



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

**Bacillus subtilis TO-A, Enterococcus faecium T-110, Clostridium
butyricum TO-A (BIO-THREE®)
(FAD-2020-0058; CRL/200025)**



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Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FAD-2020-0058 - CRL/200025**

Name of Product : ***BIO-THREE®***

Active Agent (s): **Bacillus subtilis TO-A**
Enterococcus faecium T-110
Clostridium butyricum TO-A

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

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Date: **08/12/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for a preparation containing *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A (BIO-THREE®), 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 to slaughter or to point of lay.

According to the Applicant, the *feed additive* contains as active agents viable spores of the non-genetically modified microorganism (non-GMM) *B. subtilis* TO-A, non-GMM *E. faecium* T-110 and viable spores of non-GMM *C. butyricum* TO-A. The *feed additive* is intended to be marketed as dry or liquid preparations containing a minimum content of 1×10^7 Colony Forming Unit (CFU) *B. subtilis* TO-A /g, 1×10^8 CFU *E. faecium* T-110 /g and 1×10^7 CFU *C. butyricum* TO-A /g and to be used directly in complete *feedingstuffs* or in complementary feeds and in *water* at a minimum dose of 2.0×10^6 CFU *B. subtilis* TO-A; 2.0×10^7 CFU *E. faecium* T-110 and 2.0×10^6 CFU *C. butyricum* TO-A /kg complete *feedingstuffs* and of 1.0×10^6 CFU *B. subtilis* TO-A, 1.0×10^7 CFU *E. faecium* T-110 and 1.0×10^6 CFU *C. butyricum* TO-A /l of *water*.

For the identification of the three target strains the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains.

For the enumeration of *Bacillus subtilis* TO-A and *Enterococcus faecium* T-110 in the *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant proposed the ring-trial validated spread plate methods EN 15784 and EN 15788, respectively, while for the enumeration of *Clostridium butyricum* TO-A in the *feed additive*, *premixtures* *feedingstuffs* and *water*, the Applicant proposed the pour plate method ISO 15213. Furthermore, the Applicant provided evidences of the suitability of all the mentioned methods for the enumeration of these microorganisms in the mentioned matrices.

Based on the performance characteristics reported and the applicability evidences provided by the Applicant, the EURL recommends for official control the internationally recognised standard methods EN 15784, EN 15788 and ISO 15213 for the enumeration of *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A respectively in the *feed additive*, *premixtures*, *feedingstuffs* and *water*.

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 TO-A, *Enterococcus faecium* T-110, *Clostridium butyricum* TO-A, BIO-THREE[®], gut flora stabilisers, chickens for fattening and reared for laying, turkeys for fattening and for breeding and all minor avian species to slaughter or to point of lay

1. BACKGROUND

BIO-THREE[®] is the trade name of a preparation containing the following three strains: *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A [1,2].

In the current application an authorisation is sought under Article 4(1) (new feed additive) for BIO-THREE[®], 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 requested 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 to slaughter or to point of lay [1][2].

According to the Applicant, the *feed additive* contains as active agents viable spores of the non-genetically modified microorganism (non-GMM) *B. subtilis* TO-A, non-GMM *E. faecium* T-110 and viable spores of non-GMM *C. butyricum* TO-A [1, 2]. The strains are deposited in the Japanese International Institute of Technology and Evaluation Patent Microorganisms Depository as *Bacillus subtilis* TO-A (FERM BP-07462), *Enterococcus faecium* T-110 (FERM BP-10867) and *Clostridium butyricum* TO-A (FERM BP-10866) [2]. The *feed additive* is intended to be marketed as dry or liquid preparations containing a minimum content of 1×10^7 Colony Forming Unit (CFU) *B. subtilis* TO-A / g, 1×10^8 CFU *E. faecium* T-110 / g and 1×10^7 CFU *C. butyricum* TO-A / g [2].

