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Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/ZE/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

Preparation of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609 (MixBaLac)

(FAD-2019-0090; CRL/190056)

European Union Reference Laboratory
Feed Additives

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-2019-0090 - CRL/190056**
Name of Product:	***Preparation of Bacillus subtilis CNCM I-4606, Bacillus subtilis CNCM I-5043, Bacillus subtilis CNCM I-4607 and Lactococcus lactis CNCM I-4609 (MixBaLac)***
Active Agent (s):	**Bacillus subtilis CNCM I-4606** **Bacillus subtilis CNCM I-5043** **Bacillus subtilis CNCM I-4607** **Lactococcus lactis CNCM I-4609**
Rapporteur Laboratory:	**Państwowy Instytut Weterynaryjny, Puławy, Poland**
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Report approved by: Date:	**Christoph von Holst** **13/07/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for the *preparation of Bacillus subtilis CNCM I-4606, Bacillus subtilis CNCM I-5043, Bacillus subtilis CNCM I-4607 and Lactococcus lactis CNCM I-4609 (MixBaLac)* under the category/ functional group 1(n) 'technological feed additive'/'hygiene condition enhancer', 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 as an active substance the viable spores of *Bacillus subtilis* (CNCM I-4606, CNCM I-5043, CNCM I-4607) and live cells of *Lactococcus lactis* (CNCM I-4609). The *feed additive* contains a minimum content of $2x10^9$ Colony Forming Units (CFU) of total *Bacillus subtilis* /g *feed additive* and a minimum content of $2x10^9$ CFU of *Lactococcus lactis* /g *feed additive*.

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* at a minimum dose of $1x10^9$ CFU of total *Bacillus subtilis* /kg (or L) complete *feedingstuffs* and at a minimum dose of $1x10^9$ CFU of *Lactococcus lactis* /kg (or L) complete *feedingstuffs*.

For the identification of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of the total *Bacillus subtilis* (CNCM I-4606, CNCM I-5043 and CNCM I-4607) in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread-plate CEN method EN 15784.

Based on the performance characteristics available, the EURL recommends this method for official control for the enumeration of the total *Bacillus subtilis* (CNCM I-4606, CNCM I-5043 and CNCM I-4607) in the *feed additive*, *premixtures* and *feedingstuffs*.

For the enumeration of *Lactococcus lactis* (CNCM I-4609) in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed the ISO 15214 pour-plate method dedicated for the enumeration of mesophilic lactic acid bacteria in food and feed.

Based on acceptable applicability data of the method, the EURL recommends it for official control for the enumeration of *Lactococcus lactis* (CNCM I-4609) 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Bacillus subtilis CNCM I-4606, Bacillus subtilis CNCM I-5043, Bacillus subtilis CNCM I-4607, Lactococcus lactis CNCM I4609, MixBaLac, technological additives, hygiene condition enhancers, all animal species

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for the *preparation of Bacillus subtilis CNCM I-4606, Bacillus subtilis CNCM I-5043, Bacillus subtilis CNCM I-4607 and Lactococcus lactis CNCM I-4609 (MixBaLac)* under the category/ functional group 1(n) 'technological feed additives'/'hygiene condition enhancers', 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 [1,2].

According to the Applicant, the *feed additive* contains as active substance the viable spores of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and live cells of *Lactococcus lactis* CNCM I-4609. The strains are deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) Institute Pasteur, France under the deposit numbers CNCM I-4606, CNCM I-5043, CNCM I-4607 and CNCM I-4609, respectively [3]. The *feed additive* is marketed as beige powder, containing a minimum content of $2x10^9$ Colony Forming Units (CFU) of total *Bacillus subtilis* /g *feed additive* and a minimum content of $2x10^9$ CFU of *Lactococcus lactis* /g *feed additive* [4].

The *feed additive* is intended to be to be used directly into *feedingstuffs* or through *premixtures* at a minimum dose of $1x10^9$ CFU of total *Bacillus subtilis* /kg (or L) complete *feedingstuffs* and at a minimum dose of $1x10^9$ CFU of *Lactococcus lactis* /kg (or L) complete *feedingstuffs* [5].

