



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**

***Bifidobacterium longum* (CNCM I-5642)
(FAD-2021-0031; CRL/210024)**



**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-2021-0031 - CRL/210024**

Name of Product / Feed Additive: ***Bifidobacterium longum* (CNCM I-5642)**

Active Agent (s): ***Bifidobacterium longum* (CNCM I-5642)**

Rapporteur Laboratory: **Centre Wallon de Recherches
Agronomiques (CRA-W), Gembloux,
Belgium**

Report prepared by: **Véronique Ninane**

Report checked by: **Zigmas Ezerskis**
Date: **20/09/2021**

Report approved by: **Christoph von Holst**
Date: **20/09/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *Bifidobacterium longum* (CNCM I-5642) under the category / functional group 4(e) 'zootechnical additives' / 'physiological condition stabilisers', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for cats and dogs.

According to the Applicant, the *feed additive* contains viable cells of the non-genetically modified strain *Bifidobacterium longum* (CNCM I-5642) as the active substance. The *feed additive* is to be marketed as a preparation containing a minimum content of the active substance of 5×10^{10} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used in *feedingstuffs* at minimum doses of 1.1×10^{10} and 3.5×10^9 CFU/kg complete *feedingstuffs* for cats and dogs, respectively.

For the identification of *Bifidobacterium longum* (CNCM I-5642), the EURL recommends for the official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains.

For the enumeration of *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* and *feedingstuffs*, the Applicant submitted a single-laboratory validated and further verified pour plate count method using a reinforced clostridial agar medium.

The following performance characteristics were obtained in the frame of the validation and verification studies:

- (i) for the average measured content of the active substance in the *feed additive* of 1.3×10^{11} CFU/g: a standard deviation for *repeatability* (S_r) and *intermediate precision* (S_{ip}) of $0.06 \log_{10}$ CFU/g;
- (ii) for the average measured content of the active substance in *feedingstuffs* ranging from 9.2×10^8 to 8.0×10^9 CFU/g: S_r ranging from 0.04 to 0.13 \log_{10} CFU/g and S_{ip} ranging from 0.04 to 0.17 \log_{10} CFU/g.

Furthermore, a limit of quantification (LOQ) of 100 CFU/g *feedingstuffs* can be derived following the recommendations of ISO 7218 standard.

Based on the performance characteristics and the experimental data available, the EURL recommends the pour plate count method using a reinforced clostridial agar medium for the official control for the enumeration of *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* and *feedingstuffs*.

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

Bifidobacterium longum (CNCM I-5642), PP102I, zootechnical additives, physiological condition stabilisers, cats and dogs.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *Bifidobacterium longum* (CNCM I-5642) under the category / functional group 4(e) 'zootechnical additives' / 'physiological condition 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 cats and dogs [2].

According to the Applicant, the *feed additive* contains viable cells of the non-genetically modified strain *Bifidobacterium longum* (CNCM I-5642) as the active substance [3]. The strain is deposited at the Collection Nationale de Cultures de Microorganismes (Paris) under the deposit number CNCM I-5642 [4].

The *feed additive* is to be marketed as a preparation under the trade name (PP102I) containing a minimum content of the active substance of 5×10^{10} Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be used in *feedingstuffs* at a minimum doses of 1.1×10^{10} and 3.5×10^9 CFU/kg complete *feedingstuffs* for cats and dogs, respectively [6].

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 *Bifidobacterium longum* (CNCM I-5642) 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 *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* and *feedingstuffs*, the Applicant submitted a single-laboratory validated and further verified pour plate count method using a reinforced clostridial agar medium [7,8].

According to the method, the sample is suspended and further serially diluted in a tryptone salt diluent. The appropriate dilutions are mixed in a molten reinforced clostridial agar medium poured in plates (pour plate method). The plates are then incubated anaerobically at 37 °C for 48 h [8].

The performance characteristics obtained by the Applicant in the frame of the validation [9] and verification [10] studies as re-calculated [11] by the rapporteur laboratory following the ISO 16140-4 standard [12] and Eurochem Guide [13] are summarised in Table 1. Furthermore, a limit of quantification (LOQ) of 100 CFU/g *feedingstuffs* can be derived following the recommendations of ISO 7218 standard [14].

