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DIRECTORATE GENERAL
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Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/SB/AS/Ares

**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**

***Lactobacillus hilgardii* CNCM I-4785 and
Lactobacillus buchneri CNCM I-4323
(FAD-2018-0015; CRL/180015)**



**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-2018-0015 - CRL/180015**

Name of Product ***Lactobacillus hilgardii CNCM I-4785 and
Lactobacillus buchneri CNCM I-4323***

Active Agent (s): **Lactobacillus hilgardii CNCM I-4785;
Lactobacillus buchneri CNCM I-4323**

Rapporteur Laboratory: **Centro di referenza nazionale per la
sorveglianza ed il controllo degli alimenti
per gli animali (CReAA), Torino, Italy**

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Date: **03/07/2018**

Report approved by: **Christoph von Holst**
Date: **04/07/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 under the category / functional group 1(k) 'technological additives' / 'silage additives', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the feed additive for all animal species and categories.

According to the Applicant, the active substance in the *feed additive* consists in viable cells of the non-genetically modified strains *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323. The *feed additive* is to be marketed as a powder constituted by *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in a 1:1 ratio, containing a minimum concentration of total lactobacilli 1.5×10^{11} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be added to *silage* at a minimum dose of 3×10^8 CFU/kg fresh *silage*.

For the enumeration of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in the *feed additive* and *premixtures* of silage additives, the Applicant submitted the ring-trial validated spread plate method EN 15787 which was already evaluated by EURL in the frame of previous *Lactobacillus* spp. dossiers. Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in the *feed additive* per se and *premixtures*.

Since the unambiguous determination of the content of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* initially added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend any method for official control to determine the *feed additive* in *silage*.

For the identification of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

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

Lactobacillus hilgardii CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323, technological additives, silage additives, all animal species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 under the category / functional group 1(k) 'technological additives' / 'silage additives', according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of the *feed additive* for all animal species and categories [1,2].

According to the Applicant, the *feed additive* contains, as active substance, viable freeze-dried cells of the non-genetically modified strains *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323. The strains are deposited at the Collection Nationale de Cultures de Microorganismes (CNCM), Institute Pasteur, Paris [2,3].

The *feed additive* is to be marketed as a powder constituted by *Lactobacillus hilgardii* CNCM I4785 and *Lactobacillus buchneri* CNCM I-4323 in a 1:1 ratio, containing a minimum concentration of total lactobacilli 1.5×10^{11} Colony Forming Unit (CFU)/g [4].

The *feed additive* is intended to be added to *silage* at a minimum dose of 3×10^8 CFU/kg fresh *silage* [2,5].

Note: The EURL previously evaluated the analytical methods for the determination of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in the frame of other dossiers e.g. FAD 2016-0060, FAD-2016-0050 [6].

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 *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in *feed additive* and *premixtures* of silage additives, the Applicant proposed the ring-trial validated spread plate method EN 15787 [3,7,8].

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 and must be placed in anaerobiosis for enumeration of lactobacilli. The performance characteristics of the CEN method reported after logarithmic transformation of the CFU values 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;

In addition, the EURL calculated a limit of quantification (LOQ) of 3×10^3 CFU/g applying the recommendations of ISO 7218 [9].

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in the *feed additive* and *premixtures* of silage additives.

The Applicant did not provide any experimental method or data for the determination of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in *silage*. Furthermore, the unambiguous determination of the content of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 initially added to *silage* is not achievable by analysis.

Therefore, the EURL cannot evaluate nor recommend any method for official control to determine *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in *silage*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

Evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the identification of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323, the Applicant applied a genetic characterization of the strains by restriction Pulsed Field Gel Electrophoresis (rPFGE), Random Amplification of Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR) and partial sequencing of the 16s rDNA [3].

The EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains [10].

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.

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 *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 and the ring-trial validated spread plate method EN 15787 for the enumeration of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 in the *feed additive* and *premixtures* of silage additives.

Since the unambiguous determination of the content of *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 initially added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend any method for official control to determine the micro-organisms of concern in *silage*.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in the feed additive and premixtures: 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 *Lactobacillus hilgardii* CNCM I-4785 and *Lactobacillus buchneri* CNCM I-4323 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, Reference SANCO/G1: Forw. Appl. 1831/0022-2018
- [2] *Application, Proposal for Register Entry – Annex A
- [3] *Technical dossier, Section II: 2.2 Characterisation of the Active substance(s)/agent(s)
- [4] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition
- [5] *Technical dossier, Section II: 2.5 Conditions of use of the additive
- [6] EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0050-lacto_hilgardii.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0060-lactobacillus_buchneri.pdf
- [7] EN 15787:2009. Animal feeding stuffs. Isolation and enumeration of *Lactobacillus* spp.
- [8] *Technical dossier, Section II: 2.6 Methods of Analysis
- [9] EN ISO 7218:2007 – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
- [10] 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) and Report 20873/3 EN (2002) ISBN 92-894-6252-3 (Vol. III)

*Refers to Dossier no: FAD-2018-0015

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is Centro di Referenza Nazionale per la sorveglianza ed il controllo degli Alimenti per gli Animali (CReAA), Torino, Italy. 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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