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Institute for Reference Materials and Measurements
**Community Reference Laboratory for Feed Additives**

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# CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

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| **Dossier related to:** | FAD-2009-0034<br>CRL/090018 |
| Name of Product: | *Biomin® C3* |
| Active Agent(s): | *Bifidobacterium animalis spp. animalis* **DSM 16284**<br>*Enterococcus faecium* **DSM 21913**<br>*Lactobacilus salivarius spp. salivaarius* **DSM 16351** |
| Rapporteur Laboratory: | Community Reference Laboratory for Feed Additives (CRL-FA)<br>Geel, Belgium |
| Report prepared by: | Carlos Magno Pinto (CRL-FA) |
| Report checked by:<br>Date: | Piotr Robouch (CRL-FA)<br>19/04/2010 |
| Report approved by:<br>Date: | Christoph von Holst (CRL-FA)<br>23/04/2010 |

## EXECUTIVE SUMMARY

In the current application an authorisation is sought for the microbial product Biomin® C3 under the category 'zootechnical additives', functional group 'gut flora stabilizers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorization is sought for Biomin® C3, which is composed of three microorganism strains, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Enterococcus faecium* DSM 21913 and *Lactobacillus salivarius* ssp. *salivarius* DSM 16351. The feed additive is intended to be mixed at a total viable count ranging from $1x10^8$ CFU/kg to $1x10^9$ CFU/kg of *feedingstuffs* for chickens for fattening.

For the enumeration of *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Enterococcus faecium* DSM 21913 and *Lactobacillus salivarius* ssp. *salivarius* DSM 16351 strains in *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed a ring-trial validated spread plate methods developed by Leuschner et al. All three methods are currently available as CEN method: EN 15785; EN 15788; EN 15787. The performance characteristics of the CEN methods, for plate enumeration of the three active substance in *premixtures* and *feedingstuffs*, after $\log_{10}$ conversion, are:

- a standard deviation for *repeatability* ($S_r$) ranging from 0.12 to 0.24 $\log_{10}$ CFU/g,

- a standard deviation for *reproducibility* ($S_R$) ranging from 0.23 to 0.41 $\log_{10}$ CFU/g; and

- a limit of quantification (LOQ) of $10^5$ CFU/g.

Based on these acceptable performance characteristics, the CRL recommends for official control the following CEN methods, EN 15785; EN 15788; EN 15787, for the enumeration of Bifidobacterium spp., Enterococcus spp. and Lactobacillus spp., in *feed additive, premixtures* and *feedingstuffs.*

Molecular methods were used by the applicant for strain identification. The CRL recommends for official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

Further testing or validation is not considered necessary.

**KEYWORDS**

Biomin® C3, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Enterococcus faecium* DSM 21913 , *Lactobacillus salivarius* ssp. *salivarius* DSM 16351, zootechnical additives, gut flora stabilisers, chickens for fattening.

## 1. BACKGROUND

Biomin® C3 is a feed additive for which authorisation (Article 4(1)) is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003 [1]. Biomin® C3 is a powder containing $3x10^9$ CFU/g *Bifidobacterium animalis* ssp. *animalis* DSM 16284, $6x10^9$ CFU/g *Enterococcus faecium* DSM 21913 and $1x10^9$ CFU/g *Lactobacillus salivarius* ssp. *salivarius* DSM 16351 [2].

The three strains are deposited at the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (DSMZ), Braunschweig, Germany. The feed additive is intended to be mixed at a total viable count ranging from $1x10^8$ CFU/kg to $1x10^9$ CFU/kg of *feedingstuffs* for chickens for fattening [3] and calculated as the sum of the measured CFUs from the three microorganism strains of interest.

## 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*, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application. For this dossier, the methods of analysis submitted in connection with the Biomin® C3 dossier 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 characterisation of the strains *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Enterococcus faecium* DSM 21913 and *Lactobacillus salivarius* ssp. *salivarius* DSM 16351 the applicant used Random Amplified Polymorphic DNA (RAPD) analyses [4]. This method is suitable for the purpose of analysis. However, the CRL recommends for official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [13].

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

The applicant analysed the *feed additive* for microbial contaminants (such as *Escherichia coli, Salmonella spp.*) by using appropriate EN ISO tests [5]. For undesirable substances (i.e. lead, arsenic, mercury, cadmium, aflatoxins, ochratoxin) 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 in feed additive, premixture and feedingstuffs.***

For the enumeration of total viable counts of strains (*Bifidobacterium* ssp, + *Enterococcus* spp. + *Lactobacillus* ssp.) in *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes three ring-trial validated spread plate methods developed by Leuschner et al. [6-8]. An initial suspension of 50 g of *feedingstuffs* or 20 g for *premixtures* is prepared in phosphate buffer solution. The diluted solution is then transferred to corresponding agar plates: Bile esculin azide agar (BEAA) for enterococci, and Man, Rogosa, Sharpe Agar (MRSA) supplemented with TTC for bifidobacteria and lactobacilli to allow differentiation of colonies by different coloration after anaerobic incubation. The plates are incubated at 37 °C for Enterococcus spp. aerobically for 24 h and for Bifidobacterium ssp. + Lactobacillus ssp. anaerobically for 36 h to 72h . The various strains are then counted individually to determine the total viable count in the samples.