In addition, the Applicant demonstrated the suitability of this method for the enumeration of *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* and complementary feed in the frame of stability and homogeneity studies [15]. The results from the stability study of the product were subjected to analysis of variance, obtaining value of 0.04 log₁₀ CFU/g for S_r which is within the precision range of the method as reported in Table 1.

Table 1: Performance characteristics of the pour plate count method obtained in the frame of the validation [9] and verification [10] studies as re-calculated [11] by the rapporteur laboratory for the enumeration of *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* (FA) and *feedingstuffs* (FS)

Matrix	Average measured content (CFU/g)		S _r (log ₁₀ CFU/g)		S _{ip} (log ₁₀ CFU/g)	
	Val	Ver	Val	Ver	Val	Ver
FA	-	1.3 × 10 ¹¹	-	0.06	-	0.06
FS	2.9 × 10 ⁹	9.2 × 10 ⁸ – 8.0 × 10 ⁹	0.13	0.04 – 0.08	0.17	0.04 – 0.08
Reference	[9,11]	[10,11]	[9,11]	[10,11]	[9,11]	[10,11]

S_r: standard deviation for *repeatability*; S_{ip} standard deviation for *intermediate precision*; Val: Validation; Ver: verification.

The Applicant applied also the ring-trial validated method EN 15785 [16] for the enumeration of the active substance in the *feed additive* and *feedingstuffs*. However, the provided experimental evidences revealed systematic underestimation of the CFU values (at least by factor of 10) in the mentioned matrices in comparison with the values obtained when using the pour plate count method from the Applicant [9,17]. Therefore, EN 15785 is not applicable to this product.

Even if the performance characteristics could not be established for the *feed additive* due to lack of the corresponding data in the frame of the validation studies following the ISO 16140-4 standard and the Eurochem Guide, the equivalent performance profile as the one for the *feed additive* in the frame of the stability and verification studies or in the frame of the validation and verification studies in *feedingstuffs* is expected.

Based on the performance characteristics and the experimental data available, the EURL recommends for the official control the single-laboratory validated and further verified pour plate count method using a reinforced clostridial agar medium for the enumeration of *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* 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 *Bifidobacterium longum* (CNCM I-5642), the Applicant applied a whole genome sequencing method [3].

The EURL recommends instead for official control the pulsed-field gel electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains [18]. 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 [19].

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 *Bifidobacterium longum* (CNCM I-5642) and (ii) the single-laboratory validated and further verified pour plate count method using

a reinforced clostridial agar medium for the enumeration of *Bifidobacterium longum* (CNCM I-5642) in the *feed additive* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

Identification: Pulsed Field Gel Electrophoresis (PFGE)

Enumeration in the *feed additive* and *feedingstuffs*: Pour plate method using a reinforced clostridial agar medium

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Bifidobacterium longum* (CNCM I-5642) 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. 1831-0023-2021
- [2] *Application, Annex 1 – submission number 1612348118924-2808
- [3] *Technical dossier, Section II: 2.2 Characterisation of the active substance
- [4] *Technical dossier, Section II: Annex_II_2_1_2
- [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: 2.6.1 Methods of analysis for the active substance
- [8] *Technical dossier, Section II: Annex_II_6_1d
- [9] *Technical dossier, Section II: Annex_II_6_1b
- [10] *Technical dossier, Section II: Annex_II_6_1c
- [11] *Supplementary information – CRA-W Calculation of performance characteristics
- [12] ISO 16140-4 : Microbiology of the food chain – Method validation – Part 4: Protocol for method validation in a single laboratory
- [13] B. Magnusson and U. Ornemark (eds.) Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, (2nd ed. 2014). ISBN 978-91-87461-59-0. Available from www.eurachem.org
- [14] EN ISO 7218:2007 – Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations
- [15] *Technical dossier, Section II: Annex_II_4_1 & Annex_II_4_2
- [16] EN 15785 – Animal feeding stuffs - Isolation and enumeration of *Bifidobacterium* spp.

-
- [17] *Technical dossier, Section II: Annex_II_6_1a_applicants_declaration
- [18] 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)
- [19] prEN 17697 – Animal feeding stuffs: Methods of analysis – PFGE typing of Lactobacilli, Pediococci, Enterococci and Bacilli in animal feeds

*Refers to Dossier no: FAD-2021-0031

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the 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)
- 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)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)