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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-2007-033 FAD-2006-0038
Name of Additive:	Bonvital for sows
Active Agent(s):	*Enterococcus faecium* DSM 7134
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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Date:	30/05/2007

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Bonvital under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Enterococcus faecium* DSM 7134. The additive is available in two forms (powder or granules (micro-encapsulated)) both of which contain a minimum concentration of 1 x 10^{10} colony forming units (c.f.u.) per gram. Specifically, authorisation is sought to use Bonvital for sows. The conditions of use are proposed with a recommended dosage of 0.5 to 1 x 10^{9} c.f.u./kg.

For the quantification of the active agent (*Enterococcus faecium* DSM 7134) of Bonvital in the *feed additive*, *premixtures* and *feedingstuffs*, an appropriate pour plate method using a selective enterococci agar was proposed by the applicant. The method was in-house validated and shown to be transferable to four external laboratories. The method precision data resulting from the in-house and four laboratory trials were acceptable for the intended purpose.

For official controls regarding the quantitative determination of the active agent in *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been fully ring-trial validated (J. Appl. Microbiol. 2002, 93, 781-786). The method performance characteristics include a relative standard deviation for repeatability (RSD_r) ranging between 1.5 to 3.6 % and a relative standard deviation for reproducibility (RSD_R) ranging between 2.9 to 7.4 %. The limit of quantification (LOQ) for the method is around 2 to 3 x 10^{6} c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

The identity of the bacterial strain, *Enterococcus faecium* DSM 7134, was analysed by a range of techniques including biochemistry, protein-fingerprinting and molecular methods such as polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and is considered suitable 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

Bonvital, feed additive, *Enterococcus faecium*, zootechnical additive, sows

1. BACKGROUND

Bonvital 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. Bonvital is provided in form of a powder or encapsulated granules supplemented with a strain of *Enterococcus faecium.* The strain is deposited at the German Resource Centre for Biological Materials (DSMZ), Braunschweig, Germany under the deposit number DSM 7134. Bonvital contains at least 1 x 10^{10} c.f.u. of *Enterococcus faecium* DSM 7134 per gram in the feed additive [1]. The intended use of the current application (EFSA-Q-2007-033) is for sows for the entire reproduction cycle. The proposed conditions of use are for sows at a recommended dosage of 0.5 to 1 x 10^{9} c.f.u./kg complete feedingstuffs [1].

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 (EFSA) for each application. For this particular dossier, the methods of analysis submitted in connection with the Bonvital dossier (EFSA-Q-2007-033) 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 method for quantifying the active agent as proposed by the applicant represents a pour plate method using a selective nutrient agar of which the composition is not provided. The method is suitable for Bonvital powder and Bonvital granules (microencapsulated) according to the applicant. A sample is suspended in a dilution buffer and decimally diluted. The applicant does not provide the exact compositions of the diluent or the agar. A transfer of a dilution volume of 1 ml to approximately 10 ml of warm agar is recommended. The agar plates are incubated at 37 °C for 48 h [2]. The method was in-house validated [3] and

validated by a four laboratory trial [4]. Data were obtained by inoculating three Petri dishes using one dilution. Generally the use of two successive dilutions is recommended to obtain more robust raw data for further calculation. The results of the trial are shown in the corresponding enclosures 11 – 14 [5]. The precision data for intra-laboratory repeatability and inter-laboratory reproducibility are acceptable and within the range of a published plate count method to selectively enumerate enterococci using bile esculin azid (BEA) agar which was validated by a collaborative study [6]. The ring-trial validated method is recommended for official controls in the frame of the authorisation.

The genetic identity of the strain is examined by a combination of techniques. The production strain was characterised biochemically, by protein-fingerprinting and by molecular methods such as polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE) [7]. The Department of Dairy Research and Bacteriology of the University of Agricultural Sciences in Vienna analysed the active agent by applying methods submitted by the applicant and standard methods established at the Institute [8]. PFGE is recommended for official controls in the frame of the authorisation.

