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

**Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for**
***BioPlus 2B***
**(Dossier No. FAD-2004-001)**

**Executive Summary revised**

## 1. EXECUTIVE SUMMARY

The current application for BioPlus 2B seeks the following two extensions of its use in animal feeds:

- when used for piglets, pigs for fattening and sows, to use BioPlus 2B together with feed containing the acidic growth promoter potassium diformate (Formi$^{TM}$LHS); and
- when used for turkeys for fattening, to use BioPlus 2B together with feed containing the coccidiostat lasalocid A sodium 15g/100g (Avatec 15%).

Concerning the determination of the active substances of BioPlus 2B (*Bacillus subtilis* and *Bacillus licheniformis*) *per se* (individually and together), in premixtures and feedingstuffs, a surface plate count method was proposed by the applicant to determine viable counts of the preparation. The method is quantitative and uses tryptone blood agar base (TBA) with inclusion of 5 % defibrinated blood. This method is very similar to a previously validated method (by full ring trial, according to international guidelines). The previously validated method uses tryptone soya agar (TSA) as medium and is used for quantification of the additive *per se*, in premixtures and animal feed. The applicant compared the use of TBA as medium with the use of TSA. Method performance characteristics for the method using TSA as medium included relative standard deviations for repeatability ($RSD_r$) and reproducibility ($RSD_R$) of around 1% and 6 %, respectively, according to a peer reviewed scientific journal. Upon request, the applicant provided additional documentation concerning the method validation study of TBA. According to this, the method has a limit of quantification of 1000 colony forming units (c.f.u) per gram (g) sample.

Taking into account (1) the target level of application which ranges between $10^5$ – $10^9$ c.f.u./g of feed sample, (2) the systematic and well performed validation studies for

the quantitative microbiological enumeration methodology used in the BioPlus 2B dossier showing equivalence of TBA and TSA, (3) the latter of which has acceptable performance characteristics of RSD values, in the opinion of the CRL this method is fit for purpose and suitable for official control purposes.

In summary, and taking into account the compatibility studies where the number of colonies was similar with and without the presence of Formi LHS and Avatec 15 %, indicating that these substances do not affect the method performance, the CRL finds that the proposed methods fulfil the requirements to quantitatively determine the colony forming units present in BioPlus 2B in the proposed concentration range.

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

Date: 17 October 2005

## 1. EXECUTIVE SUMMARY (original)

BioPlus 2B is a feed additive consisting of *B. subtilis* and *B. licheniformis* belonging to zootechnical additives, category 4.

The current application for BioPlus 2B seeks the following two extensions of its use in animal feeds:

- when used for piglets, pigs for fattening and sows, to use BioPlus 2B together with feed containing the acidic growth promoter potassium diformate (Formi$^{TM}$LHS); and
- when used for turkeys for fattening, to use BioPlus 2B together with feed containing the coccidiostat lasalocid A sodium 15g/100g (Avatec 15%).

Concerning the determination of the active substances of BioPlus 2B (*Bacillus subtilis* and *Bacillus licheniformis*) *per se* (individually and together), in premixtures and feedingstuffs, a surface plate count method was proposed by the applicant to determine viable counts of the preparation. The method is quantitative and uses tryptone blood agar base (TBA) with inclusion of 5 % defibrinated blood. This method is very similar to a previously validated method (by full ring trial, according to international guidelines). The previously validated method uses tryptone soya agar (TSA) as medium and is used for quantification of the additive *per se*, in premixtures and animal feed. The applicant compared the use of TBA as medium with the use of TSA. Method performance characteristics for the method using TSA as medium included relative standard deviations for repeatability ($RSD_r$) and reproducibility ($RSD_R$) of around 1% and 6 %, respectively, according to a peer reviewed scientific journal. Upon request, the applicant provided additional documentation concerning the method validation study of TBA. According to this, the method has a limit of quantification of 1000 colony forming units (c.f.u) per gram (g) sample.

Taking into account (1) the target level of application which ranges between $10^5$ – $10^9$ c.f.u./g of feed sample, (2) the systematic and well performed validation studies for the quantitative microbiological enumeration methodology used in the BioPlus 2B dossier showing equivalence of TBA and TSA, (3) the latter of which has acceptable performance characteristics of RSD values, in the opinion of the CRL this method is fit for purpose and suitable for official control purposes.

In summary, and taking into account the compatibility studies where the number of colonies was similar with and without the presence of Formi LHS and Avatec 15 %, indicating that these substances do not affect the method performance, the CRL finds that the proposed methods fulfil the requirements to quantitatively determine the colony forming units present in BioPlus 2B in the proposed concentration range.