The *feed additive* is intended to be used directly in complete *feedingstuffs* or in complementary *feeds* and in *water* at a minimum dose of 2.0×10^6 CFU *B. subtilis* TO-A; 2.0×10^7 CFU *E. faecium* T-110 and 2.0×10^6 CFU *C. butyricum* TO-A / kg complete *feedingstuffs* and of 1.0×10^6 CFU *B. subtilis* TO-A, 1.0×10^7 CFU *E. faecium* T-110 and 1.0×10^6 CFU *C. butyricum* TO-A / l of *water* [3].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus subtilis*, *Enterococcus faecium* and *Clostridium butyricum* in the frame of several dossiers [4].

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 *BIO-THREE®* 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 *Bacillus subtilis* TO-A in the *feed additive, premixtures, feedingstuffs* and *water*, the Applicant proposed the ring-trial validated spread plate EN 15784 [5] method developed to enumerate and differentiate spores of several *Bacillus* spp. in *feed matrices* which was already evaluated and recommended by the EURL in the frame of previous *Bacillus subtilis* dossiers [4].

According to the EN 15784 method, 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*). From this suspension, one new dilution is prepared with "Polysorbate 80" peptone salt solution and heat-treated at 80 °C for 10 min. For the enumeration of *Bacillus subtilis* TO-A in *water*, the Applicant directly heat-treated the sample of water. 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 [5]:

- 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.

In addition, the Applicant specified a limit of determination (LOQ) of 1000 CFU/g *feedingstuffs* and of 100 CFU/ml *water* for *Bacillus subtilis* TO-A [6]. According to the ISO 7218 standard, the experimental conditions described in the EN 15784 method allow to estimate a level of determination as low as the minimum content proposed by the Applicant for *Bacillus subtilis* TO-A in *feedingstuffs* and in *water*. Moreover, all the analyses carried out

in *feedingstuffs* and in *water* provided in the frame of this dossier revealed always a content far above the minimum content proposed by the Applicant for *Bacillus subtilis* TO-A [6].

For the enumeration of *Enterococcus faecium* T-110 in the *feed additive*, *premixtures*, *feedingstuffs* and *water*, the Applicant proposed the ring-trial validated spread plate EN 15788 [7] method which was already evaluated and recommended by the EURL in the frame of previous *Enterococcus faecium* dossiers [4].

The sample is suspended in phosphate buffered saline (PBS) and diluted in a peptone salt solution; the appropriate dilutions are then spread on bile esculin azide agar. For the enumeration of *Enterococcus faecium* T-110 in *water*, the Applicant directly analysed the water suspension. The agar plates are incubated at 37 °C for 24 hours before colony counting. The performance characteristics of this CEN method reported after logarithmic transformation (CFU) are:

- a *repeatability* standard deviation (s_r) ranging from 0.12 to 0.2 log₁₀ CFU/g,
- a *reproducibility* standard deviation (s_R) ranging from 0.23 to 0.41 log₁₀ CFU/g [7].

The Applicant specified a limit of determination (LOQ) of 1000 CFU/g *feedingstuffs* and of 100 CFU/ml *water* for *Enterococcus faecium* T-110 [6]. According to ISO 7218 standard, the experimental conditions described in the EN 15788 method allow to enumerate a level below the minimum content proposed by the Applicant for *Enterococcus faecium* T-110 in *feedingstuffs* and in *water*.

Finally, for the enumeration of *Clostridium butyricum* TO-A in the *feed additive*, *premixtures*, *feedingstuffs* and *water*, the Applicant proposed the pour plate method ISO 15213 [8], used for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions. As for the other two strains the method ISO 15213 was already evaluated and recommended by the EURL in the frame of a previous *Clostridium butyricum* dossier [4].

The sample is suspended, eventually heat-treated, and diluted in a peptone salt solution, the appropriate dilutions are then transferred into Petri dishes and poured with iron sulfite agar. For the enumeration of *Clostridium butyricum* TO-A in *water*, the Applicant directly heat-treated the sample of water. When the agar is solidified, the plates are incubated at 37° C for 48 h before colony counting [8].

