



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

Subject: Addendum to the EURL evaluation report

Reference: FEED-2021-0847 – *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 (K-9 Heritage Probiotic Blend), JRC F.5/CvH/ZE/AS/Ares(2023)1533472

Upon the request from EFSA [1], the EURL evaluated the supplementary information provided [2,3] in the frame of the dossier FEED-2021-0847 for the enumeration of total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 strains in the *feed additive*. In the frame of former evaluation [4], the EURL was not able to recommend the proposed single-laboratory validated spread-plated method using Man, Rogosa, Sharp (MRS) agar [5] as for the enumeration of the total content of the four microorganisms in the *feed additive* the Applicant presented no detailed verification data from a second independent laboratory. However, in the frame of this additional supplementary information, the Applicant submitted proper verification studies [2,3] for the enumeration of the mentioned microorganisms in the *feed additive* when analysing five batches by using the above-mentioned method [5].

The following performance characteristics were obtained (as partially re-calculated by the EURL [6]) in the frame of the verification studies for logarithmically transformed CFU values for total content of the four microorganisms ranging from 11.3 to 12.9 log₁₀ CFU / g of the *feed additive* [2,3]:

- a standard deviation for *repeatability* (S_r) of 0.01 log₁₀ CFU / g; and
- a standard deviation for *intermediate precision* (S_{ip}) ranging from 0.01 to 0.24 log₁₀ CFU / g.

Based on the experimental data, the EURL recommends for official control the single-laboratory validated and further verified spread-plated method on MRS agar for the enumeration of total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in the *feed additive*.

Recommended text for the registry entry (analytical method) (replacing the previous recommendations)

- Identification: Pulsed-Field Gel Electrophoresis (PFGE) or DNA sequencing methods

- Enumeration in the *feed additive*: spread-plated method using Man, Rogosa, Sharp (MRS) agar

References

- [1] Supplementary information – FEEDCO_EFSA-Q-2021-00383_K-9 Heritage Probiotic Blend® (CanBioCin)_FEED-2021-00847 - Request for an amendment of the EURL Final report, Ares(2023)2903736
- [2] Supplementary Information – 3rd Party Lab Method Validation Report for SOP #611.00-conf.pdf
- [3] Supplementary Information – QRA2301309_CanBiocin_Certificate of Analysis-conf.pdf
- [4] EURL report – FEED-2021-0847 (K-9 Heritage Probiotic Blend), JRC F.5/CvH/ZE/AS/Ares(2023)1533472)
- [5] Supplementary Information – Appendix-Q1-1-MICRB 611 SOP_non-conf
- [6] Supplementary Information – EURL calculation performance characteristics_amendment

Addendum

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- Reviewed and approved by María José González de la Huebra and Christoph von Holst (EURL-FA), respectively, Geel, 05/05/2023



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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Food and Feed Compliance



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**

**Lacticaseibacillus casei K9-1, Limosilactobacillus fermentum K9-2,
Levilactobacillus brevis WF-1B and Enterococcus faecium WF-3
(K-9 Heritage Probiotic Blend)**

(FEED-2021-0847; CRL/210052)



**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: **FEED-2021-0847 - CRL/210052**

Name of Product: ***Lacticaseibacillus casei K9-1,
Limosilactobacillus fermentum K9-2,
Levilactobacillus brevis WF-1B and
Enterococcus faecium WF-3 (K-9 Heritage
Probiotic Blend)***

Active Agent (s): **Lacticaseibacillus casei K9-1,
Limosilactobacillus fermentum K9-2,
Levilactobacillus brevis WF-1B and
Enterococcus faecium WF-3**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

Report prepared by: **Zigmas Ezerskis**

Report checked by: **María José González de la Huebra**
Date: **02/03/2023**

Report approved by: **Christoph von Holst**
Date: **02/03/2023**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for a lyophilised blend product of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B, *Enterococcus faecium* WF-3 (K-9 Heritage Probiotic Blend) under the category/functional group 4(b) "zootechnical additives"/"gut flora stabilisers", according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for pets and other non-food producing animals.

