporin®'s 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 lie within the ranges typically found in the analysis of probiotic micro-organisms. However, other well established and published methods for the enumeration of spore-containing probiotic feed additives are considered more suitable [2, 3]. For the official control a fully ring-trial validated method with method performance data for the enumeration of *B. subtilis* spores in premixtures and feedingstuffs which includes a heat treatment to inactivate any vegetative cells is recommended [3].

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of Calsporin® have been sent to the CRL. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] 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)

[2] Bestimmung von *Bacillus licheniformis* und *Bacillus subtilis*, Stand 2004. VDLUFA Methodenbuch III 28.2.2

[3] Leuschner, R.G.K., Bew, J., & Cruz, A. (2003). J. AOAC Int. 86, 568-575

RAPPORTEUR LABORATORY

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