In addition, the Applicant specified a limit of determination (LOQ) of 100 CFU/g *feedingstuffs* and of 10 CFU/ml *water* for *Clostridium butyricum* TO-A [6]. According to ISO 7218 standard, the experimental conditions described in the ISO 15213 method allow to enumerate a level below the minimum content proposed by the Applicant for *Clostridium butyricum* TO-A in *feedingstuffs* and in *water*.

For the enumeration of the three strains in the different matrices the Applicant provided experimental evidence obtained in the frame of the homogeneity and stability studies [9]. Furthermore, the Applicant verified the proposed standard methods in the product (BIO-THREE®), in *feedingstuffs* and in *water* [6], thereby confirming the applicability of the respective standard methods to the mentioned matrices. The verification studies provided by the Applicant for *feedingstuffs* revealed the presence of *Bacillus spp.* in the “blank”. The Applicant stated that these levels are usually found in mash feeds due to the wild *Bacillus* strains naturally present and the non-selective culture medium. However, as this level is 1-2 orders of magnitude below the expected content of *Bacillus subtilis* TO-A in the tested *feedingstuffs*, the obtained results can be considered acceptable. According to the Applicant the *premixtures* will be diluted with blank feed and further analysed as *feedingstuffs*.

Based on the performance characteristics reported and the applicability evidences provided by the Applicant, the EURL recommends for official control the internationally recognised standard methods EN 15784, EN 15788 and ISO 15213 for the enumeration of *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A respectively in the *feed additive*, *premixtures*, *feedingstuffs* and *water*.

Note: The EN 15784 and EN 15788 methods are 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 methods 28.2.2 [10] and 28.2.3 instead [11].

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) – (Include this section ONLY when relevant for the dossier evaluation e.g. probiotics)

For the identification of the three target strains, i.e. *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A, the Applicant proposed an unique taxonomical identification based on the 16S rRNA gene and the whole genome sequence [12].

The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains [13]. This methodology for bacterial identification of authorised additives at a strain level 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 subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A and (ii) the EN 15784, EN 15788 and the ISO 15213 standard methods for the respective enumeration of *Bacillus subtilis* TO-A, *Enterococcus faecium* T-110 and *Clostridium butyricum* TO-A in the *feed additive, premixtures, feedingstuffs* and *water*.

Note: The methods EN 15784 and EN 15788 are 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 methods 28.2.2 and 28.2.3 instead.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration of *Bacillus subtilis* TO-A in the *feed additive, premixtures, feedingstuffs* and *water*: Spread plate method on tryptone soya agar (EN 15784)
- Enumeration of *Enterococcus faecium* T-110 in *feed additive, premixtures, feedingstuffs* and *water*: Spread plate method using bile esculin azide agar (EN 15788)
- Enumeration of *Clostridium butyricum* TO-A in the *feed additive, premixtures, feedingstuffs* and *water*: Pour plate method using iron sulfite agar (ISO 15213)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *BIO-THREE®* 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] *Annex I Submission number 1587637312352-2588
- [2] *Technical dossier, Section II: 2.1 Identity of the additive
- [3] *Technical dossier, Section II: 2.5 Conditions of use of the additive
- [4] 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-2014-0006-fecinor.pdf>
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-20100005.pdf>

- [5] EN 15784- Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [6] *Technical dossier, Annexes II_6.1 a-d
- [7] EN 15788 'Animal feeding stuffs - Isolation and enumeration of *Enterococcus* (*E. faecium*) spp'
- [8] ISO 15213 'Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions'
- [9] *Technical dossier, Annex II_4
- [10] VDLUFA method – Enumeration of *Enterococcus faecium* (VDLUFA Methodenbuch Bd.III, 28.2.3)
- [11] VDLUFA method –Enumeration of *Bacillus licheniformis* and *Bacillus subtilis* (VDLUFA Methodenbuch Bd.III, 28.2.2)
- [12] *Technical dossier, Section II: 2.2 Characterisation of the active substance(s)/agent(s)
- [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.I)

*Refers to Dossier no: FAD-2020-0058

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:

- Centre wallon de Recherches agronomiques (CRA-W), Gembloux (BE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- 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)