Note: The EURL previously evaluated analytical methods for the enumeration of *Bacillus* spp. and *Lactococcus* spp. 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 the *preparation of Bacillus subtilis CNCM*

I-4606, Bacillus subtilis CNCM I-5043, Bacillus subtilis CNCM I-4607 and Lactococcus lactis CNCM I-4609 (MixBaLac) and their suitability 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 the total *Bacillus spp*. (CNCM I-4606, CNCM I-5043, CNCM I-4607) in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated spread-plate CEN method EN 15784 [7].

Twenty grams of the *feed additive* (or 50 g of *premixtures* or *feedingstuffs*) are suspended in a phosphate buffer saline (PBS) (or in 0.2 % sodium hydroxide solution for *premixtures* or *feedingstuffs*). From this first dilution, one new dilution is prepared with "Polysorbate 80" peptone salt solution and heat-treated at 80°C for 10 min. After the cooling down to room temperature, decimal dilutions are prepared from the heat-treated suspension, spread-plated on tryptone soya agar and incubated at 37°C for 16 to 24 h, aerobically [7].

The following performance characteristics were reported from the ring-trial validation study after a logarithmic transformation of the CFU values [7]: a standard deviation for *repeatability* (s_r) ranging from 0.07 to 0.09 $\log_{10}$CFU/g and a standard deviation for reproducibility (s_R) ranging from 0.32 to 0.35 $\log_{10}$CFU/g. In addition, the EURL calculated a limit of quantification (LOQ) of $3x10^4$ CFU/g following the recommendations of the ISO 7218 standard [8].

For the enumeration of *Lactococcus lactis* (CNCM I-4609) in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed the ISO 15214 pour-plate method [9]. This method is dedicated for the enumeration of mesophilic lactic acid bacteria in food and feed.

Following the method, a sample is suspended and diluted in a buffer solution. After appropriate dilutions the solutions are poured on plates filled with MRS (de Man, Rogosa, Sharp) agar at pH 5.7. The plates are incubated at 30 °C for 72 hours [9].

The Applicant demonstrated the fitness-for-purpose of the ISO method by providing experimental data obtained in the frame of the batch-to-batch variation for the *feed additive* in the stability and efficacy studies for *premixtures* and *feedingstuffs*, respectively [10].

Based on the performance characteristics available the EURL recommends for official control the above mentioned CEN and ISO methods, respectively, for the enumeration of the total

Bacillus spp. (CNCM I-4606, CNCM I-5043, CNCM I-4607) and *Lactococcus lactis* (CNCM I-4609) in the *feed additive*, *premixtures* and *feedingstuffs*.

Note: The EN 15784 method is not applicable to mineral feeds composed mainly of minerals and containing at least 40% crude ash. For these matrices laboratories may consider using the ring-trial validated VDLUFA method 28.2.2 instead [11].

During the review process, some NRLs suggested to recommend for the enumeration of *Lactococcus lactis* (CNCM I-4609) the EN 15787 standard method dedicated for the enumeration *Lactobacillus spp.*, instead (or in addition) to the above-mentioned ISO 15214 method. While the Applicant presented no proofs of the applicability of the EN 15787 method for the enumeration of *Lactococcus lactis* (CNCM I-4609) in the *feed additive*, *premixtures* and *feedingstuffs*, both method protocols are similar e.g. in terms of the use of the same MRS medium.

Therefore, the EURL concludes that the CEN method (EN 15787) can be considered also suitable for official control for the enumeration of *Lactococcus lactis* (CNCM I-4609), provided that the equivalence of the both methods (ISO 15214 and EN 15787), in terms of precision and content in CFU/g *feed additive* or CFU/kg *premixtures* and *feedingstuffs* regarding the analysis of samples of this dossier, has been demonstrated.

