EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
European Union Reference Laboratory for Feed Additives

JRC.D5/CvH/CMP/ago/ARES(2012)233418

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-2010-0394 - CRL/ /100119

Name of the feed additive: *Lactobacillus acidophilus D2/CSL*

Active Substance(s): *Lactobacillus acidophilus D2/CSL*

Rapporteur Laboratory: European Union Reference Laboratory for Feed Additives (EURL-FA)
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Date: 29/02/2012

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Date: 29/02/2012

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Lactobacillus acidophilus D2/CSL* under Article 4(1) and 10(2) 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 a minimum concentration of $5x10^{10}$ CFU/g *Lactobacillus acidophilus D2/CSL*. The intended use of the current application is for laying hens. The *feed additive* is intended to be incorporated in *feedingstuffs* or complementary *feedingstuffs* through *premixtures* or directly in *water*with recommended dosage of $1x10^{9}$ CFU/kg.

For enumeration of *Lactobacillus acidophilus D2/CSL,* the Applicant proposes the internationally recognised ISO 7218 pour plate method, for which no performance characteristics are available**.** The EURL identified instead the ring trial validated spread plate method EN 15787, for the enumeration of *Lactobacillus acidophilus D2/CSL* in *feedingstuffs*. The performance characteristics of the EN 15787 method reported after logarithmic transformation (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 acidophilus D2/CSL* in the *feed additive, premixtures, feedingstuffs* and *water*.

The Applicant used molecular typing probes for the identification and characterisation of *Lactobacillus acidophilus D2/CSL* 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

Lactobacillus acidophilus D2/CSL, zootechnical additives, gut flora stabiliser, laying hens.

1. BACKGROUND

In the current application authorisation is sought for *Lactobacillus acidophilus D2/CSL* under Article 4(1) (new use in water), and 10(2) (re-evaluation of the authorised additive under Commission Regulation (EC) No 2154/2004) 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 a minimum concentration of $5x10^{10}$ CFU/g of *Lactobacillus acidophilus D2/CSL* [2].

The strain is deposited in the Colección Española de Cultivos Tipo (CECT), of the University of Valencia (Spain), with a deposit number CECT 4529 [3].

The *feed additive* is intended to be incorporated in complete or complementary *feedingstuffs* through *premixtures* or directly in *water* for laying hens. The product is intended to be used mixed to *feedingstuffs* or drinking *water* with recommended dosage of $1x10^{9}$ CFU/kg [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 authorization of *feed additive*, 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 *Lactobacillus acidophilus D2/CSL,* 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 strain *Lactobacillus acidophilus D2/CSL* the Applicant used molecular typing probes (16S ribosomal RNA), Randomly Amplified Polymorphic DNA (RAPD)-PCR and API characterization [5,6]. The EURL recommends instead for official control the Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification [7].

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 [8]. 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 the enumeration of *Lactobacillus acidophilus D2/CSL,* the Applicant proposes the internationally recognised ISO 7218 pour plate method (Microbiology of food and animal feedingstuffs – General rules for microbiological examinations) [9]. The samples are inoculated into MRS agar dishes at pH 5.7, and incubated at 37 °C for 72 hours in anaerobic conditions.

No performance characteristics for the ISO 7218 method are available.

The EURL identified instead the ring trial validated spread plate method developed by CEN (EN 15787) [10] for the enumeration of *Lactobacillus acidophilus D2/CSL* in *feedingstuffs*. 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 are [10]:

- 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* [9].

Based on the performances characteristics presented the EURL recommends for official control the EN 15787 spread plate method for the enumeration of *Lactobacillus acidophilus D2/CSL* in the *feed additive, premixtures*, *feedingstuffs* and *water*.

Further testing or validation of the method 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 method EN 15787 for the enumeration of *Lactobacillus acidophilus D2/CSL* in the *feed additive, premixtures,feedingstuffs* and *water* ; and - Pulsed Field Gel Electrophoresis (PFGE) for the identification of *Lactobacillus acidophilus D2/CSL.*

Recommended text for the register entry (analytical method)

Enumeration of *Lactobacillus acidophilus D2/CSL* in the *feed additive, premixtures, feedingstuffs and water*:

- Spread plate method using MRS agar (EN 15787)

Identification of *Lactobacillus acidophilus D2/CSL*:

- 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 *Lactobacillus acidophilus D2/CSL* 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/(00182)(9301)-2010

[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, Anexex Sect. II, Encl 15a D2 AR-engl 2010.pdf

[6] *Technical Dossier, Anexex Sect. II, Encl 15b LA1 genetic stability 2010.pdf

[7] 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)*"*

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

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

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

*Refers to Dossier No: FAD-2010-0394

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

The following National Reference Laboratories contributed to this report:

- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer,DE
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, PL
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), AT
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), CZ
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), IT
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, FR
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena, DE