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Community Reference Laboratory – Feed Additives Authorisation

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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

Dossier related to:	EFSA-Q-2005-275
Name of Additive:	BioPlus 2B (for turkeys for fattening)
Active Agent(s):	*Bacillus subtilis* DSM 5750, *Bacillus licheniformis* DSM 5749
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additive Authorisation, IRMM, Geel, Belgium (CRL-FAA)
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Date:	06/06/2006

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive BioPlus 2B under the category 'zootechnical additives', functional group 'others (microorganism)', according to Annex I of Regulation (EC) No 1831/2003. The active agents of the additive are *Bacillus licheniformis* DSM 5749 and *Bacillus subtilis* DSM 5750 at a ratio of 1:1. Specifically, authorisation is sought to use BioPlus 2B for turkeys for fattening. It is proposed for use in feedingstuffs for turkeys at a concentration of 1.3 x 10^9 colony forming units (c.f.u.) of active agents *Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749 per kilogram (kg) complete feedingstuffs.

For the quantification of the two active agents (*Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749) of BioPlus 2B in the *feed additive, premixtures and feedingstuffs*, a surface plate count method was proposed by the applicant. The method was shown to perform equivalently for compound feedingstuffs containing the permitted coccidiostat maduramicin ammonium. The method is very similar to a method which uses tryptone soya agar (TSA) and which was validated by an interlaboratory study. This method is characterised by method performance data including a relative within-laboratory repeatability standard deviation (RSD_r) and relative between-laboratory reproducibility standard deviation (RSD_R) of around 1% and 6%, respectively (J. AOAC Int. 2003. 86, 568-575). The limit of quantification (LOQ) for the method is 1.0 x 10^6 c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs. For official controls the validated method is recommended.

The molecular identity of the two bacilli strains was determined by using pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and would be considered suitable for official controls.

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

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

KEYWORDS

BioPlus 2B, feed additive, *Bacillus subtilis*, *Bacillus licheniformis*, zootechnical, turkeys

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1. BACKGROUND

BioPlus 2B is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'others (microorganism)', according to Annex I of Regulation (EC) No 1831/2003. It contains spores of the strain *Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749 as active agents. The concentration of the active agent in the feed additive is at least 1.6×10^9 c.f.u./g of *B. subtilis* and 1.6×10^9 c.f.u./g of *B. licheniformis* and would not exceed 3×10^9 c.f.u./g for each strain [1]. The strain 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 [2].

The intended use of the current application (EFSA-Q-2005-275) is for turkeys for fattening, by mixing the feed additive into compound feedingstuffs at a proposed final concentration of 1.3×10^9 c.f.u./kg of complete feedingstuff [1] which may contain the permitted coccidiostats: diclazuril, halofuginone, maduramicin ammonium, monensin sodium and robenidine [3]. In the applicant's dossier [3] maduramicin ammonium is specifically highlighted as a proposed modification.

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 for each application. For this particular dossier, the suitability of control methods submitted in connection with the BioPlus 2B dossier (EFSA-Q-2005-275) was 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'. The method protocols and corresponding validation data – if applicable – are given in the Section II and Annexes of the applicant's dossier.

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

Qualitative and quantitative composition of the additive

The additive BioPlus 2B is composed of 0.5 % *Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749 spores at a ratio of 1:1. The concentration of the active agents in colony forming units (c.f.u.) per gram in the additive is at least 1.6×10^9 and at the most 3×10^9 for each strain. The method for quantifying the active agent as proposed by the applicant represents a surface plate count method using tryptone agar. Viable spores of both bacilli species are present in equal amounts in the additive and can be differentiated on the agar plates. The method protocol for the enumeration and differentiation of spores capable of germinating is provided. The results are reported as colony forming units (c.f.u.) per gram (g) additive. The method is considered suitable for the intended purpose. For official controls a similar method which was validated by an interlaboratory study is recommended [4].

The genetic identity of the two bacilli strains is examined by molecular DNA fingerprinting methodology using pulsed field gel electrophoresis (PFGE), which is

considered appropriate for the intended purpose. PFGE is considered as a suitable method for official controls.

Qualitative and quantitative composition of any impurities in the additive

The applicant provides methods of analysis for determination of microbiological contaminants such as *Salmonella*, coliforms, *Escherichia coli, Bacillus cereus*, yeast and moulds in the feed additive. The methods are provided in form of complete protocols and are considered suitable for the intended purpose. For official controls the corresponding CEN and/or ISO methods would be recommended.

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

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 active agents *Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749 in premixtures and feedingstuffs, the applicant proposes the same surface plate count method as for the feed additive. The applicant demonstrated that the performance of the enumeration method was not affected by the presence of the permitted coccidiostat maduramicin ammonium in feedingstuffs [5]. The proposed method is very similar to a method that was validated by an interlaboratory study which uses tryptone soya agar (TSA) as medium [4] and which is recommended for official controls. The method performance characteristics for the method included a relative within-laboratory repeatability standard deviation (RSD_r) and a relative between-laboratory reproducibility standard deviation (RSD_R) of around 1 % and 6 %, respectively [4]. The procedure for enumerating colony forming bacilli spores in feedingstuffs involves a reconstitution of

test samples in diluent followed by homogenisation of this suspension. To prepare the initial suspension 50 g sample are homogenised in phosphate buffered saline. Decimal serial dilutions are prepared in peptone salt diluent from the initial suspension and appropriate dilutions are heated at 80 ºC for 10 min. An aliquot of 1 ml of appropriate decimal dilutions is spread on tryptone soya agar plates which are incubated at 37 ºC overnight for determination of the final colony count. The limit of quantification (LOQ) for the method is 1.0 x 10^6 c.f.u./kg sample. The lowest target level of application is well above that limit with expected concentrations of around 10^9 c.f.u./kg in feedingstuffs. This method is therefore considered suitable for official controls. For identification of the two bacilli strains, the applicant provided a genomic DNA fingerprinting method using pulsed field gel electrophoresis (PFGE). PFGE is commonly applied for bacterial typing and would be considered suitable for official controls.

4. CONCLUSIONS AND RECOMMENDATIONS

For the quantification of the active agents (*Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749) of BioPlus 2B in the feed additive, premixtures and feedingstuffs, a surface plate count method which was validated by an interlaboratory study [4] is recommended for official controls. For identification of the correct identity of the two bacilli strains pulsed field gel electrophoresis (PFGE) is considered as an appropriate method for official control.

Official and/or standard methods are proposed by the applicant for the determination of impurities (heavy metals, microbiological quality) in the feed additive. The methods are therefore considered suitable for official controls.

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

Recommendations to the Commission

The register entry for the product should contain in the fourth column (composition, chemical formula, description, analytical method) the following:

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

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 turkeys 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] EFSA, Annex III Proposal of Register entry

[2] Technical dossier, Monograph, 2. Specifications concerning the active agent, (2) biological origin

[3] Technical dossier, Supplementary Information January 2006, Table entitled 'Proposal for inclusion of BioPlus 2B in the annex of directive 70/524/EEC, as proposed by the applicant (proposed modifications in bold)'

[4] Leuschner, R.G.K., Bew, J. & Cruz, A. (2003). J. AOAC Int. 86, 568-575

[5] Technical dossier, 2. Compatibility with maduramicin ammonium (14-10-2005); Annex 02, Technical report (24 January 2003) entitled 'Compatibility with maduramicin and BioPlus 2B'

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive Authorisation (CRL-FAA), Geel, Belgium