



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
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**

**Preparation of *Bacillus velezensis* (amyloliquefaciens) NRRL B-67647,
Bacillus pumilus NRRL B-67648, *Bacillus licheniformis* NRRL B-67649
(Microsaf®)
(FEED-2021-0928; CRL/210054)**



**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-2021-0928 - CRL/210054**

Name of Product: **Preparation of *Bacillus velezensis*
(*amyloliquefaciens*) NRRL B-67647,
Bacillus pumilus NRRL B-67648, *Bacillus*
licheniformis NRRL B-67649 (Microsaf®)**

Active Agent (s): ***Bacillus velezensis* (*amyloliquefaciens*)
NRRL B-67647, *Bacillus pumilus* NRRL B-
67648, *Bacillus licheniformis* NRRL B-
67649**

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: **22/08/2022**

Report approved by: **Christoph von Holst**
Date: **22/08/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for the *preparation of Bacillus velezensis (amyloliquefaciens) NRRL B-67647, Bacillus pumilus NRRL B-67648, Bacillus licheniformis NRRL B-67649 (Microsaf®)* under the category / functional groups 4(b, d) 'zootechnical additives' / 'gut flora stabilisers', 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for chickens for fattening, and other poultry for fattening (e.g. turkeys, ducks, geese, pheasants, quail, guinea fowl, ostrich) and ornamental birds.

According to the Applicant, the *feed additive* contains as active substances the non-genetically modified *Bacillus velezensis (amyloliquefaciens) NRRL B-67647, Bacillus pumilus NRRL B-67648* and *Bacillus licheniformis NRRL B-67649*.

The *feed additive* is to be marketed as two powder formulations containing a minimum total content of the active substances of 2.0×10^8 and of 5.0×10^9 Colony Forming Unit (CFU) / g, respectively. The *feed additive* is intended to be used in *premixtures* and *feedingstuffs* at a minimum dose of 3.0×10^7 CFU / kg complete *feedingstuffs*.

For the identification of *Bacillus velezensis (amyloliquefaciens) NRRL B-67647, Bacillus pumilus NRRL B-67648* and *Bacillus licheniformis NRRL B-67649* at a strain level, the EURL recommends for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE).

For the enumeration of total *Bacilli spp. (Bacillus velezensis (amyloliquefaciens) NRRL B-67647, Bacillus pumilus NRRL B-67648* and *Bacillus licheniformis NRRL B-67649* in the *feed additive, premixtures* and *feedingstuffs*, the EURL recommends for official control the ring-trial validated EN 15784 method.

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 velezensis (amyloliquefaciens) NRRL B-67647, Bacillus pumilus NRRL B-67648, Bacillus licheniformis NRRL B-67649, Microsaf®, zootechnical additives, gut flora stabilisers, other zootechnical additives, chickens for fattening, other poultry for fattening (turkeys, ducks, geese, pheasants, quail, guinea fowl, ostrich) and ornamental birds.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for the preparation of *Bacillus velezensis* (amyloliquefaciens) NRRL B-67647, *Bacillus pumilus* NRRL B-67648, *Bacillus licheniformis* NRRL B-67649 (*Microsaf*®) under the category / functional groups 4(b, d) 'zootechnical additives' / 'gut flora stabilisers', 'other zootechnical 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 chickens for fattening, and other poultry for fattening (e.g. turkeys, ducks, geese, pheasants, quail, guinea fowl, ostrich) and ornamental birds [1,2].

According to the Applicant, the *feed additive* contains as active substances the non-genetically modified *Bacillus velezensis* (amyloliquefaciens) NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649 [3,4].

The *feed additive* is to be marketed as two powder formulations containing a minimum total content of the active substances of 2.0×10^8 and of 5.0×10^9 Colony Forming Unit (CFU) / g, respectively [5]. The *feed additive* is intended to be used in *premixtures* and *feedingstuffs* at a minimum dose of 3.0×10^7 CFU / kg complete *feedingstuffs* [6].

Note: The EURL has evaluated analytical methods for the determination of *Bacilli* spp. in the frame of some previous dossiers [7].

