EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory – Feed Additives Authorisation

CRL Feed Additives Authorisation

D08-FSQ/CVH/AMJ/DG(2006)D 8570

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-2005-237**
Name of Additive:	**035**
Active Agent(s):	***Bacillus subtilis* DSM 17299**
Rapporteur Laboratory:	**National Veterinary Laboratory, Vilnius, Lithuania**
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Date:	**27/03/2006**
Report approved by:	**Anne-Mette Jensen (CRL-FAA)**
Date:	**07/04/2006**

EXECUTIVE SUMMARY

In the current application authorisation is sought for the feed additive '035' under the category 'zootechnical additives 4(b) – gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use '035' for chickens for fattening. The feed additive '035' is a yellowish free-flowing powdery product containing at least 1.6 x 10^9 colony forming units (c.f.u.) per gram of the additive and it is proposed for use in premixtures and feedingstuffs obtaining a concentration ranging from 0.8-1.6 x 10^9 c.f.u. of active agent per kg complete feedingstuffs.

For the determination of the active agent (*Bacillus subtilis* DSM 17299) in the *feed additive*, *premixtures* and *feedingstuffs*, a surface plate count method is proposed. The method uses Tryptose Blood Agar (TBA) base with inclusion of 5% defibrinated calf or sheep blood. The limit of quantification (LOQ) of this method is 1.0 x 10^3 c.f.u./g (1.0 x 10^6 c.f.u./kg) which is well below the anticipated minimum target level of application in the animal feedingstuffs. This method is very similar to another method that was ring-trial validated [J.AOAC Int. 2003, 86, 568-575] and which uses Tryptose Soya Agar (TSA) as medium. The applicant provides data which confirm that the use of TBA as medium results in comparable performance as when using TSA, and that there are no statistically significant differences between the two methods. The method performance characteristics for the ring trial validated method include relative standard deviations for repeatability (RSD_r) and between-laboratory reproducibility (RSD_R) of around 1% and 6%, respectively. These performance characteristics are considered acceptable. Therefore, in the opinion of the CRL, the ring trial validated method is suitable for official control purposes.

The purity and correct identity of the *Bacillus subtilis* strain (DSM 17299) is examined by molecular DNA fingerprinting methodology, which is considered to be appropriate for the intended purpose. Pulsed field gel electrophoresis would be considered a suitable technique for official control purposes.

Standard and/or official methods are proposed by the applicant for the determination of impurities (heavy metals, microbiological agents) in the feed additive. The methods are considered suitable for the intended purposes.

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

KEYWORDS

'035', feed additive, *Bacillus subtilis* DSM 17299, zootechnical, gut flora stabiliser, chickens for fattening

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1. BACKGROUND

'035' is a feed additive for which authorisation is sought under the category 'zootechnical additives 4(b) – gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. The additive is composed of 0.5% (corresponds to 1.6 x 10^9 c.f.u./g of the product) of *Bacillus subtilis* DSM 17299 spores (as the active agent), 1% of sodium alumino silicate and 98.5% of whey permeate (as carrier). The strain *Bacillus subtilis* DSM 17299 is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (D.S.M.Z.) GmbH (German Collection of Microorganisms and Cell Cultures) in Braunschweig, Germany. The intended use of the current application is to enhance the growth and feed efficiency of chickens for fattening, by mixing the feed additive into compound feedingstuffs at a proposed final concentration ranging from 0.8-1.6 x 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, 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 control methods submitted in connection with '035', *cf*. EFSA-Q-2005-237, was evaluated.

3. EVALUATION

The numbering system under this point refers to that of Section II of the "Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms (2.5 Control methods)", in the following referred as "the Guidelines". The method protocols and corresponding validation data – if applicable – are given in the Section II of the dossier.

Description of the some of the methods listed under items 2.5.1. of the Guidelines

Qualitative and quantitative composition of the additive 035

For the determination of the active agent in the *feed additive*, a surface plate count method using a Tryptose Blood Agar (TBA) as medium for the enumeration and differentiation of spores capable of germinating is proposed. The results are reported as colony forming units per gram (g) of the additive. The method is considered suitable for the intended purpose. However, for the official control purposes CRL recommends a ring-trial validated method [1].

The purity and correct identity of the *Bacillus subtilis* strain (DSM 17299) is examined by molecular DNA fingerprinting methodology, which is considered to be appropriate for the intended purpose. Pulsed field gel electrophoresis would be considered a suitable technique for official control purposes.

