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

Probiotic Lactina®

(FAD-2017-0003; CRL/160055)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2017-0003 - CRL/160055**
Name of Feed Additive:	**Probiotic lactina®**
Active Agent (s):	***Enterococcus faecium* NBIMCC 8270** ***Lactobacillus acidophilus* NBIMCC 8242** ***Lactobacillus helveticus* NBIMCC 8269** ***Lactobacillus delbrueckii* ssp. *lactis* NBIMCC 8250** ***Lactobacillus delbrueckii* ssp. *bulgaricus* NBICCM 8244** ***Streptococcus thermophilus* NBIMCC 8253**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **14/09/2017**
Report approved by: Date:	**Christoph von Holst** **15/09/2017**

EXECUTIVE SUMMARY

Probiotic lactina® is the trade name of a preparation based on viable cells from acid lactic bacteria (LAB) containing the following six strains: *Enterococcus faecium* NBIMCC 8270, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus delbrueckii* ssp. *lactis* NBIMCC 8250, *Lactobacillus delbrueckii* ssp. *bulgaricus* NBICCM 8244 and *Streptococcus thermophilus* NBIMCC 8253. This *feed additive* is currently authorized under the category/functional 4(b) 'zootechnical additives' / 'gut flora stabilisers' according to the classification system of Annex I of Regulation (EC) No 1831/2003 for sucking piglets. In the current application authorisation is sought under article 4 (1) of the Regulation (EC) No 1831/2003 for the new use for chickens for fattening and rabbits suckling and weaned. *Probiotic lactina®* is intended to be marketed as a freeze-dried powder containing a minimum total dose of the sum of the six bacterial active substances (LAB) of $5x10^9$ Colony Forming Units (CFU)/g. The *feed additive* is intended to be used directly in *feedingstuffs* with a minimum total LAB content of $2.5x10^9$ CFU/kg of complete *feedingstuffs*.

For the identification and characterisation of the different bacterial strains, the Applicant proposed a combination of classical phenotypic tests and modern molecular sequencing analysis. The EURL recommends instead for official control the Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of the sum of the six strains contained in *Probiotic lactina®(feed additive)*, the Applicant suggested the use of several International Dairy Federation (IDF) standards and the Bulgarian Standard (BS) 10945, while the ring-trial validated spread plate method developed by CEN (EN 15787) was suggested for the enumeration in *feedingstuffs*. This CEN method was already evaluated and recommended by the EURL in several dossiers. Based on the performance characteristics available, the EURL recommends for official control the EN 15787 spread plate method for the enumeration of the sum of the six strains contained in *Probiotic lactina®* in the *feed additive* 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Probiotic lactina® *Enterococcus faecium* NBIMCC 8270, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus delbrueckii* ssp. *lactis* NBIMCC 8250, *Lactobacillus delbrueckii* ssp. *bulgaricus* NBICCM 8244 and *Streptococcus thermophilus* NBIMCC 8253, zootechnical additives, gut flora stabilisers, chickens for fattteinng and rabbits suckling and weaned

1. BACKGROUND

Probiotic lactina® is the trade name of a preparation based on viable cells from acid lactic bacteria (LAB) containing the following six strains: *Enterococcus faecium* NBIMCC 8270, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus delbrueckii* ssp. *lactis* NBIMCC 8250, *Lactobacillus delbrueckii* ssp. *bulgaricus* NBICCM 8244 and *Streptococcus thermophilus* NBIMCC 8253 [1][2].

This *feed additive* is currently authorized for sucking piglets [3] under the category/functional 4(b) 'zootechnical additives' / 'gut flora stabilisers' according to the classification system of Annex I of Regulation (EC) No 1831/2003, with the identification number 4b1891 [1][3].

In the current application authorisation is sought under Article 4(1) (new use the *feed additive*) of the Regulation (EC) No 1831/2003. Authorisation is requested for chickens for fattening, and rabbits suckling and weaned [1][2].

All the strains are deposited at the Bulgarian National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC, Sofia, Bulgaria) [4].

Probiotic lactina® is intended to be marketed as a freeze-dried powder containing a minimum total dose of the sum of the six bacterial active substances (LAB) of $5x10^9$ Colony Forming Units (CFU)/g [5]. The *feed additive* is intended to be used directly in *feedingstuffs* with a minimum LAB content of $2.5x10^9$ CFU/kg of complete *feedingstuffs* [1][6].

Note: The analytical methods for the identification and quantification of lactic acid bacteria from several bacteria strains in the relevant matrices were already evaluated and recommended by the EURL in the frame of previous dossiers [7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food

Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Probiotic lactina®* 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 impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, copper, aflatoxin M1 and dioxins) are available from the respective European Union Reference Laboratories [8].