The three methods developed by Leuschner et al., are currently available as CEN methods (EN 15785; EN 15788; EN 15787). The performance characteristics of these methods are summarized in the following table.

| Microorganism | Bifidobacterium | | Enterococus | | Lactobacillus | |
|---|---|---|---|---|---|---|
| CEN method | CEN 15785 [9] | | CEN 15788 [10] | | CEN 15787 [11] | |
| Agar | MRSA | | BEAA | | MRSA | |
| Matrix | PM | FS | PM | FS | PM | FS |
| Concentration | $6.6x10^6$ CFU/g | $1.3x10^5$ CFU/g | $4x10^7$ CFU/g | $4x10^5$ CFU/g | $1x10^8$ CFU/g | $2x10^7$ CFU/g |
| $S_r$ ($log_{10}$ CFU/g) | 0.13 | 0.15 | 0.12 | 0.20 | 0.24 | 0.24 |
| $S_R$ ($log_{10}$ CFU/g) | 0.25 | 0.24 | 0.23 | 0.41 | 0.29 | 0.38 |
| LOQ [12] | $10^5$ CFU/kg | | $10^5$ CFU/kg | | $10^5$ CFU/kg | |

$S_r$ and $S_R$ = standard deviation for repeatability and reproducibility;
PM = premixtures, FS = feedingstuffs

Based on these acceptable performance characteristics, the CRL recommends for official control the following CEN methods, EN 15785; EN 15785; EN 15788, for the enumeration of Bifidobacterium spp., Enterococcus spp. and for Lactobacillus spp., in *feed additive, premixtures* and *feedingstuffs.*

## 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorization, the CRL recommends for official control the following CEN methods: EN 15785; EN 15788; EN 15787, for the enumeration of Bifidobacterium spp., Enterococcus spp. and for Lactobacillus spp. in *feed additive, premixtures* and *feedingstuffs.*

The CRL recommends for official controls pulsed field gel electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

Further testing or validation is not considered necessary.

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

- Enumeration in *feed additive, premixtures* and *feedingstuffs*:

    - for Bifidobacterium spp. - Spread plate method in MRSA (EN 15785)

    - for Enterococcus spp. - Spread plate method in BEAA(EN 15788)

    - for Lactobacillus spp. - Spread plate method in MRSA (EN 15787)

- Identification: Random Amplified Polymorphic DNA analysis (RAPD).

## 4. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive Biomin® C3 have been sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

## 5. REFERENCES

[1] *Application/Ref: SANCO/D/2: Forw.Appl.1831/032-2009

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

[3] *Technical dossier, Section 2.1.3 Qualitative and quantitative composition

[4] *Technical dossier Annex II_01; Characterisation Report II_01

[5] *Technical dossier 2.1.4. Purity

[6] *Technical dossier, Section 2, Annex -Leuschner_probiotic bifidobacteria in animal feed Leuschner R.G.K., Bew J., Simpson P., Ross P.R., Stanton C. 2003. A collaborative study for the enumeration of probiotic bifidobacteria in animal feed. Int. J. Food Microbiol. 83, 161-170

[7] *Technical dossier, Section 2, Annex –Leuschner_probiotic enterococci in animal feed. Leuschner R.G.K., Bew J.,Coeuret V., Vernoux J.P., Gueguen, M. 2003. A collaborative study of a method for the enumeration of probiotic lactobacilli in animal feed. Food Microbiol. 20, 57-66

[8] *Technical dossier, Section 2, Annex -Leuschner_probiotic lactobacilli in animal feed. Leuschner R.G.K., Bew J., Domig K.J., Kneifel W. 2002. A collaborative study of a method for the enumeration of probiotic enterococci in animal feed. J. Appl. Microbiol. 93, 781-786

[9] EN 15785:2009 "Animal feeding stuffs- Isolation and enumeration of Bifidobacterium spp."

[10] EN 15788:2009 "Animal feeding stuffs- Isolation and enumeration of Enterococcus (*E.faceium*) spp."

[11] EN 15787:2009 "Animal feeding stuffs- Isolation and enumeration of Lactobacillus spp."

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

[13] European Community Project SMT4-CT98-2235.'Methods for the Official Control of Probiotics Used as Feed Additives, Volume 1. 2002. Report 20873-1. Office for official Publications of the European Communities. ISBN 92-894-6250-7 (Vol. I)

* Refers to Dossier no: FAD-2009-0034

## 6. 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.

## 7. ACKNOWLEDGEMENTS

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