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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-2008-289 FAD-2008-0007
Name of Additive:	Bonvital
Active Agent(s):	*Enterococcus faecium* DSM 7134
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
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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 chickens for fattening. The conditions of use are proposed with a recommended dosage of 0.2 to 2.0 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 was shown to be transferable to three external laboratories. The method precision data resulting from the in-house and between-laboratory trials were acceptable for the intended purpose.

For official controls regarding the quantitative determination of the colony forming units of the active agent in the *feed additive*, *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's performance characteristics of the enumeration method are standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.12 – 0.20 $\log_{10}$ and 0.23 – 0.41 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The limits of quantification (LOQ) of this method are around 10^{4} colony forming units (c.f.u.) per gram (g) feed additive or premixture and around 10^{7} c.f.u./kg feedingstuff.

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, chickens

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-2008-289) is for chickens for fattening for the entire reproduction cycle. The proposed conditions of use are for chickens for fattening at a recommended dosage of 0.2 to 2.0 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-2008-289) 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 agar. 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. 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. The use of two successive dilutions is recommended to obtain more robust raw data for further calculation. The results of the trial are provided [5]. The reported precision data for intra-laboratory repeatability and inter-laboratory reproducibility are acceptable and within the range of data reported in a published ring-trial validation study that used bile esculin azid (BEA) agar to selectively enumerate enterococci [6]. The published ring-trial validated method is recommended for official controls to determine the colony forming units 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]. PFGE is recommended for official controls in the frame of the authorisation.

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 standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.12 – 0.20 $\log_{10}$ and 0.23 – 0.41 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. 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]. The limits of quantification (LOQ) of this method are around 10^4 colony forming units (c.f.u) per gram (g) feed additive or premixture and around 10^7 c.f.u./kg feedingstuff. 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 [8]. These recommendation were not sustained by a provision of scientific data however may be helpful information for the analysis. An initial sample dilution of a factor of 100 to suspend the sample and an addition of 500 mg imidodiacetic acid (IDES) to a liter of suspension buffer to chelate copper were suggested. A National Reference Laboratory commented that possible effects of IDES on the pH value have to be considered and confirmed use of concentrations of around 500 mg IDES per liter (350 – 875mg/l).

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

4. CONCLUSIONS AND RECOMMENDATIONS

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 chickens for fattening 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, validation report of the 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. 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. The initial evaluation report was made available for commenting to the consortium of National Reference Laboratories.

8. ACKNOWLEDGEMENTS

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- Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleißheim, Germany
- Thüringer Landesanstalt für Landwirtschaft, Jena, Germany
- National Veterinary Research Institute, Pulawy, Poland
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