Furthermore, the Applicant analysed the *feed additive* for microbial contaminants including *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, fungi and yeasts, using the methods specified in the technical dossier [9].

Qualitative and quantitative composition of the additive

The Applicant proposed a combination of classical phenotypic tests and modern molecular sequencing analysis (i.e. by 16S rDNA sequence analysis), to establish the identity for the six strains of interest (i.e. *Enterococcus faecium* NBIMCC 8270, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus delbrueckii* ssp. *lactis* NBIMCC 8250, *Lactobacillus delbrueckii* ssp. *bulgaricus* NBICCM 8244 and *Streptococcus thermophilus* NBIMCC 8253) [10]. The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains. A PFGE method for microbial identification of authorised probiotics at strain level in *feedingstuffs* [11] is currently being evaluated by the CEN Technical Committee 327 to become an European Standard.

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

For enumeration of the sum of the six strains contained in *Probiotic lactina®* (*feed additive*) the Applicant proposed two IDF standard methods (IDF 122C:1996 for the preparation of samples and dilutions for microbiological examination; and IDF 149A:1997 for the determination of lactic acid bacteria in probiotic products) in addition to the Bulgarian Standard (BS) 10945-91 [12]. For the enumeration of the sum of the six strains in the feedingstuffs the Applicant submitted the EN 15787 standard [13]. This ring-trial validated spread plate method developed by CEN was already evaluated by the EURL in the frame of several *lactobacilli* dossiers. The sample is suspended and diluted in a buffer solution; appropriate dilutions of the sample are spread on MRS (de Man, Rogosa, Sharp) agar plates and incubated anaerobically at 37 °C for 48 to 72 hours. The EURL recommended to monitor

closely the pH in order to ensure complete fermentation before performing the enumeration of the *Lactobacilli*. The following performance characteristics (expressed as precision) were reported after logarithmic transformation (CFU) [13]: (i) a standard deviation for repeatability (S_r) of 0.24 log_{10} CFU/g; and (ii) a standard deviation for reproducibility (S_R) ranging from 0.29 to 0.38 log_{10} CFU/g. In addition, the EURL calculated according to ISO 7218 a limit of quantification (LOQ) of $3x10^3$ CFU/g [14].

Based on the performance characteristics available, the EURL recommends for official control the EN 15787 method for the enumeration of the sum of the six strains of interest in the *feed additive* (product) 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 the EN 15787 method for the enumeration of the sum of six strains of interest (*Enterococcus faecium* NBIMCC 8270, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus delbrueckii* ssp. *lactis* NBIMCC 8250, *Lactobacillus delbrueckii* ssp. *bulgaricus* NBICCM 8244 and *Streptococcus thermophilus* NBIMCC 8253) in *feed additive* and *feedingstuffs*.

For the identification and characterisation of the six strains mentioned above the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE).

Recommended text for the register entry (analytical method)

- Identification of the six strains: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in *feed additive* and *feedingstuffs*: Spread plate method on MRS agar (EN 15787)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Probiotic lactina®* 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, Proposal for Register Entry, Annexes A

[2] *Application, Reference SANTE/E5: FORW. APPL. 1831-0003-2017

[3] Commission Implementing Regulation (EU) No 1077/2013 of 31 October 2013 concerning the authorisation of a preparation of *Enterococcus faecium* NBIMCC 8270, *Lactobacillus acidophilus* NBIMCC 8242, *Lactobacillus helveticus* NBIMCC 8269, *Lactobacillus delbrueckii ssp. lactis* NBIMCC 8250, *Lactobacillus delbrueckii ssp. bulgaricus* NBICCM 8244 and *Streptococcus thermophilus* NBIMCC 8253, as a feed additive for turkeys reared for suckling pigs (holder of authorisation Lactina Ltd.)

[4] *Technical dossier, Section II, Annex II.10

[5] *Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.1.3 Qualitative and quantitative composition

[6] *Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.5 Conditions of use

[7] EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2013-0048_lavipan_online.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2014-0022-procanius.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2006-0029.pdf

[8] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[9] *Technical dossier, Section II, Annex II.2

[10] *Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.2 Characterisation of the active agents

[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, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.6 Methods of analysis and reference samples

[13] EN 15787:2009 - Animal feedingstuffs - Isolation and enumeration of *Lactobacillus spp.*

[14] ISO 7218:1996, Microbiology of food and animal feedingstuffs – General rules for microbiological examinations

Refers to Dossier no: FAD-2017-0003

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)