Information on the composition of all ingredients other than the active agents, including impurities, physical state of the product, toxins and virulence factors, antibiotic production and resistance, stability of the additive, other physico-chemical or biological properties and incompatibilities with other feed ingredients, with the exception of Formi LHS and Avatec 15 %, has not been submitted for the purpose of this extension of the authorisation for the dossier.

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

Date: 24 June 2005

## 2. KEYWORDS

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

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## 4. BACKGROUND

BioPlus 2B is

- approved for piglets, pigs for fattening and sows (EU no. E1700) and
- provisionally approved for turkeys for fattening (EU No. 20). An application to make the provisional approval of BioPlus 2B for turkeys for fattening permanent was submitted to the Commission on 3 August 2004. The provisional approval of BioPlus 2B for turkeys for fattening is for use in compound feed containing the permitted coccidiostats: diclazuril, halofuginone, monensin sodium and robenidine.

The current extension for BioPlus 2B seeks authorisation for use as additive in feed products containing the approved growth promoter potassium diformate (Formi LHS) or the coccidiostat lasalocid sodium (Avatec 15 %).

## 5. 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 required to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the control methods submitted in connection with FAD – 04 – 001 were evaluated.

## 6. EVALUATION

The numbering system under this point refers to the report of the Scientific committee on Animal Nutrition on the revision of the guidelines for the assessment of additives in animal nutrition, adopted on 22 October 1999 (Guidelines for the assessment of additives in feedingstuffs Part II: Enzymes and Microorganisms). For further details regarding the structure of the dossier please see chapter 8 of this document.

Section 2.5- Control Methods

*2.5.1. Description of the methods used for the determination of the criteria listed under items 2.1.3, 2.1.4,2.1.5, 2.2.5, 2.2.6, 2.3.1, 2.3.2, and 2.3.3*

*Qualitative and quantitative composition*

The active component is a mixture of microorganisms, *Bacillus subtilis* (DSM 5750) and *Bacillus licheniformis* (DSM 5749). The numbers of viable microorganisms is given in colony forming units (c.f.u.) per unit weight in part 2 of the information provided by the applicant.

Information on other ingredients of the additive and the related methods was not provided for the purpose of this extension.

*Qualitative and quantitative composition of any impurities*

The protocol used for the routine screening of production batches for contaminants was not provided for the purpose of this extension however it is mentioned by the applicant in the 'Global ring test of BioPlus 2B (study 3791)' that the purity and correct identity of the *B. subtilis* and *B. licheniformis* strains were examined by molecular DNA fingerprinting methodology which is appropriate.

*Physical state of each form of the product*

Methods of how to reveal data on particle size, dusting potential and the use of processes such as encapsulation which affect the physical properties have not been provided for the purpose of this extension. Part 6 of the information provided by the applicant indicates that information on the topic has been provided in prior dossiers.

*Toxins and virulence factors*

Methods to test for evidence that toxins and virulence factors are absent from the two strains used in the active agents were not provided for the purpose of this extension. Part 6 of the information provided by the applicant indicates that information on the topic has been provided in prior dossiers.

*Antibiotic production and antibiotic resistance*

Methods to test the active agents for the capability to produce antimicrobial substances relevant to the use of antibiotics in humans or animals have not been provided for the purpose of this extension. Part 6 of the information provided by the applicant indicates that information on the topic has been provided in prior dossiers.

*Stability of the additive*

The method to test for the stability of the additive has not been provided for the purpose of this extension. The methods used for quantification of the additive (2.5.2) could be considered appropriate for the purpose. Part 6 of the information provided by the applicant indicates that information on the topic has been provided in prior dossiers.

*Other physico-chemical or biological properties*

Methods have not been provided for the purpose of this extension. Part 6 of the information provided by the applicant indicates that information on the topic has been provided in prior dossiers.

*Incompatibilities with other feed ingredients*

The applicant tested compatibility with Formi LHS and Avatec 15 % (Parts 4 and 5 of the dossier). The method (2.5.2) used for enumeration of the active agents was appropriate. Part 6 of the information provided by the applicant indicates that information on the topic has been provided in prior dossiers.

*2.5.2 – Description of qualitative and quantitative methods for routine control of the active agents in premixtures and feedingstuffs*

A quantitative surface plating method is used to quantify bacterial spores of the two species (*Bacillus subtilis* and *Bacillus licheniformis*) separately which are present in the active feed additive compound. Bacterial spores capable of germinating are enumerated and differentiated. Vegetative cells are not taken into account. The results are reported as colony forming units (c.f.u.) per gram (g) sample. The samples have to be representative for the product examined. The samples are initially diluted and homogenised. The homogenate is heat treated at 80 ºC for 10 minutes to inactivate any vegetative cells. Decimal dilutions are prepared from the heat treated homogenates and spread on agar plates containing tryptone blood agar base (TBA) with inclusion of 5 % defibrinated blood. The apparatus, glassware, diluents and media used are specified in the method protocol supplied by the applicant entitled 'Q-Analytical method: Enumeration of germinating spores – TBA Agar'. In addition the applicant provided the 'Test protocol for the global ring analysis for the enumeration of BioPlus 2B' where the applicant's method is compared with a very similar method validated by ring trial. A separate enumeration of *B. subtilis* and *B. licheniformis* is suggested in the document.

