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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-2006-169 FAD-2006-0028
Name of Additive:	Biomin® C5 for chickens for fattening
Active Agent(s):	***Pediococcus acidilactici*** **DSM 16210,** **Enterococcus faecium DSM 16211,** ***Bifidobacterium animalis*** **ssp.** ***animalis*** **DSM 16284,** ***Lactobacillus reuteri*** **DSM 16350,** ***Lactobacillus salivarius*** **ssp.** ***salivarius*** **DSM 16351**
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EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Biomin® C5 under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agents in the additive are viable cells of five microorganism strains, *Pediococcus acidilactici* DSM 16210, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Lactobacillus reuteri* DSM 16350, *Lactobacillus salivarius* ssp. *salivarius* DSM 16351. The additive is a whitish powder containing 5 x 10^{10} colony forming units (cfu)/g microorganisms, whereby each strain is represented in a different concentration: *P. acidilactici* 1.3 x 10^{10} cfu/g, *E. faecium* 3.0 x 10^{10} cfu/g, *B. animalis* 5.0 x 10^{9} cfu/g, *L. reuteri* 1.0 x 10^{9} cfu/g, *L. salivarius* 1.0 x 10^{9} cfu/g. Specifically, authorisation is sought to add Biomin® C5 to drinking water for chickens for fattening until the slaughter age of about 42 days. The conditions of use are proposed with a recommended dosage of the additive resulting in 1.0 x 10^{9} to 1.0 x 10^{10} c.f.u. per liter drinking water.

For the quantification of the total viable counts in the *feed additive* Biomin® C5 and in its 'drinking water suspension', the applicant provides a validated pour plate method which is appropriate for the purpose. Validated plate count methods are further provided for an enumeration of each strain prior to addition to the final product which are appropriate.

For the quantification of the c.f.u. of each strain in the feed additive and in its 'drinking water suspensions' for official controls in the frame of the authorisation ring-trial validated methods are recommended, whereby it may be difficult to differentiate c.f.u. of the two *Lactobacillus* strains. (J. AOAC Int. 2003. 86, 4, 791-801, J. Appl. Microbiol. 2002, 93, 781-786, Food Microbiol. 2003, 20, 57-66, Int. J. Microbiol. 2003, 83, 161-170). The performance characteristics of the published methods were relative standard deviations for repeatability (RSD_r) between 0.5 to 6 % and relative standard deviations for between-laboratory reproducibility (RSD_R) of 1 to 9 %. The limit of quantification (LOQ) for the method is around 2 to 3 x 10^{6} c.f.u./kg sample or liter suspension which is well below the minimum anticipated target level of application.

The identity of the bacterial strains was demonstrated by a range of morphological, physiological and genotypic methods. Pulsed-field gel electrophoresis is a generally recognised standard methodology for microbial identification and is considered as a suitable technique 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

Biomin® C5, *Pediococcus acidilactici*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Lactobacillus reuteri*, *Lactobacillus salivarius* ssp. *salivarius*, zootechnical feed additive, chickens

1. BACKGROUND

Biomin® C5 is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Biomin® C5 is provided in form of a powder containing five strains at different concentrations, containing 1.3 x 10^{10} cfu/g *Pediococcus acidilactici* DSM 16210, 3.0 x 10^{10} c.f.u. *Enterococcus faecium* DSM 16211, 5.0 x 10^{9} c.f.u. *Bifidobacterium animalis* ssp. *animalis* DSM 16284, 1.0 x 10^{9} c.f.u. *Lactobacillus reuteri* DSM 16350, 1.0 x 10^{9} c.f.u. *Lactobacillus salivarius* ssp. *salivarius* per gram in the feed additive and in total 5 x 10^{10} c.f.u./g [1, 2]. The five strains are deposited at the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH' (DSMZ), Braunschweig, Germany [3]. The intended use of the current application (EFSA-Q-2006-169) is for chickens for fattening to slaughter age of around 42 days. The proposed conditions of use are a recommended dosage of 1 x 10^{9} to 1 x 10^{10} c.f.u./l drinking water [1, 2, 3, 4].

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 particular dossier, the methods of analysis submitted in connection with the Biomin® C5 dossier (EFSA-Q-2006-169) 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 the colony forming units of the strains *Pediococcus acidilactici* DSM 16210, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Lactobacillus reuteri* DSM 16350, *Lactobacillus salivarius* ssp. *salivarius* DSM 16351 in the additive and in the water/additive suspension by applying enumeration methods using appropriate media [5, 6]. Individual strains are enumerated at the end of the production process by five appropriate plate count methods which were validated by an external laboratory according to International IDF Standard 169 (1994). The total viable counts were determined in the feed additive and its water suspension by using an appropriate pour plate method which was in-house validated by an external laboratory. Some information appears to be missing in the methods provided by the applicant. This refers to the handling and duration of the analysis of total viable counts in the drinking water where the stability of the active agents appears to be guaranteed for at least 24 h. The enumeration method for *Bifidobacterium animalis* appears not to outline measures to take account of the oxygen sensitivity of the species. The enumeration methods for *L. reuteri* and *L. salivarius* are the same and no reference is made with regards to colony morphology of both strains. The proposed methods are suitable for the intended purpose. However, fully ring-trial validated methods are recommended for official controls in the frame of the authorisation, whereby if may be difficult to distinguish between the two *Lactobacillus* strains depending on their colony morphology [7-10].

