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European Union Reference Laboratory for Feed Additives

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Feed Additives

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EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of Feed Additives according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2010-0305 - CRL/100233**
Name of Feed Additive:	**Provita LE**
Active Substance(s):	***Enterococcus faecium* DSM 7134** ***Lactobacillus rhamnosus* DSM 7133**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
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Date:	**02/10/2012**
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Date:	**02/10/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 10(2) for *Provita LE,* consisting of two active agents: *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133*,* under the category/functional group 4(b), "zootechnical additives/gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive* will be marketed in a powdered form containing a minimum concentration of $7x10^9$ CFU/g of *Enterococcus faecium* DSM 7134 and $3x10^9$ CFU/g of *Lactobacillus rhamnosus* DSM 7133, with dextrose anhydrous, maltodextrin and whey powder as carriers. Specifically, authorisation is sought for the use of the *feed additive* for calves for rearing and it is intended to be mixed at a total concentration of the two microorganisms ranging from $1x10^9$ to $5x10^9$ CFU/kg of complete *feedingstuffs*.

For the enumeration of the two microorganisms in the *feed additive, premixtures* and *feedingstuffs* the Applicant proposed two internationally recognised ring-trial validated spread plate method issued by the European Committee for Standardization (CEN): (i) for the enumeration of Enterococcus spp (EN 15788), using Bile Esculin Azide Agar and for enumeration of Lactobacillus spp (EN 15787), using MRS agar. The performance characteristics reported for CEN methods after logarithmic transformation (CFU) are:

- a repeatability standard deviation (s_r) ranging from 0.12 to 0.24 $\log_{10}$ CFU/g,
- a reproducibility standard deviation (s_R) ranging from 0.23 to 0.41 $\log_{10}$ CFU/g; and
- a limit of detection (LOD) of $1x10^5$ CFU/kg *feedingstuffs*.

Based on the performance characteristics presented, the EURL recommends for official control, the two CEN methods (EN 15788 and EN 15787) for the determination of *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133 in the *feed additive, premixtures* and *feedingstuffs*.

Molecular methods were used by the Applicant to identify the active agent in the *feed additive*. The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

Enterococcus faecium DSM 7134, *Lactobacillus rhamnosus* DSM 7133, E 1706, zootechnical additives, gut flora stabilisers, calves for rearing.

1. BACKGROUND

In the current application authorisation is sought under Article 10(2) (re-evaluation of already authorised additives) for *Provita LE,* consisting of two active agents: *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133*,* under the category/functional group 4(b), "zootechnical additives/gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. The *feed additive* (E 1706) is already authorised under the Commission Regulation (EC) No 1288/2004 for calves; and under the Commission Regulation (EC) No 2148/2004 for weaned piglets. The strains are deposited in the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)' in Braunschweig, Germany [2,3].

The *feed additive* will be marketed in a powdered form containing a minimum concentration of $7x10^9$ CFU/g of *Enterococcus faecium* DSM 7134 and $3x10^9$ CFU/g of *Lactobacillus rhamnosus* DSM 7133, with dextrose anhydrous, maltodextrin and whey powder as carriers [4,5].

Specifically, authorisation is sought for the use of the *feed additive* for calves for rearing and it is intended to be mixed at a total concentration of the two microorganisms ranging from $1x10^9$ to $5x10^9$ CFU/kg of complete *feedingstuffs* [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 European Union Reference Laboratory concerning applications for authorisations of feed additives, as last amended by Regulation (EC) No 885/2009, the EURL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application, or for each group of applications. For this particular dossier, the methods of analysis submitted in connection with the *Provita LE* 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 strains *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133 the Applicant used molecular methods such as Pulsed Field Gel Electrophoresis (PFGE), Polymerase Chain Reaction (PCR) amplifications and sequencing of 16S rRNA, as well as repetitive sequence-based PCR (rep-PCR) [6-10]. These methods are suitable for the purpose of analysis. However, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [11].

Qualitative and quantitative composition of impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (such as coliforms, *Escherichia coli*, Salmonella spp., yeasts and moulds) by using appropriate internationally recognised tests [5]. For undesirable substances (i.e. arsenic, cadmium, mercury, lead, selenium, copper, zinc, chrome, aflatoxins) internationally recognised standard methods are available at the respective European Union Reference Laboratory, in accordance with Commission Regulation (EC) No 776/2006.