The present evaluation report is specifically based on the information included in the method protocol ('Q-Analytical method for enumeration of germinating spores – TBA agar') and the validation studies that were submitted for evaluation to the CRL.

Information relevant to validation of the method proposed by the company is submitted in the form of a report entitled 'Global ring test of BioPlus 2B (study 3791)'. The study reveals that the agar TBA and tryptone soya agar (TSA), an agar suggested in a Standard method (BSI (1986) BS4285-3.3. British Standards Institution, London UK) and following and international collaborative study (4) performed equivalently. Statistical data did not reveal any significant difference between the two agars ($p=0.867$; $p_{critical}=0.05$). The method testing was not carried out in the presence of Avatec and Formi. However two compatibility studies (BioPlus 2B with Avatec 15 % and BioPlus 2B with Formi LHS) were carried out using the method. The results showed that the method for enumeration of the active substance in BioPlus 2B performed appropriately in both studies.

The purity and correct identity of the *B. subtilis* and *B. licheniformis* strains used was examined by molecular DNA fingerprinting methodology which is appropriate.

The methods described under 2.5.2 may be used as routine method. The method using TSA is recommended for official control purposes as it was formally validated following international guidelines (2). The validation was carried out using samples which represented main categories of animal feed and ensured an appropriate range of concentration levels of the active substances. Feed samples and premixtures containing viable counts of the active substances of $10^9$ c.f.u./g and $10^5$ c.f.u./g, respectively were used in the validation study. Method precision data were established and published in the Scientific Community (4). Relative standard deviations of repeatability ($RSD_r$) and reproducibility ($RSD_R$) of 1 % and 6 % were determined. The performance characteristics are within the range of other studies reported in the scientific literature (3, 5).

The quantification limit of the enumeration method provided by the applicant in the 'Test protocol for global ring analysis' is $10^3$ c.f.u./g is well below concentrations in feed of about $10^{5-6}$ c.f.u./g or premixtures of around $10^{8-9}$ c.f.u./g.

**CHECK LIST – Part I**

| | | | Y | N | N/A | Comments |
|---|---|---|---|---|---|---|
| 1. | A. | Description of the Qualitative and Quantitative analytical method/s for routine control of the active substance in | X | | | Quantitative |
| | | - Premixtures | X | | | |
| | | - Feedingstuffs | X | | | |
| | B. | The method has been validated[1]: | | | | |
| | | - In a ring test involving at least four laboratories | X | | | |
| | | - In-house following harmonised guideline | X | | | |
| | C. | The validation study contains the following parameters[2]: | | | | |
| | | - Applicability | X | | | |
| | | - Selectivity | X | | | |
| | | - Calibration | X | | | |
| | | - Accuracy | X | | | |
| | | - Precision | X | | | |
| | | - Range | X | | | |
| | | - Limit of detection | | | X | |
| | | - Limit of quantification | X | | | |
| | | - Sensitivity | | | X | |
| | | - Robustness | X | | | |
| | | - Practicability | X | | | |
| | D. | Is there evidence available that the characteristics listed above have been assessed? | X | | | |
| 2. | Description of the Qualitative and Quantitative analytical method/s to determine the marker residue(s) of the active substance: | | | | X | |
| | - In target tissue/s | | | | | |
| | - In animal products | | | | | |

