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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 the Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2009-0023 CRL/090008
Product Name:	Bioplus® 2B
Active Substance(s):	*Bacillus subtilis* DSM 5750 *Bacillus licheniformis* DSM 5749
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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Date:	11/10/2010
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Date:	29/10//2010

EXECUTIVE SUMMARY

In the current application authorisation is sought for BioPlus® 2B under the category 'zootechnical additives', functional group 'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003. The product consists of two active substances, namely *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749,* mixed at a 1:1 ratio. Specifically, authorisation is sought for the *feed additive* to be placed on the market in the powder form containing minimum concentration of $1.6x10^{10}$ CFU/g of each strain. The intended use of the current application is for chickens for fattening, piglets for fattening, sows, turkeys for fattening and calves. A total dosage including both strains of $1.3x10^{9}$ CFU/kg in complete *feedingstuffs* for all above mentioned species is proposed by applicant. Furthermore, the applicant suggested a minimum content of BioPlus® 2B ranging from 11 to 270 g (depending of the species) per 1000 animals in drinking *water*. For the enumeration of the sum of the two strains of the active agents in *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant proposes the ring trial validated a spread plate method (EN 15784) using tryptone soya agar. The performance characteristics of the CEN method reported after logarithmic transformation of measured CFU values are:

- a standard deviation for *repeatability* (s_r) ranging from 0.07 to 0.09 $\log_{10}$ CFU/g
- a standard deviation for *reproducibility* (s_R) ranging from of 0.32 to 0.35 $\log_{10}$ CFU/g, and
- a limit of detection (LOD) of 10^5 CFU/kg of *feedingstuffs.*

Based on these performance characteristics, the CRL recommends for official control the ring trial validated method EN 15784 for the determination of *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749* in *feed additive*, *premixtures*, *feedingstuffs* and *water.*

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.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

BioPlus® 2B*, Bacillus subtilis DSM 5750, Bacillus lichenforms* DSM 17236, zootechnical additives, chicken for fattening, piglets for fattening, sows, turkeys for fattening and calves, gut flora stabilisers.

1. BACKGROUND

In the current application authorisation is sought for BioPlus® 2B under the category 'zootechnical additives', functional group 'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003[1]. Authorisation is sought under Article 4(1) for new use in water and under Article 10(2). The *feed additive* was previously authorised under the following Commission Regulations (EC) No 1453/2004, 2148/2004, 600/2005, 2028/2006 and 202/2009.

The product consists of two active substances, namely *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749,* mixed at a 1:1 ratio. The strain *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749* were originally isolated from the soil and are deposited in Deutsche Sammlung von Mikro-organismem und Zelkulturen (DSMZ) [2].

Specifically, authorisation is sought for the *feed additive* to be placed on the market in the powder form containing a minimum concentration of $1.6x10^{10}$ CFU/g of each strain [3].

The intended use of the current application is for chickens for fattening, piglets for fattening, sows, turkeys for fattening and calves up to 3 months. . A total dosage including both strains of $1.3x10^{9}$ CFU/kg in complete *feedingstuffs* for all above mentioned species[3] is proposed by applicant. Furthermore, the applicant suggested a minimum content of BioPlus® 2B ranging from 11 to 270 g (depending of the species) per 1000 animals in drinking *water*.

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*, as last amended by Regulation (EC) No 885/2009, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application, or for each group of applications. For this particular dossier, the methods of analysis submitted in connection with the *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749* 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 and characterization of the strain *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749* the applicant used DNA fingerprinting and plasmid analyses. This method is suitable for the purpose of analysis [5]. However, the CRL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [6].

Qualitative and quantitative composition of any impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (such as Enterobacteria, *Escherichia coli*, Salmonella spp. and yeasts) by using appropriate EN ISO tests [7]. For undesirable substances (i.e. arsenic, cadmium, mercury, lead, selenium, copper, zinc, chrome, aflatoxins) internationally recognised standard methods are available at the respective Community Reference Laboratories, in accordance with Commission Regulation (EC) No 776/2006.

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

For the enumeration of the sum of the two strains of the active agents (i.e. *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749), in the *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant proposes a spread plate method based on the ring trial validated CEN method EN 15784 [8]. Twenty grams of additive (or 50g of *premixtures* or 50g of *feedingstuffs*) are suspended in 0.2% Na OH solution. Decimal dilutions are prepared in peptone salt diluent and subjected to a heat treatment at 80 ^{o}C for 10 minutes. Subsequently, appropriate dilutions are spread on tryptone soya agar and plates are incubated at 37 ^{o}C for 16-24 h. When using KOH, care must be taken when preparing the appropriate dilutions to avoid losses by spore precipitation.

The performance characteristics of the CEN method reported after logarithmic transformation of measured CFU values are:

- a standard deviation for *repeatability* (s_r) ranging from 0.07 to 0.09 $\log_{10}$ CFU/g
- a standard deviation for *reproducibility* (s_R) ranging from of 0.32 to 0.35 $\log_{10}$ CFU/g, and
- a limit of detection (LOD) of 10^5 CFU/kg of *feedingstuffs* [10].

Based on these acceptable performance characteristics, the CRL recommends for official control the ring trial validated CEN method EN 15784 for the determination of *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749*4 in the *feed additive, premixtures, feedingstuffs* and *water.*

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

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 agent *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749* in the *feed additive*.

For the analysis of the identity of the bacterial strain, *Bacillus subtilis DSM 5750* and *Bacillus licheniformis DSM 5749,* the CRL recommends Pulsed Field Gel Electrophoresis (PFGE) for official control.

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

- Enumeration: Spread plate method using tryptone soya agar (EN 15784)
- 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 DSM 5750 and Bacillus licheniformis DSM 5749,* for chickens for fattening, piglets for fattening, sows, turkeys for fattening and calves, 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.1831/0016-2009

[2] *Technical Dossier, Annex II_2.1.2a

[3] *Application, Annex A, Proposal for register entry

[4] *Technical Dossier, Section II 2.5.1 Conditions of use of the additive

[5] *Technical Dossier, Annex II 2.1.2b

[6] European Community Project SMT4-CT98-2235.*'Methods for the Official Control of Probiotics Used as Feed Additives*, Report 20873/1 EN (2002) ISBN 92-894-6250-7 (Vol. I)

[7] *Technical Dossier, Section II 2.1.3 Qualitative and quantitative composition

[8] *Technical Dossier, Section II 2.6 Methods of analysis and reference samples

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

[10] ISO 7218:2007: Microbiology of food and animal feeding stuffs- General requirements and guidance for microbiological examinations

*Refers to Dossier no: FAD-2009-0023

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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Austria
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Czech Republic
- Plantedirektoratet, Laboratorium for Foder og Gødning, Denmark
- Laboratoire de Rennes, Service Commun des Laboratoires, France