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Community Reference Laboratory for Feed Additives

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

Dossier related to:	FAD-2009-0013 CRL/080037
Name of Additive:	CALSPORIN
Active Agent(s):	*Bacillus subtilis C-3102*
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive *Bacillus subtilis C-3102, DSM 15544* under the category 'zootechnical additive', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) 1831/2003. Specifically, authorisation is sought for the use of *Bacillus subtilis C-3102* for piglets (weaned). The feed additive consists of a minimum of $1x10^{10}$ colony forming units (CFU) per gram of viable spores of *Bacillus subtilis C-3102.* The feed additive is a pale granular powder intended to be mixed into complete *feedingstuffs* at a final concentration of $3x10^{8}$ CFU/kg.

For the enumeration of *Bacillus subtilis C-3102* in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes the CEN method - EN 15784:2009 – an internationally recognised spread plate method. This method was ring-trial validated using the *premixtures* and *feedingstuffs* samples containing *Bacillus subtilis* spores. The performance characteristics of the CEN method - reported after logarithmic transformation of measured values (CFU) - are:

- For the *premixtures*: - a standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ CFU/g and - a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$ CFU/g.
- For the *feedingstuffs*: - s_r = 0.07 $\log_{10}$ CFU/g; - s_R = 0.35 $\log_{10}$ CFU/g and - a limit of quantification (LOQ) of $2x10^{7}$ CFU/kg of *feedingstuffs*, well bellow the minimum content proposed by the applicant ($3x10^{8}$ CFU/kg).

Molecular methods were used by the applicant for identification of the active agent. The CRL recommends for official control pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification. The CEN Technical Committee 327 is currently occupied with the harmonization of a European Standard for this identification method.

Further testing or validation is not considered necessary.

KEYWORDS

Bacillus subtilis C-3102, zootechnical, gut flora stabiliser, piglets weaned.

1. BACKGROUND

Bacillus subtilis C-3102 DSM 15544 is a feed additive for which authorisation is sought under the category of 'zootechnical additives' functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003 [1]. *Bacillus subtilis C-3102* is provided in the form of a pale granular powder containing a minimum of $1x10^{10}$ CFU/g of viable spores [2]. The strain of *Bacillus subtilis C-3102* was originally derived from the soil in Japan in 1985 and deposited at a culture collection, in the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (DSMZ, Braunschweig, Germany) under accession number DSM 15544 [3]. The intended use of the current application is for piglets (weaned). The proposed dosage is of $3x10^{8}$ CFU/ kg of complete *feedingstuffs* [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 *Bacillus subtilis C-3102* dossier and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Qualitative and quantitative composition of the additive

For the identification of the strain *Bacillus subtilis C-3102* molecular methods such as pulsed Field Gel Electrophoresis (PFGE) and nucleotide sequencing were used. These methods are suitable for the purpose of analysis [4].

The CRL recommends for official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification. The CEN Technical Committee 327 is currently developing a European Standard for this identification method.

Qualitative and quantitative composition of any impurities in the additive

The applicant analysed the *feed additive* for microbial contaminants (such as coliform bacteria, *Escherichia coli*, *Salmonella* and moulds) by using appropriate EN ISO tests [5]. For undesirable substances (i.e. lead, arsenic, mercury, cadmium, aflatoxins, ochratoxin) internationally recognised standard methods are available at the respective Community Reference Laboratories.

Description of the analytical methods for the determination of active agent(s) in feed additive, premixtures and feedingstuffs

For the enumeration of *Bacillus subtilis C-3102* in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes the CEN method - EN 15784:2009 – an internationally recognised spread plate method [6]. The applicant used the same surface plate count method [7] applying a heat treatment of the initial sample suspension at 80°C for 10 min to reduce the vegetative background flora. Subsequently, appropriate dilutions were spread on non-selective tryptone soya agar and the plates were incubated at 37°C for 16 – 24 h.

This method was ring-trial validated using the *premixtures* and *feedingstuffs* samples containing *Bacillus subtilis* spores. The performance characteristics of the CEN method reported after logarithmic transformation of measured values (CFU) are [6]:

- For the *premixtures*: - a standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ CFU/g and - a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$ CFU/g.
- For the *feedingstuffs*: s_r = 0.07 $\log_{10}$ CFU/g; s_R = 0.35 $\log_{10}$ CFU/g and - a limit of quantification (LOQ) of $2x10^7$ CFU/kg of *feedingstuffs*, well bellow the minimum content proposed by the applicant ($3x10^8$ CFU/kg).

For the determination of the active agent *Bacillus subtilis* in the *product*, method performance characteristics are not available from interlaboratory studies. However, the CRL estimated the precision of the method from results of stability studies [8], obtaining for the repeatability standard deviation a value of 0.07 $\log_{10}$, which is in agreement with the results previously obtained for *premixture.*

Based on this acceptable performance characteristic CRL recommends the standard method EN 15784:2009 for the enumeration of the active *Bacillus subtilis C-3102* in the *feed additive, premixtures* and *feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the CRL recommends the CEN method (EN 15784:2009) for the enumeration of the active *Bacillus subtilis C-3102* in the *feed additive, premixtures* and *feedingstuffs*. Further testing or validation is not considered necessary.

For the analysis of the identity of the bacterial strain, *Bacillus subtilis C-3102DSM* 15544 the CRL recommends pulsed-field gel electrophoresis (PFGE) for official control.

Further testing or validation is not considered necessary.

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

- Enumeration: Spread plate method using tryptone soya agar in all target matrices (EN 15784:2009)
- 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 *Bacillus subtilis C-3102* for piglets and pigs for fattening 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] * Application, Ref: SANCO/D/2: Forw.Appl.1831/011-2009

[2] * Application, Proposal of Registry Entry EFSA Annex A

[3] * Technical dossier, Section 2.2 Characterisation of the active substance/agent

[4] * Technical dossier, Annex II.2.1.2g

[5] * Technical dossier, Section II-Identity, Table II.6 Analytical Methods

[6] EN 15784:2009: Animal feeding stuffs – Isolation and enumeration of presumptive Bacillus spp.

[7] * Technical dossier, Section II, Identity (2.6.2.5)

[8] * Technical dossier, Annex II.4.1.b

* Refers to Dossier no: FAD-2009-0013

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005.

8. ACKNOWLEDGEMENTS

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