Qualitative and quantitative composition of any impurities in the additive

Copies of the protocols for the proposed methods were provided by the applicant without any corresponding method performance data for the field of application. The methods for determination of *Salmonella* species and coliforms in the *feed additive* are the standard methods (Nos 71 and 44) of the Nordic Committee on Food Analysis (Nordisk Metodikkomité for Næringsmidler: NMKL). Analysis of *Salmonella* is performed by a method including a non-selective pre-enrichment (medium: buffered peptone water), followed by a selective enrichment phase (medium: Rappaport-Vassiliades broth). Afterwards the organisms are plated out on a selective indicative (xylose lysine desoxycholate) medium. Verification of both presumptive and typical colonies is done using triple sugar iron (TSI) agar. Coliforms are enumerated at 37^{o}C using violet red bile lactose (VRBL) agar. For the analysis of *Bacillus cereus,* a *B. cereus*-selective Mac Conkey agar is used as described in the Microbiological Manual (Merck, 1996). Yeasts

and moulds are enumerated using yeast glucose chloramphenicol (YGC) agar according to the standard method No 094 of the International Dairy Federation (corresponds to ISO 6611). The methods are considered suitable for the intended purposes. However, for official control purposes the corresponding standard methods (e.g. ISO) are recommended by the CRL.

For the analysis of heavy metals, the applicant proposes an atomic absorption spectrometry (AAS) method. The AAS methods used for determination of arsenic (As) and mercury (Hg) are described by the Association of the Analytical Community International (AOAC) whereas the standard AAS methods of the National Food Agency of Denmark are used for the analysis of cadmium (Cd) and lead (Pb). The methods are considered to be suitable for official control purposes.

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* DSM 17299 in *premixtures* and *feedingstuffs*, the applicant proposes the same surface plate count method as for the feed additive, just twenty grams of sample instead of ten grams (as for the additive) are weighed and suspended in sterile diluent. The suspension is treated in a stomacher for 4 minutes. One ml of the suspension is transferred to each of two tubes containing 9.0 ml of sterile diluent and mixed. After mixing, the tubes are treated at 80°C ± 1°C water bath for 10 minutes and then cooled in 10°C water. Decimal dilutions are prepared from the heat treated samples, spread on TBA agar plates and incubated at 37^{o}C for 17-20 hours. After incubation, TBA plates containing 10-80 colonies per plate are used for the final calculations. The limit of quantification (LOQ) of the method is 1.0 x 10^3 c.f.u./g (1.0 x 10^6 c.f.u./kg).

The proposed method is very similar to another ring-trial validated method [1] which uses Tryptose Soya Agar (TSA) as medium. The applicant provides data which confirm that the use of TBA as medium results in a comparable performance profile as when using TSA and that there are no statistically significant differences ($p=0.3301$; $p_{critical}=0.05$) between the two methods. The method performance characteristics for the method which uses TSA as medium include relative standard deviations for repeatability (RSD_r) and between-laboratory reproducibility (RSD_R) of around 1% and 6%, respectively [1]. Taking into consideration the acceptable performance characteristics and the target level

of application (about 10^9 c.f.u./kg of feedingstuff), in the opinion of the CRL the ring-trial validated method [1] is suitable for official control purposes.

CHECKLIST of the methods for determination of the active substance (*Bacillus subtilis* DSM 17299)

		Y	N	N/A	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 detection	**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 detection	**X**			
	- Limit of quantification	**X**			
	- Validation procedure used	**X**			

4. CONCLUSIONS AND RECOMMENDATIONS

Concerning the enumeration of active agent (*Bacillus subtilis* DSM 17299) in the *feed additive* '035', *premixtures* and *feedingstuffs*, a surface plate count method is proposed. The method uses Tryptose Blood Agar (TBA) base with inclusion of 5% defibrinated calf or sheep blood. The limit of quantification (LOQ) of this method is 1.0×10^3 c.f.u./g (1.0×10^6 c.f.u./kg). This method is very similar to a method that was ring-trial validated [J AOAC Int. 2003, 86, 568-575] and which uses Tryptose Soya Agar (TSA) as medium. The applicant provides data which confirm that the use of TBA as medium results in comparable performance as when using TSA, and that there are no statistically significant differences between the two methods. The method performance characteristics for the ring trial validated method include relative standard deviations for repeatability (RSD_r) and between-laboratory reproducibility (RSD_R) of around 1% and 6%, respectively [1]. These performance characteristics are considered acceptable. Therefore, in the opinion of the

CRL, the method which is ring-trial validated, is suitable for official control purposes. Taking into consideration the acceptable performance characteristics and the target level of application (about 10^9 c.f.u./kg of feedingstuff), in the opinion of the CRL the ring trial validated method [1] is suitable for official control purposes.

The purity and correct identity of the *Bacillus subtilis* strain (DSM 17299) is examined by molecular DNA fingerprinting methodology, which is considered to be appropriate for the intended purpose. Pulsed field gel electrophoresis would be considered a suitable technique for official control purposes.

Standard and/or official methods are proposed by the applicant for the determination of impurities (heavy metals, microbiological agents) in the feed additive. The methods are suitable for the intended purposes.

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of '035' 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. 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.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was National Veterinary Laboratory (NVL), Vilnius, Lithuania.