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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 Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

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| Dossier related to: | EFSA-Q-2007-166<br>FAD-2007-0028 |
| Name of Additive: | BioPlus 2B® for rabbits |
| Active Agent(s): | *Bacillus subtilis* DSM 5750 and<br>*Bacillus licheniformis* DSM 5749 |
| Rapporteur Laboratory: | Community Reference Laboratory for Feed Additives (CRL-FA) |
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| Report revised by:<br>Date: | Christoph von Holst (CRL-FA)<br>26/06/2008 |
| Report approved by:<br>Date: | Christoph von Holst (CRL-FA)<br>26/06/2008 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive BioPlus® 2B under the category 'zootechnical additives', functional group 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive are viable cells of two microorganism strains, *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749. The additive contains equivalent numbers of both strains and a minimum total concentration of $1.6 \times 10^9$ colony forming units (c.f.u.) per gram additive of each of the strains. Specifically, authorisation is sought to use BioPlus® 2B for rabbits for fattening. The conditions of use are proposed with a recommended total dosage of $1.28 \times 10^9$ c.f.u./kg complete feedingstuffs including both strains.

For the quantification of the active agents (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749) of BioPlus® 2B in the *feed additive*, *premixtures* and *feedingstuffs* the applicant uses tryptose blood agar. This is appropriate for the intended purpose.

For the quantitative determination of the colony forming units of the active agents for official controls in the *feed additive*, *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been ring-trial validated using premixtures and feedingstuff samples (J. AOAC Int. 2003. 86, 568-575). The method is similar to that used by the applicant using tryptone soya agar. The method's performance characteristics are standard deviations for repeatability ($s_r$) and reproducibility ($s_R$) of around 0.07 – 0.28 $\log_{10}$ and 0.32 – 0.58 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The limit of quantification (LOQ) for the method is around 2 to $3 \times 10^6$ c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

The identity of the bacterial strains, *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749, was analysed by pulsed-field gel electrophoresis (PFGE) which showed a sufficient degree of differentiation. PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

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

## KEYWORDS

BioPlus® 2B, feed additive, *Bacillus subtilis* DSM 5750, *Bacillus licheniformis* DSM 5749, zootechnical additive, rabbits

## 1. BACKGROUND

BioPlus® 2B is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. BioPlus® 2B is provided in form of a powder containing two strains in equal numbers, *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749, containing at least 1.6 x $10^9$ c.f.u. of each strain per gram in the feed additive [1, 2]. The two strains are deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (D.S.M.Z.) GmbH, Braunschweig, Germany. The intended use of the current application (EFSA-Q-2007-166) is for rabbits for fattening at a recommended dosage of 1.28 x $10^9$ c.f.u./kg complete feedingstuffs [1,2].

## 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 authorisations 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 BioPlus® 2B dossier (EFSA-Q-2007-166) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

## 3. EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5 Control methods), in the following referred to as 'the Guidelines'.

### *Description of some of the methods listed under item 2.5.1. of the Guidelines*

*Qualitative and quantitative composition of the additive*

The applicant analyses colony forming units of the strains *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749 in the additive by an enumeration method using tryptose blood agar [3, 4]. The proposed method is suitable for the intended purpose and was validated in a three laboratory ring-trial by the applicant [5]. For official controls in the frame of the authorisation another very similar method is recommended which was validated in a full collaborative study [6]. The two active agents can be distinguished and separately enumerated based on their different colony morphology on the agar plates.

The genetic identity of the strains was examined however method details were not provided [2]. Colonies of the two *Bacillus* strains can be subjected to identification methods following the enumeration. Pulsed-field gel electrophoresis (PFGE) is a generally recognised standard methodology for microbial identification and is suggested for official controls in the frame of the authorisation.

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

The applicant analyses the feed additive for microbial contaminants such as coliforms, *Salmonella* species, *Bacillus cereus*, yeasts and moulds. Various contaminants including heavy metals (Cd, Hg, Pb) and aflatoxin B1 are monitored [3, 4]. Method descriptions or protocols are not provided. Therefore, internationally recognised standardised methods such as from the International Organization for Standardization (ISO) and the European Committee for Standardisation (CEN) where available are recommended for official controls in line with current EU legislation.

***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 the two strains of the active agents, *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749, in *premixtures* and *feedingstuffs*, the applicant uses a diluent containing 0.2 % KOH for the initial suspension of the sample and heat inactivation of vegetative cells prior to an enumeration on tryptose blood agar [4, 8]. The method is suitable for the intended purpose. Care has to be taken to prepare appropriate dilutions from the initial suspension in particular when using KOH as the precipitate may contain higher concentrations of spores than the remaining supernatant. The applicant conducted a three-laboratory ring trial to investigate the effects that the use of tryptone soya agar (TSA) instead of tryptone blood agar (TBA) may have and concluded from their data there were no significant differences observed between the two agars [5].

For official controls the fully ring-trial validated method as cited above is recommended [6, 7]. This validated method includes a preparation of an initial sample suspension using 20 g for premixtures and 50 g for feedingstuff samples in 0.2 % KOH solution. Decimal dilutions are prepared in peptone salt diluent and subjected to a heat treatment at 80 ºC for 10 min. Subsequently, appropriate dilutions are spread on tryptone soya agar and plates are incubated at 37 °C for 24 – 48 h. The method's performance characteristics are standard deviations for repeatability ($s_r$) and reproducibility ($s_R$) of around 0.07 – 0.28 $\log_{10}$ and 0.32 – 0.58 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively.

Pulsed-field gel electrophoresis (PFGE) is considered as a suitable method for official controls in the frame of the authorisation to identify *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749.

## 4. CONCLUSIONS AND RECOMMENDATIONS

The applicant uses appropriate conventional methods to enumerate the active agent. A ring-trial validated method using tryptone soya agar and a heat treatment of the sample dilutions is recommended for official controls in the frame of the authorisation [6]. Pulsed-field gel electrophoresis (PFGE) is widely used by reference laboratories to identify bacterial isolates and it is therefore recommended as a suitable methodology in this context for official controls.

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

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

Enumeration: Spread plate method using tryptone soya agar with pre-heat treatment of feed samples

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 BioPlus® 2B for rabbits for fattening have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

## 6. REFERENCES

[1] Proposal of Register entry, Annex III
[2] Technical dossier, section II, 2.2.2. Biological origin
[3] Technical dossier, section II, 2.5. Control methods
[4] Technical dossier, section II, Annex 05, Q Analytical method QC-511
[5] Technical dossier, section II, Annex 19a, Global ring test of product 035

[6] 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] Technical dossier, section II, Annex 19b
[8] Technical dossier, section II, Annex 05, Assay Procedure 205-02

## 1. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium. The initial evaluation report was made available for commenting to the consortium of National Reference Laboratories.

## 8. ACKNOWLEDGEMENTS

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- Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleiβheim, Germany
- Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany
- Thüringer Landesanstalt für Landwirtschaft, Jena, Germany