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CRL Evaluation Report on the Analytical Methods submitted in
connection with Section 2.5 (Control Methods) of the Application for
Authorisation as a Feed Additive
according to Regulation (EC) No 1831/2003

Dossier No.: FAD-2005-0002

Name of Additive: Biomin® IMB 52

Active Substance(s): *Enterococcus faecium* DSM 3530

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Date: 22/08/2005

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Date: 24/08/2005

1. EXECUTIVE SUMMARY

Bioimin[®] IMB 52 is a feed additive, consisting of the active probiotic strain *Enterococcus faecium* DSM 3530 and other non-active components, belonging to zootechnical additives, category 4.

The current application for Bioimin[®] IMB 52 seeks the following extension for its use as feed additive in feedingstuffs when used for chicken for fattening as a gut flora stabilizer, in the EU.

Concerning the determination of the active substance of Bioimin[®] IMB 52 (*Enterococcus faecium*), in the feed additive, in premixtures and in feedingstuffs, a pour plate count method was proposed by the applicant to determine viable counts of the probiotic bacterium of the preparation. The method is quantitative and uses KEA-Agar (Kanamycine-Esculine-Azide Agar). The method used has a limit of quantification of 100 colony forming units (c.f.u) per gram (g) sample which is considered sufficiently sensitive. “In-house” validation studies demonstrate that the method is suitable for quantification of *Enterococcus faecium* taking into account validation performance characteristics obtained. The suitability of the method has been evaluated against an international guideline for enumeration of *E. faecium* in premixture and feed. The obtained performance characteristics fulfilled the criteria defined in this guideline. A collaborative study of the method and corresponding reproducibility data were not provided, however, the presented method is very similar to a previously full ring trial validated method, which uses Bile Esculine Azide (BEA) Agar for quantification of the active substance in the feed additive, in premixtures and in feedingstuffs. The enumeration of enterococci on BEA agar showed a relative deviation on repeatability (RSD_r) of 1.5 – 3.6% and a relative deviation on reproducibility (RSD_R) between 2.9-7.4%. BEA agar was selective for enterococci in the presence of other probiotic micro-organisms such as pediococci, lactobacilli and yeast. Characterisation and identification of the microbial strain of the active substance, *E. faecium* IMB 52 (DSM 3530), was carried out using appropriate methodologies.

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, have been submitted for the purpose of this extension of the authorisation for the dossier.

In summary, and taking into account the results presented, the CRL finds that the proposed methods fulfil the requirements for routine control methods to quantitatively determine the colony forming units present in Biomin[®] IMB 52 in the proposed concentration range.

For official control we recommend a similar method which was ring-trial validated (J. Appl. Microbiol. 2002, 93, 781-786).

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

2. KEYWORDS

Bioimin[®] IMB 52, *Enterococcus faecium*, gut stabilizer, feed additive

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4. BACKGROUND

Bioimin[®] IMB 52 actually is approved for calves (EU no. E 21). The authorisation was valid until the 28th of February 2005.

The current extension for Bioimin[®] IMB 52 seeks authorisation for use as feed additive as a gut flora stabilizer for chicken for fattening in EU.

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 and related validation studies submitted in connection with FAD – 2005 – 0002 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), in the following referred to as “the Guidelines.”

For further details regarding the structure of the dossier please see chapter 8 of this document.

General methods

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 of the Guidelines.

Qualitative and quantitative composition

The active component is a culture of viable cells of micro-organisms *Enterococcus faecium* (its depositing number is DSM 3530). The number of viable microorganisms is given in colony forming units (c.f.u.) per unit weight in section II of the information provided by the applicant. Minimum concentration of *Enterococcus faecium* in the pure culture of the feed additive Biomin® IMB 52 should be at least 1.0×10^{11} c.f.u./g (granulate). *E. faecium* belongs to the family *Enterobacteriaceae* which belongs together with *Lactobacillaceae* to the group of ‘lactic acid bacteria’. The method protocols used for the routine enumeration of production batches for the active substance was provided. The method used represents an aerobic pour plate count technique using a selective agar. Using this protocol some routine analyses have been performed for the feed additive, premixtures and feedingstuffs and suitable certificates delivered by the applicant to this extent. The species and strain of *Enterococcus faecium* IMB 52 (DSM 3530) was characterised and identified by microscopy, growth characteristics, biochemical fermentation and electrophoretic protein banding patterns. The methods were appropriate and able to distinguish *E. faecium* IMB 52 from other *E. faecium* strains.

(Cf. the requirements listed in point 2.1.3 of the Guidelines.)