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 velezensis* (amyloliquefaciens) NRRL B-67647, *Bacillus pumilus* NRRL B-67648, *Bacillus licheniformis* NRRL B-67649 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 *Bacilli spp.* (*Bacillus velezensis (amyloliquefaciens)* NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649) in the *feed additive, premixtures* and *feedingstuffs* the Applicant proposed [8] the ring-trial validated spread plate CEN method EN 15784 [9], which was recently revised by CEN. During the revision of the CEN method it was adjusted to VDLUFA method 28.2.2 [10] and completed with validation data from interlaboratory studies using commercial feed products. The revision resulted in the updated CEN method dedicated for the enumeration of *bacilli spp.* in *feedingstuffs* (additives, *premixtures* and compound feeds including mineral feeds) that contain bacilli as a single microorganism component or in a mixture with other microorganisms [11].

Following the protocol of the updated CEN method, the sample (5 to 50 g) is suspended in 0.2 % sodium hydroxide solution containing Polysorbate 80 (Tween® 80) (tPBS). Decimal dilutions are prepared from the suspension using the above mentioned solution, spread plated on tryptone soya agar and incubated, aerobically at 37 °C for 16 to 24 h [11].

The following performance characteristics were reported from the ring-trial validation studies of non-transformed logarithmically CFU values of *bacilli spp.* ranging from 9.0×10^8 to 4.45×10^{14} / kg *feed additives, premixtures* and *feedingstuffs* (including a mineral feed) [11]: a relative standard deviation for *repeatability* ranging from 9.1 to 19.6 %; and a relative standard deviation for *reproducibility* (RSD_R) ranging from 17.1 to 33.9 %.

In addition, a limit of quantification (LOQ) of 3×10^7 CFU / kg can be calculated following the considerations of the ISO 7218 standard [12].

Furthermore, in the frame of the stability and homogeneity studies [13], the Applicant provided experimental evidences demonstrating the applicability of the above mentioned CEN method for the enumeration of the total *Bacilli* strains in the *feed additive, premixtures* and *feedingstuffs*.

Based on the performance characteristics and experimental data available, the EURL recommends for official control the ring-trial validated EN 15784 method for the enumeration of total *Bacilli spp.* (*Bacillus velezensis (amyloliquefaciens)* NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649) in the *feed additive, premixtures* and *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)

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 *Bacillus velezensis (amyloliquefaciens)* NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649 at strain level, the Applicant applied Pulsed-Field Gel Electrophoresis (PFGE) and DNA sequencing methods such a comparative rRNA sequencing of the 16S region and Whole Genome Sequencing [14]. 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 [15]. Furthermore, this methodology has been recently ring-trial validated and is supposed to become a CEN Technical Specification [16].

The EURL considers that both methodologies (PFGE and DNA sequencing methods, such as Whole Genome Sequencing - WGS) are suitable for official control for the bacterial identification of *Bacillus velezensis (amyloliquefaciens)* NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649 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) DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) for the identification of *Bacillus velezensis (amyloliquefaciens)* NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649; and (ii) the ring-trial validated spread-plate method EN 15784 for the enumeration of the total *Bacilli* spp. (*Bacillus velezensis (amyloliquefaciens)* NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649) in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

- Identification: DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE)
- Enumeration in the *feed additive, premixtures, feedingstuffs*: Spread-plate method on tryptone soya agar (EN 15784)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Bacillus velezensis* (*amyloliquefaciens*) NRRL B-67647, *Bacillus pumilus* NRRL B-67648 and *Bacillus licheniformis* NRRL B-67649 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/1402>
<https://open.efsa.europa.eu/questions/EFSA-Q-2021-00449>
- [2] *Application, Annex 1
- [3] *Technical dossier, Section II: 2.2.1.2.1 Origin of the strains
- [4] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition
- [5] *Technical dossier, Section II: 2.1.4.1.5. Proposal for a specification of the additive
- [6] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition
- [7] EURL Evaluation Reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2020-0049_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0086_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0074_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0009_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0064_en
- [8] *Technical dossier, Section II: 2.6.1. Methods of analysis for the active substance
- [9] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [10] VDLUFA method – Enumeration of *Bacillus licheniformis* and *Bacillus subtilis* (VDLUFA Methodenbuch Bd.III, 28.2.2)
- [11] EN 15784:2021 - Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of *Bacillus* spp. used as feed additive
- [12] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations
- [13] *Technical dossier, Section II: 2.4. Physico-chemical and technological properties of the additive
- [14] *Technical dossier, Section II: 2.2. Characterisation of the active substance(s) / agent(s)
- [15] EURL reports: https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en
- [16] 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

*Refers to Dossier no: FEED-2021-0928

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:

- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)