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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

Preparation of Propionibacterium freudenreichii DSM 33189 and Lactobacillus buchneri DSM 12856

(FAD-2021-0007; CRL/200015)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2021-0007 - CRL/200015**
Name of Product:	**Preparation of Propionibacterium freudenreichii DSM 33189 and Lactobacillus buchneri DSM 12856**
Active Agent (s):	**Propionibacterium freudenreichii DSM 33189 and Lactobacillus buchneri DSM 12856**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**María José González de la Huebra** **24/11/2021**
Report approved by: Date:	**Christoph von Holst** **29/11/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *a preparation of Propionibacterium freudenreichii* DSM 33189 *and Lactobacillus buchneri* DSM 12856 under the category/functional group 1(k) "technological additives"/"silage additives", according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all animal species.

According to the Applicant, the *feed additive* contains a non-genetically modified strains of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856 as *active substances* with a minimum content of 1 x 10^{11} and 4 x 10^{11} Colony Forming Units (CFU) / g *feed additive*, respectively.

The *feed additive* is intended to be added into *silage* through its aqueous suspension at a minimum dose of the *active substances* of 1 x 10^{8} CFU / kg fresh *silage*.

For the genetic identification of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856 the EURL recommends for official control the pulsed-field gel electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains.

For the enumeration of *Lactobacillus buchneri* DSM 12856 in the *feed additive* the EURL recommends for official control the ring-trial validated EN 15787 method.

For the enumeration of *Propionibacterium freudenreichii* DSM 33189 in the *feed additive* the EURL recommends for official control the pour plate method on caseine peptone, yeast extract, sodium lactate and L-cysteine agar (based on ISO 27205 standard method).

Since the unambiguous enumeration of content of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856 initially added to *silage* is not experimentally achievable, the EURL is not able to evaluate or recommend any method for official control for the enumeration of *these microorganisms* in *silage*.

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

Propionibacterium freudenreichii DSM 33189, *Lactobacillus buchneri* DSM 12856, technological additives, silage additives, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for a *preparation of Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856 under the category/functional group 1(k) "technological additives"/"silage additives", according to Annex I of Regulation (EC) No 1831/2003 [1,2]. The authorisation is sought for the use of the *feed additive* for all animal species [2].

According to the Applicant, the *feed additive* contains a non-genetically modified strains of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856 as active substances with a minimum content of 1 x 10^{11} and 4 x 10^{11} Colony Forming Units (CFU) / g *feed additive*, respectively [3,4]. The strains are deposited at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under the following deposit numbers: Propionibacterium freudenreichii (Lac 1425) - DSM 33189 and Lactobacillus buchneri 218 - DSM 12856 [4].

The *feed additive* is intended to be added into *silage* through its aqueous suspension at a minimum dose of the *active substances* of 1 x 10^{8} CFU / kg fresh *silage* [5].

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 a *preparation of Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856 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)

Lactobacillus buchneri DSM 12856

For the enumeration of *Lactobacillus buchneri* DSM 12856 in the *feed additive*, the Applicant proposed [6] the ring-trial validated spread plate method EN 15787 [7].

According to the protocol of the method, the sample is suspended in phosphate buffered saline and further diluted in peptone salt solution; the appropriate dilutions are then spread on MRS (de Man, Rogosa, Sharp) agar plates. The agar plates are incubated anaerobically at 37 ºC for 48 to 72 h [7].

The performance characteristics reported from the validation study of the EN 15787 method after logarithmic transformation of the CFU values are the following: a standard deviation for *repeatability* (S_r) of 0.24 $\log_{10}$ CFU / g and a standard deviation for *reproducibility* (S_R) ranging from 0.29 to 0.38 $\log_{10}$ CFU / g [7]. In addition, following the recommendations of ISO 7218 [8], a limit of quantification (LOQ) of 3 x 10^3 CFU / g can be calculated.

The Applicant performed the analysis of the *feed additive* samples in the frame of shelf life stability studies [9] and standard deviation for *repeatability* (S_r) ranging from 0.02 to 0.05 $\log_{10}$ CFU / g was derived for the content of *Lactobacillus buchneri* DSM 12856 in the *feed additive* of 4 x 10^{11} CFU / g [10]. In addition, the Applicant performed the stability studies of the *feed additive* in water [11] and S_r ranging from 0.04 to 0.05 $\log_{10}$ CFU / g was calculated for the content of *Lactobacillus buchneri* DSM 12856 in water ranging from 2.6 x 10^{10} to 3.2 x 10^{10} CFU / ml [10].

Furthermore, the EURL previously evaluated and recommended the above mentioned method for the enumeration of the *Lactobacillus buchneri* DSM 12856 strain [12], which was further authorised as a silage additive by the Commission Implementing Regulation (EU) No 1263/2011 [13].

Based on the above mentioned performance characteristics and the available information, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lactobacillus buchneri* DSM 12856 in the *feed additive.*

For the enumeration of *Lactobacillus buchneri* DSM 12856 in *silage*, the Applicant proposed the above mentioned EN 15787 method [6, 7]. However, the unambiguous enumeration of content of *Lactobacillus buchneri* DSM 12856 initially added to *silage* is not experimentally achievable. Therefore, the EURL is not able to evaluate or recommend any method for official control for the enumeration of *Lactobacillus buchneri* DSM 12856 in *silage*.

<u>*Propionibacterium freudenreichii* DSM 33189</u>

For the enumeration of *Propionibacterium freudenreichii* DSM 33189 in the *feed additive*, the Applicant submitted a pour plate method [14] based on ISO 27205 standard method [15].

According to the method of the Applicant, the sample (1 g) is suspended in 9 ml of an aqueous buffer for dilution containing nutrient broth (8 g / L), potassium hydrophosphate (2 g / L) and potassium dihydrophosphate (1 g / L). The aliquots, after the appropriate dilutions of the initial suspension with the above mentioned buffer, are poured on agar plates,

containing caseine peptone, yeast extract, sodium lactate and L-cysteine. The plates are incubated anaerobically at 30 ºC for 72 h [14].

The Applicant performed the analysis of the *feed additive* samples in the frame of shelf life stability studies [9] and standard deviation for *repeatability* (S_r) ranging from 0.04 to 0.11 $\log_{10}$ CFU / g was derived for the content of *Propionibacterium freudenreichii* DSM 33189 in the *feed additive* of 1 x 10^{11} CFU / g [10]. In addition, the Applicant performed the stability studies of the *feed additive* in water [11] and S_r ranging from 0.07 to 0.10 $\log_{10}$ CFU / g was calculated for the content of *Propionibacterium freudenreichii* DSM 33189 in water ranging from 8.3 x 10^9 to 1.1 x 10^{10} CFU / ml [10].

Based on the above mentioned performance characteristics and the overall available information, the EURL recommends for official control the pour plate method on caseine peptone, yeast extract, sodium lactate and L-cysteine agar (based on ISO 27205 standard method) for the enumeration of *Propionibacterium freudenreichii* DSM 33189 in the *feed additive*.

For the enumeration of *Propionibacterium freudenreichii* DSM 33189 in *silage*, the Applicant proposed a slightly modified protocol [6] of the above mentioned pour plate method [16].

However, the unambiguous enumeration of content of *Propionibacterium freudenreichii* DSM 33189 initially added to *silage* is not experimentally achievable. Therefore, the EURL is not able to evaluate or recommend any method for official control for the enumeration of *Propionibacterium freudenreichii* DSM 33189 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)

An 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 genetic identification of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856, the Applicant proposed a whole genome sequencing [17].

The EURL recommends instead for official control the pulsed-field gel electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains [18]. This methodology for bacterial identification of authorised additives at a strain level is currently being evaluated by the CEN Technical Committee 327 to become a European Standard [19].