The genetic identity of the strains of *Pediococcus acidilactici* DSM 16210, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Lactobacillus reuteri* DSM 16350, *Lactobacillus salivarius* ssp. *salivarius* DSM 16351 is examined using a polyphasic approach combining morphological, physiological and genotypic methods [11, 12].

Qualitative and quantitative composition of any impurities in the additive

The applicant analyses the feed additive for microbial contaminants such as coliforms, *Salmonella* species, *E. coli*, yeasts and moulds. Heavy metals including lead, arsenic, mercury, cadmium and other impurities are further monitored. The applicant uses Food and Agriculture Organization (FAO), Association of Analytical Communities (AOAC International) methods which are appropriate [13, 14]. Internationally recognised standardized methods such as International Organization for Standardization (ISO) and European Committee for Standardisation (CEN) standards 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)

The feed additive is applied to chickens for fattening via the drinking water and is not added to feeding stuffs [15]. For the enumeration of the five strains of the active agents, *Pediococcus acidilactici* DSM 16210, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Lactobacillus reuteri* DSM 16350, *Lactobacillus salivarius* ssp. *salivarius* DSM 16351 the methods were described and discussed above [5, 6]. The methods were in-house validated by the applicant and are suitable for the intended purpose.

For official controls the same ring-trial validated methods as cited above are recommended [7-10]. The methods were validated using feed samples however appear applicable for the analysis of the feed additive and its 'drinking water suspension' as indicated above. The ring-trial validated methods include a preparation of an initial sample suspension using 20 g for premixtures and 50 g for feed samples in phosphate buffered saline. Decimal dilutions in peptone salt diluent were prepared. The next step includes a transfer of dilutions to agar plates containing medium suitable for each microorganism species. Subsequently, appropriate dilutions were spread on de Man, Rogosa, Sharpe (MRS) agar and plates were incubated at 37 °C for 24 – 48 h for lactobacilli. An addition of 0.01 % triphenyl tetrazolium chloride to MRS agar is recommended in particular to enhance differentiation between the two *Lactobacillus* strains. For enterococci bile esculin azide agar was used. A supplementation of MRS agar with cysteine hydrochloride was recommended for bifidobacteria or alternatively the use of a selective bifidobacterium medium. Following incubation of agar plates under

appropriate conditions for each species, viable counts are determined on each medium and added to a final count. The published methods revealed relative standard deviations for repeatability (RSD_r) between 0.5 – 6 % and for inter-laboratory reproducibility (RSD_R) between 1 – 9 %.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant uses appropriate conventional methods to enumerate the active agents. Ring-trial validated methods using MRS agar for lactobacilli and pediococci, MRS agar supplemented with cysteine hydrochloride for bifidobacteria and bile esculin azid agar for enterococci is recommended for official controls in the frame of the authorisation [7-10]. As pulsed-field gel electrophoresis is already widely used by reference laboratories to identify bacterial isolates it is 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 methods using MRS agar supplemented with 0.01 % triphenyl tetrazolium chloride for lactobacilli and pediococci, MRS agar supplemented with cysteine hydrochloride for bifidobacteria and bile esculin agar for enterococci and an incubation temperature of 37 °C.

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 Biomin® C5 for chickens 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] Proposal of Register entry
[2] Summary for publication 2.1.3 Qualitative and quantitative composition
[3] Technical dossier section I, 2.2.1 Nomenclature

[4] Technical dossier, section II, 2.4.Conditions of use
[5] Technical dossier, section II, .5. Control methods, general methods
[6] Technical dossier, supplement S9, Standing operating procedures (SOP's)
[7] Leuschner R.G.K., Bew J., Simpson P.J., Ross P.R., Stanton C. 2003. Enumeration of probiotic pediococci in animal feed: Interlaboratory study. J. AOAC Int. 86, 791-801
[8] 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] 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
[10] 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
[11] Technical dossier, section II, 2.1. Identity of the additive
[12] Technical dossier, supplement S1, Product identity
[13] Technical dossier, section II, 5. Control methods, determination of impurities
[14] Technical dossier, supplement S2, Product specifications
[15] Technical dossier, section II, 2.4. Zootechnical additives

7. RAPPORTEUR LABORATORY

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

8. ACKNOWLEDGEMENTS

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- Agricultural Research Centre, Saku, Harjumaa, Estonia
- Laboratoire de Rennesa, Rennes, France
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- Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany
- National Research Institute of Animal Production, Krakow, Poland
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