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 morphological characterisation and isolation of the individual *Bacillus subtilis* and *Lactococcus lactis* strains in the *feed additive*, the Applicant proposed blood agar media (for the *Bacillus subtilis* strains) and reinforced clostridial agar for microbiology (RCM) combined with De Man, Rogosa and Sharpe (MRS) agar for the *Lactococcus lactis* strain [12]. For the genetic identification of *Bacillus subtilis* (CNCM I-4606, CNCM I-5043, CNCM I-4607) and *Lactococcus lactis* (CNCM I-4609) at strain level, the Applicant proposed a Pulsed-Field Gel Electrophoresis (PFGE) method [12].

The EURL recommends for official control PFGE, a generally recognised methodology for genetic identification of bacterial strains. This methodology for microbial identification of authorised probiotics at strain level is currently being evaluated by the CEN Technical Committee 327 to become an European Standard [13].

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 (i) Pulsed Field Gel Electrophoresis (PFGE) for the genetic identification of *Bacillus subtilis* CNCM I-4606, *Bacillus subtilis* CNCM I-5043, *Bacillus subtilis* CNCM I-4607 and *Lactococcus lactis* CNCM I-4609; (ii) ring-trial validated spread-plate method EN 15784 for the enumeration of the total *Bacillus spp.* (CNCM I-4606, CNCM I-5043, CNCM I-4607) in the *feed additive*, *premixtures* and *feedingstuffs*; and (iii) the pour-plate ISO 15214 method for the enumeration of *Lactococcus lactis* CNCM I-4609 in the *feed additive*, *premixtures* and *feedingstuffs*.

Note: The EN 15784 method is not applicable to mineral feeds composed mainly of minerals and containing at least 40% crude ash. For these matrices, laboratories may consider using the ring-trial validated VDLUFA method 28.2.2 instead.

In addition, the EURL concludes that the CEN method (EN 15787) can be considered also suitable for official control for the enumeration of *Lactococcus lactis* (CNCM I-4609), provided that the equivalence of the both methods (ISO 15214 and EN 15787), in terms of precision and content in CFU/g *feed additive* or CFU/kg *premixtures* and *feedingstuffs* regarding the analysis of samples of this dossier, has been demonstrated.

Recommended text for the register entry (analytical method)

Identification of *Bacillus subtilis* (CNCM I-4606, CNCM I-5043, CNCM I-4607) and *Lactococcus lactis* (CNCM I-4609):

- Pulsed Field Gel Electrophoresis (PFGE)

Enumeration in the *feed additive*, *premixtures* and *feedingstuffs*:

- Spread-plate method on tryptone soya agar (TSA) (EN 15784) for the total *Bacillus subtilis* strains (CNCM I-4606, CNCM I-5043, CNCM I-4607) and
- Poured-plate method on De Man, Rogosa and Sharpe (MRS) agar – ISO 15214 for the *Lactococcus lactis* strain (CNCM I-4609)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *the preparation of Bacillus subtilis CNCM I-4606, Bacillus subtilis CNCM I-5043, Bacillus subtilis CNCM I-4607 and Lactococcus lactis CNCM I-4609 (MixBaLac)* 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. 1831-0002-2020

[2] *Application, Annex I – submission number 1576506592582

[3] *Technical dossier, Section II, 2.2. Characterisation of the active substance(s)/agent(s)

[4] *Technical dossier, Section II, 2.1. Identity of the additive

[5] *Technical dossier, Section II, 2.5. Conditions of the use of the additive

[6] #EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0074_correlink.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0044_syncra.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0024-technospore.pdf
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https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2013-0048_lavipan_online.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0032.pdf

[7] EN 15784:2009 – Animal feeding stuffs – Isolation and enumeration of presumptive *Bacillus spp.*

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

[9] ISO 15214:1998 – Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of mesophilic lactic acid bacteria

[10] *Supplementary information – CRL-190056 Cover letter 02.06.2020

[11] VDLUFA method – Enumeration of *Bacillus licheniformis* and *Bacillus subtilis* (VDLUFA Methodenbuch Bd. III, 28.2.2)

[12] *Technical dossier, Section II, 2.6. Methods of analysis and reference samples

[13] 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. 1) and Report 20873/3 EN (2002) ISBN 92-894-6252-3 (Vol. 3)

*Refers to Dossier no: FAD-2019-0090

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the Państwowy Instytut Weterynaryjny, Puławy, Poland. 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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- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)