Description of the analytical methods for the determination of active substance in feed additive, premixtures and feedingstuffs

For the enumeration of *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133 in the *feed additive, premixtures* and *feedingstuffs* the Applicant proposed a single laboratory validated pour plate method using MRS (de Man, Rogosa, Sharp) agar [12,13]. The samples are suspended and diluted in a nutrient bouillon buffer (with added Tween-80 for *premixtures* and *feedingstuffs* samples); appropriate dilutions are then transferred into Petri dishes and MRS agar is added. When the agar is solidified, plates are incubated at 37 °C for 72 hours before colony counting.

Additionally, the Applicant proposed two internationally recognised ring-trial validated spread plate method issued by the European Committee for Standardization (CEN): (i) for the enumeration of Enterococcus spp (EN 15788) [14] and for enumeration of Lactobacillus spp (EN 15787) [15] in the *feed additive, premixtures* and *feedingstuffs*.

In the CEN method 15788, the sample is suspended in phosphate buffered saline (PBS) and diluted in a peptone salt solution; the appropriate dilutions are then spread on Bile Esculin Azide Agar. The agar plates are incubated at 37 °C for 24 hours before colony counting. The

performance characteristics of the EN 15788 method reported after logarithmic transformation (CFU) are [14]:

- a repeatability standard deviation (s_r) ranging from 0.12 to 0.2 $\log_{10}$ CFU/g,
- a reproducibility standard deviation (s_R) ranging from 0.23 to 0.41 $\log_{10}$ CFU/g; and
- a limit of detection (LOD) of $1x10^5$ CFU/kg *feedingstuffs* [16].

In the CEN method 15787 the sample is suspended and diluted in a phosphate buffered saline (PBS); the appropriate dilutions are then spread on MRS agar plates. The agar plates are incubated at 37 °C for 48 to 72 hours. The performance characteristics of the EN 15787 method reported after logarithmic transformation are [15]:

- $s_r = 0.24$ $\log_{10}$ CFU/g;
- s_R ranging from 0.29 to 0.38 $\log_{10}$ CFU/g; and
- LOD = 10^5 CFU/kg *feedingstuffs* [16].

Based on the performance characteristics presented the EURL recommends for official control the two CEN methods (EN 15788 and EN 15787) for the enumeration of *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133 in the *feed additive, premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE) for the identification of the strains and the respective CEN methods (EN 15788 and EN 15787) for the enumeration of *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133 in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- of *Enterococcus faecium* DSM 7134: Spread plate method using Bile Esculin Azide Agar (EN 15788)

– of *Lactobacillus rhamnosus* DSM 7133: Spread plate method using MRS agar (EN 15787)

Identification of *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133:

– Pulsed Field Gel Electrophoresis (PFGE)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of *Provita LE* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application/Ref: SANCO/G1: Forw.Appl.1831/0029-2012
[2] *Technical Dossier, Section II, Annex II.2-1 Safe deposit 7134
[3] *Technical Dossier, Section II, Annex II.2-2 Safe deposit 7133
[4] *Application, Proposal for Register Entry, Annex A
[5] *Technical dossier, Section II, 2.1 Identity of the additive
[6] *Technical dossier, Section II, Annex II.2-3 PFGE 7134
[7] *Technical dossier, Section II, Annex II.2-4 16S rRNA 7134
[8] *Technical dossier, Section II, Annex II.2-5 PFGE 7133
[9] *Technical dossier, Section II, Annex II.2-6 rep_PCR 7133
[10] *Technical dossier, Section II, Annex II.2-7 16S rRNA 7133
[11] European Community Project SMT4-CT98-2235.*"Methods for the Official Control of Probiotics Used as Feed Additives,* Report 20873/1 EN (2002) ISBN 92-894-6250-7 (Vol. I)*"*
[12] *Technical Dossier, Annex II.6-3 SOP inhouse
[13] *Technical dossier, Section II, Annex II.6-4 trial report
[14] EN 15788:2009 - Animal feeding stuffs - Isolation and enumeration of Enterococcus *(E. faecium*) spp
[15] EN 15787:2009 - Animal feeding stuffs - Isolation and enumeration of Lactobacillus spp
[16] ISO 7218:1996 - Microbiology of food and animal feedingstuffs – General rules for microbiological examinations

*Refers to Dossier No: FAD-2010-0305

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union 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, as last amended by Regulation (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)