Qualitative and quantitative composition of any impurities

Methods for analysis of possible microbial contaminants in the product such as total aerobic plate counts, coliforms and *E. coli*, *Salmonella*, yeast and moulds were provided. The

microbiological methods are appropriate. In the frame of the quality control process the most important microbial contaminants (TPC, coliforms, *E. coli*, *Salmonella*) are analysed using appropriate methods. Heavy metals have been determined applying standardised European methods. The quality of each batch of the feed additive is controlled according to a quality control plan. Microbiological, chemical and physical parameters are controlled systematically. The analyses are done in the quality control laboratories of the company. Differences of the results obtained when compared to the specifications result in corrective action.

Traces of fermentation substrates (glucose, yeast extract) and traces of fermentation products (lactate) are described to be less than 1% in total.

Levels of heavy metals are checked and results presented.

(Cf. the requirements listed in point 2.1.4 of the Guidelines.)

Physical state of each form of the product

Methods of how to determine 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.

(Cf. the requirements listed in point 2.1.5 of the Guidelines.)

Toxins and virulence factors

The microbiological purity of the feed additive's active strain during the fermentation process is guaranteed by the applicant due to the existence of contamination-free cell banks (strain checked at stored at -80°C) as well as to a rigorously controlled fermentation process. Studies on the pathogenicity and toxicity and the results obtained proved that the strain is not pathogenic and not toxic when used as a feed additive. The biological risk for the environment of the strain is considered negligible according to studies performed by the applicant. A toxicological study demonstrated that a 10-fold overdose of *Enterococcus faecium* Biomin® IMB 52 has not have any negative effect on the performance parameters of broilers.

(Cf. the requirements listed in point 2.2.5 of the Guidelines.)

Antibiotic production and antibiotic resistance

While information was provided, 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. Additional studies on the susceptibility proved that the strain *Enterococcus faecium* Biomin[®] IMB 52 is sensitive to a broad range of antibiotics used in human medicine. The broth dilution method used for determination of the minimum inhibitory concentration (MIC) according to The National Committee for Clinical Laboratory Standards (NCCLS) should be recognised as suitable for this purpose.

(Cf. the requirements listed in point 2.2.6 of the Guidelines.)

Characterisation of the additive: physico-chemical and technological properties

Stability of the additive

The methods and procedures to test for the stability of the additive have been provided for the purpose of this extension. The methods used for quantification of the additive (2.5.2 of the Guidelines) could be considered appropriate for the purpose. In the course of a short and long term storage experiment the stability of the additive regarding colony count was investigated after exposure to different environmental conditions, including temperature, light, moisture and pH-value. Stability studies of the different batches of the feed additive Biomin[®] IMB 52 alone, and mixtures with premixtures and feedingstuffs were performed using suitable laboratory methods.

(Cf. the requirements listed in point 2.3.1 of the Guidelines.)

Other physico-chemical or biological properties

Several properties were examined e.g. the ability to obtain a homogenous mixture, resistance in the digestive tract. Methods have been provided for the purpose of this extension.

(Cf. the requirements listed in point 2.3.2 of the Guidelines.)

Incompatibilities with other feed ingredients

The applicant stated that none are known at present and no method has been proposed.

(Cf. the requirements listed in point 2.3.3 of the Guidelines.)

Control methods

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

A quantitative pour plating method is used by the applicant to quantify bacterial cells of the one species (*Enterococcus faecium*) present in the premixture and feed. Bacterial cells are enumerated and differentiated. The results are reported as colony forming units (c.f.u.) per gram (g) sample. Representative samples for the product are examined. The samples are initially diluted and homogenised. Further decimal dilutions are prepared from initial dilution and agar plating by pour plate technique. The method uses a special selective agar, the KEA-Agar (Kanamycine-Esculine-Azide Agar) which is appropriate. The test is performed on triplicates by pouring 1 ml of each dilution into a Petri dish (e.g. dishes 95/15 LMG.MN, Greiner) and mixing it with a minimum of 10 ml of the respective agar (agar temperature = 50°C). Some method parameters i.e. the agar temperature and amount used are different in comparison with the Standard Method ISO 7218 however this is not expected to pose any problems. The general rules for calculation of the results obtained are shown in the standard ISO 7218:1996 [1]. After incubation all plates with more than 10 but less than 700 colonies are evaluated by colony counting. In agreement with ISO 7218 the maximum number of colonies should be no more than 300 when determining colony forming units. In the applicant documents related to 2.2.2 of the Guideline it is mentioned that microscopic image and generation time were examined after incubation in MRS (De Man, Rogosa, Sharp) broth. It should be mentioned that in principle the MRS medium is used for cultivation of *Enterococcus* and *Lactobacillus* species. The above described methods can be considered as routine method and was in-house validated following international guidelines [4]. The obtained performance characteristics fulfilled the criteria defined in this guideline. The in-house validation was carried out using samples which represented typical premixtures and feedingstuffs and ensured an appropriate range of concentration levels of the active substances. Feed samples (around 10^5 - 10^6 c.f.u./g), premixtures containing viable counts of the active substances of 10^8 - 10^9 c.f.u./g were used in the validation study. The results obtained in this in-house validation study proved that the method is suitable for quantification of *Enterococcus faecium* taking into account validation performance criteria obtained. A collaborative study of the method and corresponding reproducibility data were not provided, however, the presented method is very similar to a previously validated method (by full ring trial, according to international guidelines) [2], which uses Bile Esculine Azide (BEA) Agar for quantification of the active substance in the feed additive, in premixtures and in feedingstuffs. The enumeration of enterococci on BEA agar showed a relative standard deviation on repeatability (RSD_r) of 1.5 – 3.6% and a relative standard deviation on