According to the Applicant, the *feed additive* contains a naturally occurring strains of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 as active substances with a minimum total content of 6.7×10^8 Colony Forming Units (CFU) / g *feed additive*. The *feed additive* is intended to be sprinkled on top of a pet feed daily at the dose corresponding to the total content of the active substances ranging from 0.5 to 3.0×10^9 CFU / animal / day.

For the identification of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 at a strain level, the EURL recommends Pulsed-Field Gel Electrophoresis (PFGE) or DNA sequencing methods.

For the enumeration of total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 strains in the *feed additive* the Applicant submitted a single-laboratory validated spread-plated method using Man, Rogosa, Sharp (MRS) agar and provided the EURL with the corresponding validation data. However, no detailed verification data from a second independent laboratory for the determination of the total content of the four microorganisms in the product were submitted by the Applicant.

Based on the experimental data available, the EURL is not able to recommend for official control the method proposed by the Applicant or any other method for the enumeration of total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in the *feed additive*.

For the enumeration of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in *premixtures* and *compound feed* the Applicant did not submit any method or data. Furthermore, in the conditions of use the Applicant did not express the dose of the product in *premixtures* or *compound feed* as CFU of the target microorganisms per mass of the mentioned matrices.

Therefore, the EURL is not able to recommend any method for the enumeration of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in *premixtures* and *compound feed*.

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

Lacticaseibacillus casei K9-1, Limosilactobacillus fermentum K9-2, Levilactobacillus brevis WF-1B, Enterococcus faecium WF-3, K-9 Heritage Probiotic Blend, zootechnical additives"/>"gut flora stabilisers", pets and other non-food producing animals.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for a lyophilised blend product of *Lacticaseibacillus casei K9-1, Limosilactobacillus fermentum K9-2, Levilactobacillus brevis WF-1B, Enterococcus faecium WF-3 (K-9 Heritage Probiotic Blend)* under the category / functional group 4(b) "zootechnical additives" / "gut flora stabilisers", according to Annex I of Regulation (EC) No 1831/2003 [1,2]. The authorisation is sought for the use of the *feed additive* for pets and other non-food producing animals [1,2].

According to the Applicant, the *feed additive* contains a naturally occurring strains of *Lacticaseibacillus casei K9-1, Limosilactobacillus fermentum K9-2, Levilactobacillus brevis WF-1B* and *Enterococcus faecium WF-3* as active substances with a minimum total content of 6.7×10^8 Colony Forming Units (CFU) / *g feed additive* [3]. The strains are deposited at the International Depository Authority of Canada (IDAC) under the following deposit numbers: *Lacticaseibacillus casei K9-1* - IDAC accession No. 210415-01, *Limosilactobacillus fermentum K9-2* - IDAC Accession No. 210415-02, *Levilactobacillus brevis WF-1B* - IDAC Accession No. 051120-02 and *Enterococcus faecium WF-3* - IDAC Accession No. 181218-03 [4].

The *feed additive* is intended to be sprinkled on top of a pet feed daily at the dose corresponding to the total content of the actives substances ranging from 0.5 to 3.0×10^9 CFU /animal / day [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 *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 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 total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 strains in the *feed additive* the Applicant proposed [6] and submitted a single-laboratory validated spread-plated method using Man, Rogosa, Sharp (MRS) agar [7].

According to the method, the sample of the *feed additive* is suspended in 0.1 % aqueous peptone solution. Decimal dilutions are prepared from the suspension, spread-plated on MRS agar and incubated, aerobically at 37 °C for 24 to 48 h [7].

The Applicant performed a single-laboratory validation study of the above described method using several batches of the product [8]. The following performance characteristics were obtained (as partially re-calculated by the EURL [9]) for logarithmically transformed CFU values for total content of the four microorganisms ranging from 9.0 to 9.6 log₁₀ CFU / g of the product [8,9]:

- a standard deviation for *repeatability* (S_r) ranging from 0.07 to 0.10 log₁₀ CFU / g; and
- a standard deviation for *intermediate precision* (S_{ip}) ranging from 0.07 to 0.14 log₁₀ CFU / g.