N/A: Not applicable

[1, 2] See references 2 and 4 in section 9

**CHECK LIST – Part II**

| | | Y | N | N/A | Comments |
|---|---|---|---|---|---|
| 1.1 | Is/Are the method(s) mentioned in Part I (1.- A. Premixtures) accompanied by information on: | | | | |
| | - Sampling Method used | | X | | |
| | - Percentage Recovery | | | X | |
| | - Specificity | X | | | |
| | - Accuracy | X | | | |
| | - Precision | X | | | |
| | - Limits of detection | | | X | |
| | - Limits of quantification | X | | | |
| | - Validation procedure used | X | | | |
| 1.2 | Is/Are the method(s) mentioned in Part I (1.- A. Animal feed) accompanied by information on: | | | | |
| | - Sampling Method used | | X | | |
| | - Percentage Recovery | | | X | |
| | - Specificity | X | | | |
| | - Accuracy | X | | | |
| | - Precision | X | | | |
| | - Limits of detection | | | X | |
| | - Limits of quantification | X | | | |
| | - Validation procedure used | X | | | |
| 2.1 | Is/Are the method(s) mentioned in Part I (2. – Target tissues) accompanied by information on: | | | X | |
| | - Sampling Method used | | | | |
| | - Percentage Recovery | | | | |
| | - Specificity | | | | |
| | - Accuracy | | | | |
| | - Precision | | | | |
| | - Limits of detection | | | | |
| | - Limits of quantification | | | | |
| | - Validation procedure used | | | | |
| 2.2 | Is/Are the method(s) mentioned in Part I (2. – Animal products) accompanied by information on: | | | X | |
| | - Sampling Method used | | | | |
| | - Percentage Recovery | | | | |
| | - Specificity | | | | |
| | - Accuracy | | | | |
| | - Precision | | | | |
| | - Limits of detection | | | | |
| | - Limits of quantification | | | | |
| | - Validation procedure used | | | | |
| 3. | If the method(s) has/have been devised, consideration has been given to the fact that their limits of quantification must be below the MRLs. | | | X | |

N/A: Not applicable

## 7. CONCLUSIONS AND RECOMMENDATIONS

Overall, systematic and well performed validation studies were conducted for the quantitative microbiological enumeration methodology used in the BioPlus 2B dossier. The applicant provided additional documentation concerning the method validation study upon request.

Performance characteristics for the method described in the applicant's dossier for BioPlus 2B are within the ranges of method precision data described in other scientifically peer reviewed studies. The limit of quantification of the method for enumeration of the two bacilli species is considered sensitive enough to be suitable for official control purposes.

In summary, and taking into account the compatibility studies where the number of colonies was similar with and without the presence of Formi LHS and Avatec 15 %, indicating that these substances do not affect the method performance, the CRL finds that the proposed methods fulfil the requirements to quantitatively determine the colony forming units present in BioPlus 2B in the proposed concentration range.

Information on the composition of all ingredients other than the active agents, including impurities, physical state of the product, toxins and virulence factors, antibiotic production and resistance, stability of the additive, other physico-chemical or biological properties and incompatibilities with other feed ingredients, with the exception of Formi LHS and Avatec 15 %, has not been submitted for the purpose of this extension of the authorisation for the dossier.

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

## 8. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

The general information is provided by the applicant in several parts:

- Part 1. Introduction to dossier
- Part 2. Proposals for inclusion into list of approval
- Part 3. Summary of content of dossier
- Part 4. BioPlus 2B compatibility with the permitted acidic growth promoter potassium diformate (Formi LHS) in feedingstuff for pigs

- Part 5. BioPlus 2B compatibility with the permitted coccidiostat lasalocid A sodium (Avatec 15%) in feedingstuff for turkeys
- Part 6. Overview – BioPlus 2B dossier submissions

In addition information on compatibility and efficacy of using BioPlus 2 B is found in:

- Annex 7.A1. No.1: Technical Report no. TR03132 'Compatibility of BioPlus 2B with Formi (K-diformiate)'
- Annex 8a.A2. No.2: Efficacy report, Poland 2004: 'Efficacy of using BioPlus 2B in feeding of turkeys' Project no. 2003626, part 1
- Annex 8b.A2. No.2: Efficacy report, Poland 2004: 'efficacy of using BioPlus 2B in feeding of turkeys' Project no. 2003026, part 2
- Annex 8c.A2. No.2: Efficacy report, Poland 2004: 'Efficacy of using BioPlus 2B in feeding turkeys' Project no. 2003026, part 3

Further information provided by applicant upon request:

- A method protocol (Q-Analytical method) entitled 'Enumeration of germinating spores – TBA agar'
- Test protocol for global ring analysis
- Report: Global ring test of BioPlus 2B, study 3791

Product samples have been made available to the CRL on 24.01.2005.

The dossier has been made available to the CRL by EFSA.

## 9. REFERENCES

(1) BSI (1986) BS 4285-3.3., British Standards Institution, London, UK

(2) Horwitz, W. (1995) Pure Appl. Chem. 76, 331-343

(3) Leuschner, R.G.K., Bew, J., Domig, K., & Kneifel, W. (2002) J. Appl. Microbiol. 93, 781-786

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

(5) Schulten, S.M., in't Veld, P.H., Nagelkerke, N.J.D., Scotter, S., de Buyser, M.L., Rollier, P., & Lahallec, C. (2000) Int. J. Food Microbiol. 57, 53-61

## 10. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive Authorisation, IRMM, Geel, Belgium.

Responsible person for the evaluation is Christoph von Holst.

## 11. APPENDIX

Not applicable.