



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

***Bacillus amyloliquefaciens* TOA5001 (NITE BP-01844)**
(FAD-2020-0049; CRL/200016)



**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-2020-0049 – CRL/200016**

Name of Product: ***Bacillus amyloliquefaciens* TOA5001
(NITE BP-01844)**

Active Agent (s): ***Bacillus amyloliquefaciens* TOA5001**

Rapporteur Laboratory: **Centre Wallon de Recherches
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Belgium**

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Date: **17/12/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *Bacillus amyloliquefaciens* TOA5001 (NITE BP-01844) 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 chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and all minor avian species.

According to the Applicant, the *feed additive* contains as *active substance* viable spores of the non-genetically modified strain *Bacillus amyloliquefaciens* TOA5001. The *feed additive* is to be marketed as a preparation (BA-KING®) containing a minimum content of *active substance* of 1×10^8 Colony Forming Unit (CFU)/g. The *feed additive* is intended to be added directly in *feedingstuffs* or through *premixtures* at a minimum dose of 5×10^7 CFU/kg complete *feedingstuffs* and in *water* for drinking at a minimum dose of 2.5×10^7 CFU/L.

For the identification of *Bacillus amyloliquefaciens* TOA5001, 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 *Bacillus amyloliquefaciens* TOA5001 in the *feed additive*, *premixtures*, *feedingstuffs* and *water* the EURL recommends for official control the ring-trial validated spread-plate method EN 15784.

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 amyloliquefaciens TOA5001 (NITE BP-01844), zootechnical additives, gut flora stabilisers, chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, all minor avian species

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *Bacillus amyloliquefaciens* TOA5001 (NITE BP-01844) under the category / functional group 4(b) 'zotechnical 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 chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding and all minor avian species [2].

According to the Applicant, the *feed additive* contains as *active substance* viable spores of the non-genetically modified strain *Bacillus amyloliquefaciens* TOA5001 [3].

The strain is deposited at the Japanese National Institute of Technology and Evaluation Patent Microorganisms Depository (Chiba, Japan) under the deposit number NITE BP-01844 [4].

The *feed additive* is to be marketed as a preparation (BA-KING[®]) containing a minimum content of *active substance* of 1×10^8 Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be added directly in *feedingstuffs* or through *premixtures* at a minimum dose of 5×10^7 CFU/kg complete *feedingstuffs* and in *water* for drinking at a minimum dose of 2.5×10^7 CFU/L [2,6,7].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus* spp. in the frame of several dossiers [8].

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 *Bacillus amyloliquefaciens* TOA5001 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 water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of *Bacillus amyloliquefaciens* TOA5001 in the *feed additive, premixtures, feedingstuffs* and *water* the Applicant proposed [9] and submitted the standard method EN 15784 [10]. This is the ring-trial validated spread-plate method developed by CEN for the analysis of *Bacillus* spp. [10], which was already evaluated and recommended by the EURL in the frame of previous *Bacillus* spp. dossiers [8].

According to EN 15784, 20 g of the *feed additive* (or 50 g of *premixtures* or *feedingstuffs*) are suspended in a phosphate buffered saline (or in 0.2 % sodium hydroxide solution for *premixtures* or *feedingstuffs*). For the enumeration of *Bacillus amyloliquefaciens* TOA5001 in *water*, the Applicant prepared the first dilution from 50 mL of water [11]. From this first dilution for all matrices, a new dilution is prepared with “Polysorbate 80” peptone salt solution and heat-treated at 80 °C for 10 min. Further decimal dilutions are prepared from the heat-treated suspension, spread-plated on tryptone soya agar and incubated at 37 °C for 16-24 h aerobically. The following performance characteristics were reported from the validation study after logarithmic transformation of the CFU values [10]:

- a repeatability standard deviation (S_r) ranging from 0.07 to 0.09 \log_{10} CFU/g and
- a reproducibility standard deviation (S_R) ranging from 0.32 to 0.35 \log_{10} CFU/g.

The limit of quantification (LOQ) of *Bacilli* in *feedingstuffs* specified in EN 15784 is 2×10^4 CFU/g. This LOQ is below the minimum content indicated for *Bacillus amyloliquefaciens* TOA5001 in complete *feedingstuffs* and *water*. Furthermore, according to ISO 7218, the enumeration under the experimental conditions described in EN 15784 is achievable at the level of 6×10^3 CFU/g *feedingstuffs* (or mL of *water*) [12].

In addition, the Applicant provided experimental evidence demonstrating the applicability of the EN 15784 standard method to the targeted matrices [11,13].

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated EN 15784 method for the enumeration of *Bacillus amyloliquefaciens* TOA5001 in the *feed additive, premixtures, feedingstuffs* and *water*.

Note: The method EN 15784 is not applicable to mineral feeds composed mainly of minerals and containing at least 40 % crude ash. For these matrices laboratories may consider using instead the ring-trial validated VDLUFA method 28.2.2 [14].

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 amyloliquefaciens* TOA5001 at strain level, the Applicant applied a Whole Genome Sequencing method [15].

The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains [16]. This methodology is currently being evaluated by the CEN Technical Committee 327 to become a European Standard.

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) for the identification of *Bacillus amyloliquefaciens* TOA5001 and (ii) the ring-trial validated spread-plate method EN 15784 for the enumeration of *Bacillus amyloliquefaciens* TOA5001 in the *feed additive, premixtures, feedingstuffs* and *water*.

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

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE);
- Enumeration in the *feed additive, premixtures, feedingstuffs* and *water*: 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 amyloliquefaciens* TOA5001 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. 1831/0045-2020
- [2] *Application, Annex 1 – submission number 1583341913039-2556
- [3] *Technical dossier, Section II: 2.1.2. Proposal for classification
- [4] *Technical dossier, Section II: 2.2.1.2. Micro-organisms
- [5] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition
- [6] *Technical dossier, Section II: 2.5. Conditions of use of the additive
- [7] *Technical dossier, Section II: Section II: 2.4.1. Stability
- [8] #EURL Evaluation Reports:
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0007.pdf>
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0013.pdf>
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0023.pdf>
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0041.pdf>
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<https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0009-galliprofit.pdf>
- [9] *Technical dossier, Section II: 2.6. Methods of analysis and reference samples
- [10] EN 15784 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [11] *Technical dossier, Section II: Annex II_6_1b
- [12] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations
- [13] *Technical dossier, Section II: Annex II_6_1a
- [14] VDLUFAMethod – Enumeration of *Bacillus licheniformis* and *Bacillus subtilis* (VDLUFAMethodenbuch Bd.III, 28.2.2)
- [15] *Technical dossier, Section II: 2.2 Characterisation of the active substance
- [16] 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-2020-0049

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Centre Wallon de Recherches Agronomiques (CRA-W), Gembloux, 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)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)