However, the Applicant presented no detailed verification data from a second independent laboratory for the determination of the total content of the four microorganisms in the product. Instead, one of the products of the Applicant was analysed in another laboratory applying the above mentioned method and the reported total content of lactobacilli microorganisms was higher than 8.48 log₁₀ CFU / g of the sample [10].

Based on the experimental data available, the EURL is not able to recommend for official control the method of the Applicant or any other method for the enumeration of total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in the *feed additive*.

For the enumeration of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in *premixtures* and

compound feed the Applicant did not submit any method or data. Furthermore, in the conditions of use the Applicant did not express the dose of the product in *premixtures* or *compound feed* as CFU of the total content of the target microorganisms per mass of the mentioned matrices [5].

Therefore, the EURL is not able to recommend any method for the enumeration of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in *premixtures* and *compound feed*.

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 identification of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 at strain level, the Applicant proposed Pulsed-Field Gel Electrophoresis (PFGE) [6] and has submitted the specific protocol including the results of the identification [11]. The PFGE methodology, which is a generally recognised methodology for the genetic identification of bacterial strains has been already recommended by the EURL in former reports for similar dossiers [12]. Furthermore, this methodology has been recently ring-trial validated [13] and is supposed to become a CEN Technical Specification [14]. In addition, for the identification of the microorganisms at strain level, the Applicant used DNA sequencing methods such as sequencing of the conserved regions of 16S ribosomal RNA gene and whole genome sequencing [15].

The EURL considers that both methodologies (PFGE and DNA sequencing methods) are suitable for official control for the bacterial identification of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 at a strain level.

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 the official control: (i) Pulsed-Field Gel Electrophoresis (PFGE) or ii) DNA sequencing methods for the identification of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3.

Based on the experimental data available, the EURL is not able to recommend for official control the method proposed by the Applicant or any other method for the enumeration of total content of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in the feed additive.

For the enumeration of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in premixtures and compound feed the Applicant did not submit any method or data, and in the conditions of use the dose of the product in premixtures or compound feed was not expressed as CFU of the total content of the target microorganisms per mass of the mentioned matrices. Therefore, the EURL is not able to recommend any method for the enumeration of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 in premixtures and compound feed.

Recommended text for the register entry (analytical method)

- Identification: Pulsed-Field Gel Electrophoresis (PFGE) or DNA sequencing methods

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Lacticaseibacillus casei* K9-1, *Limosilactobacillus fermentum* K9-2, *Levilactobacillus brevis* WF-1B and *Enterococcus faecium* WF-3 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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform – <https://webgate.ec.europa.eu/esfc/#/applications/1341>
<https://open.efsa.europa.eu/questions/EFSA-Q-2021-00383>
- [2] *Application, Annex 1
- [3] *Technical dossier, Section II: II.1.4. Purity
- [4] *Technical dossier, Section II: II.1.1. Name of the additive
- [5] *Technical dossier, Section II: II.5.1. Proposed mode of use in animal nutrition
- [6] *Technical dossier, Section II: II.6. Methods of analysis and reference samples
- [7] *Supplementary information – Appendix-Q1-1-MICRB 611 SOP_non-conf
- [8] *Supplementary information – Appendix-Q1-4-MVR-1100.00 Method Validation Report for SOP #611.00_conf
- [9] *Supplementary information – EURL calculation performance characteristics

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- [10] *Supplementary information – Appendix-Q1-2-MVR-1000 Validation report_conf
- [11] *Technical dossier, Section II – Annex_II_13
- [12] EURL reports: https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en
- [13] CEN project TC 327 WI00327127 (2020): DNA fingerprinting of lactobacilli, pediococci, enterococci and bacilli in animal feeds by pulsed field gel electrophoresis (PFGE) Draft Report of a validation trial
- [14] prEN 17697 – Animal feeding stuffs: Methods of analysis - PFGE typing of Lactobacilli, Pediococci, Enterococci and Bacilli in animal feeds
- [15] *Technical dossier, Section II – Annex_II_5

*Refers to Dossier no: FEED-2021-0847

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

The following National Reference Laboratories contributed to this report:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)