




EUROPEAN COMMISSION  
JOINT RESEARCH CENTRE

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

 Ref. Ares(2019)4748771 - 22/07/2019

JRC.F.5/CvH/ZE/AS/Ares (2019)

**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**

**GalliPro® Fit**  
*(FAD-2019-0009; CRL/180055)*





**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-2019-0009 – CRL/180055**

Name of Product: ***GalliPro® Fit***

Active Agent (s): ***Bacillus subtilis DSM32324,*  
*Bacillus subtilis DSM32325,*  
*Bacillus amyloliquefaciens DSM25840***

Rapporteur Laboratory: **Centre wallon de Recherches  
agronomiques (CRA-W), Gembloux,  
Belgium**

Report prepared by: **Véronique Ninane**

Report checked by: **Zigmas Ezerskis**  
Date: **22/07/2019**

Report approved by: **Christoph von Holst**  
Date: **22/07/2019**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for a preparation of *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM32325 and *Bacillus amyloliquefaciens* DSM25840 (*GalliPro® Fit*) under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. 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 other growing poultry species.

According to the Applicant, the *feed additive* contains as *active substances* viable spores of non-genetically modified strains *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840. The *feed additive* is to be marketed as a powder containing a minimum content of total *active substances* of  $3.2 \times 10^9$  Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of  $8 \times 10^8$  CFU/kg complete *feedingstuffs* and in drinking *water* at a minimum dose of  $2.7 \times 10^8$  CFU/L.

For the identification of *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM32325 and *Bacillus amyloliquefaciens* DSM25840, 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 overall *Bacillus* spp. (DSM 32324, DSM 32325 and DSM 25840) in the *feed additive*, *premixtures*, *feedingstuffs* and drinking *water* 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 overall *Bacillus* spp. (DSM 32324, DSM 32325 and DSM 25840) 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* DSM 32324, *Bacillus subtilis* DSM32325, *Bacillus amyloliquefaciens* DSM25840, zootechnical additives, gut flora stabilisers, chickens for fattening, chickens reared for laying, turkeys for fattening, turkeys reared for breeding, other growing poultry species.

## 1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new feed additive) for a preparation of *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840 (*GalliPro® Fit*) under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1]. 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 other growing poultry species [2].

According to the Applicant, the *feed additive* contains as *active substances* viable spores of non-genetically modified strains *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840 [3]. The strains are deposited at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under the deposit numbers DSM 32324, DSM 32325 and DSM 25840, respectively [4].

The *feed additive* is to be marketed as a powder containing a minimum content of total *active substances* of  $3.2 \times 10^9$  Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of  $8 \times 10^8$  CFU/kg complete *feedingstuffs* and in drinking *water* at a minimum dose of  $2.7 \times 10^8$  CFU/L [6].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus* spp. in the frame of several 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 subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840 (*GalliPro® Fit*) 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 the overall *Bacillus* spp. (DSM 32324, DSM 32325 and DSM 25840) in the *feed additive, premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated spread plate CEN method EN 15784 [8] which was already evaluated and recommended by the EURL in the frame of previous *Bacillus* spp. dossiers [7].

Twenty grams 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 first dilution, one new dilution is prepared and heat-treated at 80 °C for 10 minutes. 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. The following performance characteristics were reported from the validation study after logarithmic transformation of the CFU values [8]:

- 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 EURL calculated a limit of quantification (LOQ) of  $3 \times 10^4$  CFU/g following the recommendations of the ISO 7218 standard [9].

In the frame of the stability studies, the Applicant provided additional experimental evidences demonstrating the applicability of the CEN method for the enumeration of the overall *Bacillus* spp. (DSM 32324, DSM 32325 and DSM 25840) in *water* [10]. For this, the Applicant prepared the first dilution from 10 g of *water* [11] and further applied the CEN method.

Based on the performance characteristics and experimental data available, the EURL recommends for official control the ring-trial validated 15784 method for the enumeration of the overall *Bacillus* spp. (DSM 32324, DSM 32325 and DSM 25840) in the *feed additive, premixtures, feedingstuffs* and *water*.

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 instead the ring-trial validated VDLUFA method 28.2.2 [12].

***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 subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840 at strain level, the Applicant proposed a Pulsed-Field Gel Electrophoresis (PFGE) method [13].

The EURL recommends for official control PFGE, a generally recognised methodology for genetic identification of bacterial strains [14]. This methodology for microbial identification of authorised probiotics at 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 official control Pulsed Field Gel Electrophoresis (PFGE) for the identification of *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840, and the ring-trial validated spread plate method EN 15784 for the enumeration of the overall *Bacillus* spp. (DSM 32324, DSM 32325 and DSM 25840) in the *feed additive, premixtures, feedingstuffs* and *water*.

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 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 the preparation of *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquefaciens* DSM 25840 (*GalliPro® Fit*) 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: FWD. APPL. 1831/0010-2019 & Annex I – submission number 1544531351162-2330
- [2] \*Application, Proposal for Register Entry, Annex A
- [3] \*Technical dossier, Section II: 2.2 Characterisation of the Active Substance
- [4] \*Technical dossier, Section II: Annex II.2.1.2a
- [5] \*Technical dossier, Section II: 2.1. Identity of the additive
- [6] \*Technical dossier, Section II: 2.5 Conditions of use of the additive
- [7] #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>  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2015-0006-bacillus\\_subtilis.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2015-0006-bacillus_subtilis.pdf)  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad\\_2015\\_0008\\_enviva\\_pro202gt.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad_2015_0008_enviva_pro202gt.pdf)  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0070-baci\\_subtilis.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0070-baci_subtilis.pdf)  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0060-cinergy\\_life.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0060-cinergy_life.pdf)  
<https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0064-optimize.pdf>
- [8] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive Bacillus spp.
- [9] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations
- [10] \*Technical dossier, Section II: 2.4.1 Stability
- [11] \*Supplementary information: SOP-03532 v7 draft
- [12] VDLUFA method – Enumeration of Bacillus licheniformis and Bacillus subtilis (VDLUFA Methodenbuch Bd.III, 28.2.2)
- [13] \*Technical dossier, Section II: Annex II.2.1.2d
- [14] 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-2019-0009

# <https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports>

## 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:

- 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)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- 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)