EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/MGH /ACS/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

Lactobacillus reuteri NBF-1 (DSM 32203)

(FAD-2017-0001; CRL/160035)

European Union Reference Laboratory
Feed Additives

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-2017-0001 - CRL/160035**
Name of Product:	**Lactobacillus reuteri NBF-1 (DSM 32203)**
Active Agent (s):	***Lactobacillus reuteri NBF-1*** **(DSM 32203)**
Rapporteur Laboratory:	**Centre wallon de Recherches agronomiques (CRA-W), Gembloux, Belgium**
Report prepared by:	**Véronique Ninane**
Report checked by: Date:	**María José González de la Huebra & Piotr Robouch (EURL-FA)** **14/09/2017**
Report approved by: Date:	**Christoph von Holst** **15/09/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Lactobacillus reuteri NBF-1* (DSM 32203) 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 dogs.

According to the Applicant, the *feed additive* contains as active substance viable cells of the strain *Lactobacillus reuteri NBF-1* (DSM 32203). The *feed additive* is to be marketed as a lyophilised powder containing a minimum *Lactobacillus reuteri NBF-1* (DSM 32203) content of 10^{11} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be administered as complementary *feedingstuffs* with a maximum content of $6x10^{10}$ CFU/kg or to be mixed with usual feed.

For the identification of *Lactobacillus reuteri NBF-1* (DSM 32203) the Applicant applied 16S rRNA gene sequence analysis and random amplified polymorphic DNA analysis. The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

For the enumeration of *Lactobacillus reuteri NBF-1* (DSM 32203) in the *feed additive* and *feedingstuffs*, the Applicant submitted the ring-trial validated spread plate method EN 15787. Based on the performance characteristics available, the EURL recommends this method for official control.

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

Lactobacillus reuteri NBF-1 (DSM 32203), zootechnical additives, gut flora stabilisers, dogs

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Lactobacillus reuteri NBF-1* (DSM 32203) 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 dogs [1][2].

According to the Applicant, the *feed additive* contains as *active substance* viable cells of the strain *Lactobacillus reuteri NBF-1* DSM 32203 [3]. The strain is deposited at the Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) under the deposit number DSM 32203 [4].

The *feed additive* is to be marketed as a lyophilised powder with a minimum *Lactobacillus reuteri NBF-1* (DSM 32203) content of 10^{11} Colony Forming Unit (CFU)/g [5].

The *feed additive* is intended to be administered as complementary *feedingstuffs* with a maximum content of $6x10^{10}$ CFU/kg or to be mixed with usual feed [2][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 *Lactobacillus reuteri NBF-1* (DSM 32203) and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. cadmium, lead, mercury and aflatoxin M1) are available from the respective European Union Reference Laboratories [7].

Furthermore, the Applicant analysed the *feed additive* for microbial contaminants (e.g. yeast and mould, non-lactic acid bacteria, *Enterobacteriaceae*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella*) using the methods mentioned in the technical dossier [8].

Qualitative and quantitative composition of the additive

For the identification of *Lactobacillus reuteri NBF-1* (DSM 32203), the Applicant applied 16S rRNA gene sequence analysis and random amplified polymorphic DNA analysis [3].

The EURL recommends instead for official control the Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification. A PFGE method for microbial identification of authorised probiotics at strain level in *feedingstuffs* [9] is currently being evaluated by the CEN Technical Committee 327 to become an European Standard.

Description of the analytical methods for the determination of the active substance in the feed additive and feedingstuffs

For the enumeration of *Lactobacillus reuteri NBF-1* (DSM 32203) in the *feed additive* and *feedingstuffs*, the Applicant submitted the ring-trial validated spread plate method EN 15787 developed by CEN for *Lactobacillus* spp. [10][11].

The sample is suspended and diluted in a buffer solution; the appropriate dilutions are then spread on MRS (de Man, Rogosa, Sharp) agar plates. The agar plates are incubated anaerobically at 37 °C for 48 to 72 hours.

The following performance characteristics, expressed in terms of precision, were reported after logarithmic transformation of the CFU values [11]:

- a standard deviation for *repeatability* (S_r) of 0.24 $\log_{10}$ CFU/g, and
- a standard deviation for *reproducibility* (S_R) ranging from 0.29 to 0.38 $\log_{10}$ CFU/g.

In addition, the EURL calculated according to ISO 7218 a limit of quantification (LOQ) of $3x10^3$ CFU/g [12].

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lactobacillus reuteri NBF-1* (DSM 32203) 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.

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 *Lactobacillus reuteri NBF-1* (DSM 32203) and the ring-trial validated spread plate method EN 15787 for the enumeration of this strain in the *feed additive* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in *feed additive* and *feedingstuffs*: Spread plate method on MRS agar (EN 15787)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Lactobacillus reuteri NBF-1* (DSM 32203) 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. 1831-0001-2017

[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.1

[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] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[8] *Technical dossier, Section II: 2.1.4 Purity

[9] 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)

[10] *Technical dossier, Section II: 2.6 Methods of analysis

[11] EN 15787:2009 - Animal feeding stuffs - Isolation and enumeration of *Lactobacillus spp.*

[12] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations

*Refers to Dossier no: FAD-2017-0001

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:

- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT))