



**EUROPEAN COMMISSION**  
DIRECTORATE GENERAL  
JOINT RESEARCH CENTRE  
Directorate F – Health, Consumers and Reference Materials  
**European Union Reference Laboratory for Feed Additives**

JRC F.5/CvH/MGH/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**

**Pediococcus acidilactici DSM 13943, Lactobacillus plantarum DSM 8862  
and Lactobacillus plantarum DSM 8866  
(FAD-2018-0002; CRL/180002)**





**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-2018-0002 - CRL/180002**

Name of Product ***Pediococcus acidilactici* DSM 13943,  
*Lactobacillus plantarum* DSM 8862  
and *Lactobacillus plantarum* DSM 8866**

Active Agent (s): ***Pediococcus acidilactici* DSM 13943,  
*Lactobacillus plantarum* DSM 8862  
and *Lactobacillus plantarum* DSM 8866**

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: **29/10/2018**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for a preparation of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 (*Minalac*) under the category / functional group 4(d) 'zootechnical additives' / other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for minks (species type pets and other non-food producing animals).

According to the Applicant, the active substance in the *feed additive* consists in viable cells of the non-genetically modified strains *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866.

The *feed additive* "Minalac" is a free flowing, off-white slightly yellow powder constituted by a mixture of the three strains at a final culture preparation of  $1 \times 10^{10}$  CFU/g and using lactose as carrier. The *feed additive* is intended to be added to *feed* at a minimum dose of  $6 \times 10^8$  CFU/kg and a maximum dose of  $2.1 \times 10^9$  CFU/kg complete *feedingstuffs*.

For the enumeration of *P. acidilactici* DSM 13943; *L. plantarum* DSM 8862 and *L. plantarum* DSM 8866 in the *feed additive*, the Applicant proposes the internationally recognised ISO 15214 spread plate method, specifically designed for the enumeration of mesophilic lactic acid bacteria, for which no performance characteristics are available.

The EURL identified instead the ring-trial validated spread plate method EN 15786:2009 which was already evaluated by EURL in the frame of previous *Pediococcus spp.* dossiers.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated method EN 15786 for the enumeration of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 or the internationally recognised ISO 15214:1998 spread plate method for the enumeration of mesophilic lactic acid bacteria in the *feed additive* and in *feedingstuffs*.

For the identification of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866, 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

*Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862, *Lactobacillus plantarum* DSM 8866, Minalac, zootechnical additives, other zootechnical additives', mink.

## 1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 (Minalac) under the category / functional group 4(d) 'zootechnical additives' / 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of the *feed additive* for mink (*Neovison vison*) [1,2].

According to the Applicant, the *feed additive* contains, as active substance, viable cells of the non-genetically modified strains *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866. The strains are deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig - Germany [2,3].

The *feed additive* "Minalac" is a free flowing, off-white slightly yellow powder constituted by a mixture of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 at a final culture preparation of  $1 \times 10^{10}$  CFU/g and using lactose as carrier [2, 4].

The *feed additive* is intended to be added to *feed* at a minimum dose of  $6 \times 10^8$  CFU/kg and a maximum dose of  $2.1 \times 10^9$  CFU/kg *complete feedingstuffs* [2,5].

Note: The EURL previously evaluated the analytical methods for the determination of *Lactobacillus plantarum* and for the determination of *Pediococcus acidilactici*, in the frame of several dossiers [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 *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 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 *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 in feed additive, the Applicant proposed the 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). The dry product is homogenized in a peptone and Tween 80 buffered solution, then, according to the expected cell count, serial dilutions are performed in phosphate buffered saline solution and finally 1 mL of the final dilution is inoculated into MRS agar plates, at pH 5.7, and incubated at 30 °C for 72 hours [7,8]. Due to the growth rate and size of the colonies on MRS agar, *Pediococcus* species are easily distinguished from *Lactobacillus* species, because the latter growth faster, forming larger colonies. No performance characteristics for the method ISO 15214 are available.