Qualitative and quantitative composition of any impurities in the additive

The applicant analyses the feed additive for microbiological contaminants such as *Escherichia coli*, yeast and moulds, *Salmonella* species, *Staphylococcus aureus*, *Bacillus cereus* and sulphite reducing bacteria using standard methods EN ISO 9308-1, EN ISO 7954, ISO 6579, LMGB L02.07-2, DIN 10198-1,2 and DIN 38411, respectively [9]. These methods are considered suitable for the intended purpose.

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 *Enterococcus faecium* DSM 7134 in *premixtures* and *feedingstuffs*, the applicant proposes the same pour plate method as for the feed additive which was described above [2, 6].

The applicant validated the method in-house using samples of Bonvital powder, Bonvital granulate, complete feed and milk replacer supplemented with Bonvital. An equivalent set of samples was used for a four laboratory ring-trial. The method precision data for within- and inter-laboratory repeatability and reproducibility were in the range of those obtained by a full collaborative study in which a different selective enterococci agar was used [3, 4]. The fully ring-trial validated method used Bile Esculine Azide (BEA) agar for quantification of the active substance in premixtures and feedingsstuff. The enumeration of enterococci on BEA agar showed relative standard deviation deviations (RSD_r) between 1.5 – 3.6 % for

repeatability and (RSD_R) between 2.9-7.4 % for reproducibility. The statistical analysis was carried out on log_{10} transformed raw data of the enumeration results. BEA agar was selective for enterococci in the presence of other probiotic micro-organisms such as pediococci, lactobacilli and yeast. The results of the collaborative study were published [6]. This method is recommended by the CRL for official controls in the frame of the authorisation.

The applicant made some recommendations regarding the preparation of an initial suspension of a sample when trace elements, in particular copper, are present in premixtures or mineral feeds which may affect the viable counts [10]. An initial dilution of a factor of 100 to suspend the sample and an addition of 500 mg imidodiacetic acid to a liter of suspension buffer to chelate copper were suggested. These recommendation were not sustained by scientific data however may be helpful information for the analysis.

The applicant has used a range of techniques to identify the *Enterococcus faecium* strain used as active agent as described above [7, 8]. Pulsed-field gel electrophoresis (PFGE) is considered as a suitable method for official controls.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant uses appropriate conventional methods to enumerate the active agent and potential impurities in the product. These are considered appropriate for the purpose, however, the CRL recommends for official controls in the frame of the authorisation ISO and/or CEN methods in line with current European legislation. A ring-trial validated spread plate method using BEA agar to enumerate the active agent is recommended for official controls in the frame of the authorisation [6].

For the analysis of the identity of the bacterial strain, *Enterococcus faecium* DSM 7134, the applicant uses also a range of techniques which are appropriate to identify the strain. As pulsed-field gel electrophoresis (PFGE) 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 method using bile esculin azid agar.

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 Bonvital for sows have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Proposal of Register entry, Annex III

[2] Dossier Section II. 5. Control methods, sections 5.1. and 5.2. Qualitative and quantitative analysis of the feed additive, premixtures and feedingstuffs using method Lac-DO-Ef-1a_1

[3] Enclosure 10a, in-house validation of method Lac-DO-Ef-1a_1

[4] Enclosure 10b, between-laboratory validation of method Lac-DO-Ef-1a_1

[5] Enclosures 11 – 14, results of the between-laboratory validation trial

[6] 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

[7] Dossier Section II. 2. Section 2 'Specification of the active ingredient'

[8] Dossier Section II. 'Genetic stability'

[9] Enclosure 9, 2.5.1. General methods

[10] Dossier Section II. 5. Control methods. 7. Annotations

7. RAPPORTEUR LABORATORY

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

8. ACKNOWLEDGEMENTS

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- National Reference Laboratory RO Praha, Praha, Czech Republic
- Laboratoire de Rennes, Rennes, France
- Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany
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