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 the current authorisation, the EURL recommends for the official control: (i) Pulsed-Field Gel Electrophoresis (PFGE) for the identification of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856; (ii) the ring-trial validated spread plate method EN 15787 for the enumeration of *Lactobacillus buchneri* DSM 12856 in the *feed additive*; and (iii) pour plate method on caseine peptone, yeast extract, sodium lactate and L-cysteine agar (based on ISO 27205 standard method) for the enumeration of *Propionibacterium freudenreichii* DSM 33189 in the *feed additive*.

The unambiguous enumeration of content of *Lactobacillus buchneri* DSM 12856 and of *Propionibacterium freudenreichii* DSM 33189 initially added to *silage* is not experimentally achievable. Therefore, the EURL is not able to evaluate or recommend any method for official control for the enumeration of *Lactobacillus buchneri* DSM 12856 and *Propionibacterium freudenreichii* DSM 33189 in *silage*.

Recommended text for the register entry (analytical method)

For the identification of *Propionibacterium freudenreichii* DSM 33189 and *Lactobacillus buchneri* DSM 12856:

- Pulsed-Field Gel Electrophoresis (PFGE)

For the enumeration of *Lactobacillus buchneri* DSM 12856 in the *feed additive*:

- Spread plate method on MRS agar (EN 15787)

For the enumeration of *Propionibacterium freudenreichii* DSM 33189 in the *feed additive*:

- Pour plate method on caseine peptone, yeast extract, sodium lactate and L-cysteine agar

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of a *preparation of Propionibacterium freudenreichii* DSM 33189 *and Lactobacillus buchneri* DSM 12856 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 SANTE/E5: FORW.APPL. 1831-0012-2021

[2] *Application, Annex 1 – submission number 1612854785941-2816

[3] *Technical dossier, Section II: II.1. Identity of the additive

[4] *Technical dossier, Section II: II.2. Characterisation of the Microorganisms

[5] *Technical dossier, Section II: II.5. Conditions of use

[6] *Technical dossier, Section II: II.6.1. Methods of analysis of the active substances

[7] EN 15787 – Animal feeding stuffs - Isolation and enumeration of *Lactobacillus* spp.

[8] EN ISO 7218:2007 – Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations

[9] *Technical dossier, Section II – Annex II_4_1

[10] Supplementary information – EURL calculations

[11] *Technical dossier, Section II – Annex II_4_2

[12] EURL Evaluation Report : https://ec.europa.eu/jrc/sites/default/files/FinRep-uorg-silage-group1.pdf

[13] COMMISSION IMPLEMENTING REGULATION (EU) No 1263/2011 of 5 December 2011 concerning the authorisation of *Lactobacillus buchneri* (DSM 16774), *Lactobacillus buchneri* (DSM 12856), *Lactobacillus paracasei* (DSM 16245), *Lactobacillus paracasei* (DSM 16773), *Lactobacillus plantarum* (DSM 12836), *Lactobacillus plantarum* (DSM 12837), *Lactobacillus brevis* (DSM 12835), *Lactobacillus rhamnosus* (NCIMB 30121), *Lactococcus lactis* (DSM 11037), *Lactococcus lactis* (NCIMB 30160), *Pediococcus acidilactici* (DSM 16243) and *Pediococcus pentosaceus* (DSM 12834) as feed additives for all animal species, OJ L 322, 6.12.2011

[14] *Technical dossier, Section II – Annex_II_6_6

[15] ISO 27205:2010 – Fermented milk products - Bacterial starter cultures - Standard of identity

[16] *Technical dossier, Section II – Annex_II_6_7

[17] *Technical dossier, Section II: II.6.3. Methods of the analysis relating to the identity and characterisation of the additive

[18] 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)

[19] prEN 17697 – Animal feeding stuffs: Methods of analysis – PFGE typing of Lactobacilli, Pediococci, Enterococci and Bacilli in animal feeds

*Refers to Dossier no: FAD-2021-0007

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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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)