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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-2010-0405 - CRL/100355
Product Name:	*Probiomix B*
Active Substance(s):	- *Lactobacillus plantarum KKP 593/p* - *Lactobacillus rhamnosus KKP 825*
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Date:	10/11/2011

EXECUTIVE SUMMARY

Authorisation is sought for Probiomix B under Article 4(1) under category/functional group 4(b), 'zootechnical additive'/'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the *feed additive* to be placed on the market as a powder, containing minimum concentration of $1.9x10^9$ CFU/g of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825*. The intended use of the current application is for broiler chicken. The product is intended to be used directly or mixed with *feedingstuffs* with a proposed recommended dosage ranging from $1x10^7$ to $1x10^8$ CFU/kg.

For enumeration of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* in *feed additive*, the Applicant proposes the internationally recognised ISO 15214 spread plate method, for which no performance characteristics are available**.** The EURL identified instead the ring trial validated spread plate method EN 15787. The performance characteristics of the EN 15787 method reported after logarithmic transformation of measured values (CFU) are:

- a standard deviation for repeatability (S_r) of 0.24 $\log_{10}$ CFU/g;
- a standard deviation for reproducibility (S_R) ranging from 0.29 to 0.38 $\log_{10}$ CFU/g;
- a limit of detection (LOD) of 10^5 CFU/kg of *feedingstuffs* .

Based on these performances characteristics the EURL recommends for official control the EN 15787 spread plate method for the determination of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* in the *feed additive, premixtures* and *feedingstuffs*.

The Applicant used molecular typing probes for the identification and characterisation of the two *lactobacilli* of interest in the *feed additive*. The EURL recommends instead for official control the 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

Probiomix B, *Lactobacillus plantarum KKP 593/p, Lactobacillus rhamnosus KKP 825*, zootechnical additives, gut flora stabiliser, broiler chicken.

1. BACKGROUND

Authorisation is sought for Probiomix B under Article 4(1) under category/functional group 4(b), 'zootechnical additive'/'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003 [1].

Specifically, authorisation is sought for the *feed additive* to be placed on the market as a powder, containing minimum concentration of $1.9x10^9$ CFU/g of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* [2].

Both strains are deposited in the Culture Collection of Industrial Microorganisms Institute of Agriculture and Food Biotechnology (Warsaw, Poland) [3].

The intended use of the current application is for broiler chicken. The product is intended to be used directly or mixed to *feedingstuffs* with a proposed recommended dosage ranging from $1x10^7$ to $1x10^8$ CFU/kg [2, 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 authorizations 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 *Probiomix B,* 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 characterization of the strains *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* the Applicant used molecular typing probes (16S ribosomal RNA) and API characterization [5]. This method is suitable for the purpose of analysis. The EURL recommends instead for official control the Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [6].

Qualitative and quantitative composition of impurities in the additive

The Applicant analysed the *feed additive* for microbial contaminants (such as Enterobacteria, *Escherichia coli*, Salmonella spp.) by using appropriate EN ISO tests [7]. For undesirable substances (i.e. arsenic, cadmium, mercury, lead and 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 enumeration of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* in *feed additive*, the Applicant proposes internationally recognised ISO 15214 pour plate method (Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of mesophilic lactic acid bacteria -- Colony-count technique at 30°C) [8]. The samples are inoculated into MRS agar dishes, at pH 5.7, and incubated for 30°C for 72 hours.

No performance characteristics for the ISO 15214 are available.

The EURL identified instead the ring trial validated spread plate method developed by CEN (EN 15787) [9]. The sample is suspended and diluted in a buffer solution; the appropriate dilutions are then spread on MRS (de Man, Rogosa, Sharp) 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 of measured values (CFU) are [9]:

- a standard deviation for repeatability (S_r) of 0.24 $\log_{10}$ CFU/g;
- a standard deviation for reproducibility (S_R) ranging from 0.29 to 0.38 $\log_{10}$ CFU/g; and
- a limit of detection (LOD) of 10^5 CFU/kg of *feedingstuffs* [10].

Based on the performances characteristics presented the EURL recommends for official control the EN 15787 spread plate methods for the enumeration of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* 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 the international standard methods EN 15787 for the enumeration of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* in the *feed additive, premixtures* and *feedingstuffs*.

The EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE) for the identification of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825.*

Recommended text for the register entry (analytical method)

Enumeration of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825* in the *feed additive*:

- Spread plate method using MRS agar (EN 15787)

Identification of *Lactobacillus plantarum KKP 593/p* and *Lactobacillus rhamnosus KKP 825*:

- 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 the additive *Probiomix B* 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/D/2: Forw.Appl.1831/(004)(10527)-2011

[2] *Application, Annex A, Proposal for register entry

[3] *Technical Dossier, 2.2. Characterization of the active substance(s)/agent(s)

[4] *Technical Dossier, Section II.2.5.1. Conditions of use

[5] *Technical Dossier, References-Sect_II-III-IV, Ref.8

[6] 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)"

[7] *Technical Dossier, Section II.2.1.4. Purity

[8] ISO 15214- Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of mesophilic lactic acid bacteria -- Colony-count technique at 30°C

[9] EN 15787 : " Animal feeding stuffs- Isolation and enumeration of Lactobacillus spp."

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

*Refers to Dossier No: FAD-2010-0405

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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Austria

- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, France

- Sächsische Landesanstalt für Landwirtschaft, Germany