reproducibility (RSD_R) between 2.9 - 7.4%. BEA agar was selective for enterococci in the presence of other probiotic micro-organisms such as pediococci, lactobacilli and yeast.

The quantification limit of the enumeration method provided by the applicant is 10^2 c.f.u./g is well below concentrations in feedingstuffs of about 10^{5-6} c.f.u./g and premixtures of about 10^{8-9} c.f.u./g or in the feed additive of around 10^{11} c.f.u./g.

(Cf. the requirements listed in point 2.5.2 of the Guidelines.)

CHECK LIST

		Y	N	N/ A	Comments
1.	Is/Are the method(s) mentioned in Part I (1.- A. Premixtures)				
1	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.	Is/Are the method(s) mentioned in Part I (1.- A. Animal feed)				
2	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.	Is/Are the method(s) mentioned in Part I (2. – Target tissues)			X	
1	accompanied by information on:				
	- Sampling Method used				
	- Percentage Recovery				
	- Specificity				
	- Accuracy				
	- Precision				
	- Limits of detection				
	- Limits of quantification				
	- Validation procedure used				
2.	Is/Are the method(s) mentioned in Part I (2. – Animal products)			X	
2	accompanied by information on:				
	- 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

Concerning the determination of the active substance of Biomin[®] IMB 52 (*Enterococcus faecium* DSM 3530), in the feed additive, in premixtures and in feedingstuffs, a pour plate count method was proposed by the applicant to determine viable counts of the probiotic bacterium of the preparation. The method is quantitative and uses KEA-Agar (Kanamycine-Esculine-Azide Agar). The method used has a limit of quantification of 100 colony forming units (c.f.u) per gram (g) sample, which is considered sufficiently sensitive. In-house validation studies demonstrate that the method is suitable for quantification of *Enterococcus faecium* in premixture and feed taking into account validation performance characteristics obtained. The suitability of the method has been evaluated against an international guideline for enumeration of *E. faecium* in premixture and feed. The obtained performance characteristics fulfilled the criteria defined in this guideline. No data concerning collaborative studies of the method (reproducibility data) were provided, however, the presented method is similar to a previously full ring trial validated method (according to international guidelines) which uses Bile Esculine Azide (BEA) Agar for quantification of the active substance in the feed additive, in premixtures and in feedingstuffs. The enumeration of enterococci on BEA agar showed a relative deviation on repeatability (RSD_r) of 1.5 – 3.6% and a relative deviation on reproducibility (RSD_R) between 2.9 - 7.4%. BEA agar was selective for enterococci in the presence of other probiotic micro-organisms such as pediococci, lactobacilli and yeast. Characterisation and identification of the microbial strain of the active substance, *E. faecium* IMB 52 (DSM 3530), was carried out using appropriate methodologies.

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 and appropriate methodology for their assessment have been submitted for the purpose of this extension of the authorisation for the dossier.

In summary, and taking into account the results presented, the CRL finds that the proposed methods fulfil the requirements for routine control methods to quantitatively determine the colony forming units present in Biomin[®] IMB 52 in the proposed concentration range.

For official control we recommend a similar method which was ring-trial validated (J. Appl. Microbiol. 2002, 93, 781-786).

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

Product samples have been made available to the CRL on 17.01.2005.

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

Further information provided by applicant upon request Data concerning MRS medium composition

9. REFERENCES

- (1) ISO 7218:1996 and ISO 7218:1996/Amd 1:2001. Microbiology of food and animal feedingstuffs – General rules for microbiological examinations.
- (2) Leuschner, R.G.K., Bew, J., Domig, K., & Kneifel, W. (2002) J. Appl. Microbiol. 93, 781-786.
- (3) Bergey's Manual of Determinative Bacteriology, 1984. The Williams & Wilkins Co, Baltimore.
- (4) IDF Standard 169:1994. Quality control in the microbiological laboratory – Analyst performance assessment for colony count. International Dairy Federation.

10. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the National Reference Laboratory for Feed Additive Authorisation, National Veterinary Research Institute, Pulawy, Poland.