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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-2008-0056  
CRL/080010

Name of Additive: *ANIMAVIT*

Active Agent(s): *Bacillus subtilis KBL001 CBS117162*

Rapporteur Laboratory: Community Reference Laboratory for  
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## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive *Bacillus subtilis* KBL 001 CBS 117162 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* KBL 001 CBS 117162 for piglets (weaned) and pigs for fattening. The feed additive consists of a minimum of  $4 \times 10^9$  colony forming units (CFU) per gram of viable spores of *Bacillus subtilis* KBL 001 CBS 117162. The feed additive is in the form of granulate and is intended to be mixed into complete *feedingstuffs* at a final concentration of  $2 \times 10^9$  to  $1 \times 10^{10}$  CFU/kg of *feedingstuffs*.

For the enumeration of *Bacillus subtilis* KBL 001 CBS 117162 in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes the draft CEN method - prEN 15784:2008 – 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 draft CEN method reported after logarithmic transformation of measured values (CFU) are:

- For the *premixtures*: (1) a standard deviation for repeatability ( $s_r$ ) of  $0.09 \log_{10}$  CFU/g and (2) a standard deviation for between-laboratory reproducibility ( $s_R$ ) of  $0.32 \log_{10}$  CFU/g.
- For the *feedingstuffs*: (1) a  $s_r = 0.07 \log_{10}$  CFU/g and (2) a  $s_R = 0.35 \log_{10}$  CFU/g and (3) a limit of quantification (LOQ) of  $2 \times 10^7$  CFU/kg, well below the minimum content proposed by the applicant ( $2 \times 10^9$  CFU/kg of *feedingstuffs*)

Molecular methods were used by the applicant for identification of the active agent. For official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification, is recommended.

Further testing or validation is not considered necessary.

## KEYWORDS

*Bacillus subtilis* KBL 001 CBS 117162, zootechnical, gut flora stabiliser, piglets and pigs for fattening.

## 1. BACKGROUND

*Bacillus subtilis* KBL 001 CBS 117162 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* KBL 001 CBS 117162 is provided in the form of granulates containing at least  $4 \times 10^9$  CFU/g spores of *Bacillus subtilis* as active agent [2]. The strain of *Bacillus subtilis* KBL 001 CBS 117162 was isolated in the vicinity of Novo mesto from the soil in 2004 and the strain was deposited in the Centraalbureau voor Schimmelcultures in the Netherlands [3]. The intended use of the current application is for piglets (weaned) and pigs for fattening. The proposed recommend dosage is of  $2 \times 10^9$  to  $1 \times 10^{10}$  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* KBL001 CBS 117162 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 identification of the strain *Bacillus subtilis* KBL 001 CBS 117162 molecular methods such as gene sequence analysis and nucleotide sequence 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.

##### *Qualitative and quantitative composition of any impurities in the additive*

The applicant analyses the *feed additive* for microbial contaminants such as coliform bacteria, *Escherichia coli*, *Salmonella* and moulds by using appropriate European Pharmacopoeia 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 [6].

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

For the enumeration of *Bacillus subtilis* KBL 001 CBS 117162 in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes the draft CEN method - prEN 15784:2008 – an internationally recognised spread plate method [7].

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

- For the *premixtures*: (1) a standard deviation for repeatability ( $s_r$ ) of  $0.09 \log_{10}$  CFU/g and (2) a standard deviation for between-laboratory reproducibility ( $s_R$ ) of  $0.32 \log_{10}$  CFU/g.
- For the *feedingstuffs*: (1)  $s_r = 0.07 \log_{10}$  CFU/g and (2)  $s_R = 0.35 \log_{10}$  CFU/g.

For the enumeration of spores of *Bacillus subtilis* KBL 001 CBS 117162 in *feed additive*, *premixtures* and *feedingstuffs* the applicant uses the same surface plate count method [7]. This method for the enumeration of *Bacillus subtilis* spores in the *premixtures* and *feedingstuffs* applies 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.

The applicant used the above mentioned method to analyse the various matrices containing *Bacillus subtilis* KBL 001 CBS 117162 spores and reported the following results: (a)  $11.6 \times 10^9$  to  $12.19 \times 10^9$  CFU/g for *feed additive* [8]; (b)  $2.4 \times 10^{11}$  to  $4.2 \times 10^{11}$  CFU/kg for *premixtures* [9] and (c)  $5.09 \times 10^9$  to  $7.34 \times 10^9$  CFU/kg for *feedingstuffs* [10]. These values confirmed the suitability of the suggested method of analysis.

The applicant investigated the repeatability in the *feed additive*, containing  $9.69 \times 10^9$  CFU/g and reported  $s_r = 0.66 \times 10^9$  CFU/g; the CRL derived after logarithmic transformation  $s_r = 0.03 \log_{10}$  CFU/g [11]. The CRL considers this repeatability standard deviation acceptable for the intended purpose.

The results obtained for *feed additive*, *premixtures* and *feedingstuffs* are considered acceptable; the draft CEN method is therefore recommended for official controls for the *feed additives*, *premixtures* and *feedingstuffs* in the frame of the authorisation.

#### 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the CRL recommends the draft CEN method (prEN 15784:2008) for the enumeration of the active agent *Bacillus subtilis* KBL001 117162 in the *feed additive*, *premixtures* and *feedingstuffs*. 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.
- 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* KBL 001 CBS 117162 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.1831/026-2008
- [2] \*Proposal of Register entry EFSA Annex A
- [3] \*Technical dossier, section II, Identity of the additive p. 6/365
- [4] \*Technical dossier, section II, 2.2 characterisation of the active substance(s)/ agent(s)  
p. 31/365
- [5] European Pharmacopoeia 5.0; Total viable aerobic count; 2.6.12 Microbiological examination of non-sterile products (total viable aerobic count) and 2.6.13 Microbiological examination of non-sterile products (test for specified micro-organisms)
- [6] COMMISSION REGULATION (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards Community reference laboratories, Official Journal of the European Union L 136
- [7] Draft CEN method (prEN 15784:2008): Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp. (version April 2008)

[8] \*Annex II.7\_2.4.1. Stability - Stability after opening/ in –use

[9] \*Annex II.8\_2.4.1. Stability - Stability in premixture

[10] \*Annex II.9\_2.4.1. Stability- Stability in feed

[11] \*Annex II. 19\_2.6.3 Method of analysis for the finished product-validation report-  
Concentration

\*Refers to Dossier no: FAD-2008-0056

## **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**

The following National Reference Laboratories Contributed to this report:

- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, CZ
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