Additionally, the EURL identified the ring-trial validated spread plate method EN 15786:2009 [10] dedicated to the isolation and enumeration of *Pediococcus* spp. in samples containing pediococci as a single bacterial component or in a mixture with other microorganisms, such as lactobacilli.

The sample is suspended and diluted in a buffer solution; the appropriate dilutions are then spread on the appropriate media. As in this feed additive (*Minalac*) pediococci is expected in combination with lactobacilli, MRS (de Man, Rogosa, Sharp) agar plates supplemented with Triphenyl Tetrazodium Chloride (TTC) should be used in order to allow the differentiation

between pediococci and lactobacilli colonies by different colouration after anaerobic incubation. The agar plates supplemented with TTC are incubated at 37 °C for 48 hours before enumeration. The following performance characteristics of the CEN method, expressed in terms of precision, were calculated after logarithmic transformation of the CFU values obtained in the ring-trial validation study in samples where pediococci were present in the sample in combination with lactobacilli:

- a standard deviation for repeatability ( $S_r$ ) ranging from 0.04 to 0.16  $\log_{10}$  CFU/g;
- a standard deviation for reproducibility ( $S_R$ ) ranging from 0.14 to 0.22  $\log_{10}$  CFU/g;

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

The Applicant, upon request of the EURL, confirmed the equivalence between the standard mentioned above i.e. EN 15786:2009 and the ISO 15214, and thus both can be considered suitable for the enumeration of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 in the feed additive (Minalac) and in the feedingstuffs.

Consequently the EURL recommends for official control the ring-trial validated EN 15786 method for the enumeration of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 or the internationally recognised ISO 15214:1998 method for enumeration of mesophilic lactic acid bacteria in the feed additive and in feedingstuffs.

***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)***

The 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 *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 in feed additive, the Applicant applied a genetic characterization of the strains based on 16S rDNA sequence similarity for *Pediococcus acidilactici* DSM 13943 and on amplification of variable regions of bacterial 16S rRNA (nested PCR approach and denaturing gradient gel electrophoresis of PCR amplicons) for both *Lactobacillus plantarum* strains [3].

The EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains [11] that contrary to the genetic-based methods, does not require for its application any specific material to be provided by the Applicant.

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 the ring-trial validated spread plate method EN 15786 for the enumeration of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 or the internationally recognised ISO 15214:1998 pour plate method for enumeration of mesophilic lactic acid bacteria in the *feed additive* and in *feedingstuffs*.

##### ***Recommended text for the register entry (analytical method)***

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in the *feed additive* and in the *feedingstuffs*: Spread plate method on MRS agar supplemented with TTC (EN 15786) or pour plate method on MRS agar (ISO 15214:1998)

#### **5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL**

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Pediococcus acidilactici* DSM 13943, *Lactobacillus plantarum* DSM 8862 and *Lactobacillus plantarum* DSM 8866 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. 1831/0005-2018
- [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-2011-0004.pdf>  
<https://ec.europa.eu/jrc/sites/default/files/finrep-FAD-2015-0013-lactobacillus%20plantarum.pdf>  
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2008-0015.pdf>  
<http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD2009-0025.pdf>  
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0109.pdf>  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad\\_2015\\_0035\\_lactob\\_plantarum.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad_2015_0035_lactob_plantarum.pdf)

- [7] \*Technical dossier, Section II: 2.6 Methods of Analysis
- [8] ISO 15214:1998. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of mesophilic lactic acid bacteria.
- [9] EN 15786:2009 "Animal feeding stuffs – Isolation and enumeration of *Pediococcus spp*"
- [10] EN ISO 7218:2007 – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
- [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) and Report 20873/3 EN (2002) ISBN 92-894-6252-3 (Vol. III)

\*Refers to Dossier no: FAD-2018-0002

## 7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the 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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centre wallon de Recherches agronomiques (CRA-W), Gembloux (BE)