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Feed Additives

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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

NS AH007
(FAD-2021-0001; CRL/210049)

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-2021-0001 - CRL/210049**
Name of Product:	***NS AH007***
Active Agent (s):	***Bacillus velezensis NRRL-B-50910*** ***Pediococcus acidilactici NRRL-B-50959*** ***Pediococcus acidilactici NRRL-B-50964***
Rapporteur Laboratory:	**Centre wallon de Recherches agronomiques (CRA-W), Gembloux, Belgium**
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Report approved by: Date:	**Christoph von Holst** **26/08/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for NS AH007 under the category / functional groups 1(a & j) 'Technological additives' / 'preservatives' & 'acidity regulators', 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 and chickens reared for laying.

According to the Applicant, the *feed additive* contains as active substances viable spores of the non-genetically modified strain *Bacillus velezensis* NRRL-B-50910, and viable cells of the non-genetically modified strains *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964. The *feed additive* is to be marketed as a powder containing a minimum content of *Bacillus velezensis* NRRL-B-50910 of $3x10^{10}$ Colony Forming Unit (CFU) / g and of <u>total</u> *Pediococcus acidilactici* (sum of the two strains) of $1x10^{10}$ CFU / g. The *feed additive* is intended to be used in *feedingstuffs* at a minimum dose of <u>total active substances</u> (sum of the three strains) of 4×10^8 CFU / g complete *feedingstuffs*.

For the identification of *Bacillus velezensis* NRRL-B-50910, *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964, 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 *Bacillus velezensis* NRRL-B-50910 in the *feed additive*, the Applicant submitted the ring-trial validated spread plate CEN method EN 15784. For the enumeration of <u>total</u> *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 in the *feed additive*, the Applicant submitted the ISO 15214 method which is not ring trial validated but it is similar to the ring trial validated spread plate method EN 15786 using acidified MRS agar (AMRSA).

The Applicant did not provide any method or experimental data for the quantification of the <u>total active substances</u> in *feedingstuffs*. Both CEN methods previously mentioned, EN 15784 and EN 15786 using AMRSA, were however also ring trial validated for the enumeration of respectively *Bacillus* spp. and *Pedioccocus* spp. in *feedingstuffs*.

Based on the performance characteristics and the experimental data available, the EURL recommends for the official control the EN 15784 spread plate method for the enumeration of *Bacillus velezensis* NRRL-B-50910 in the *feed additive* and *feedingstuffs* and the EN 15786 spread plate method using AMRSA or the ISO 15214 method for the enumeration of <u>total</u> *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 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

Bacillus velezensis NRRL-B-50910, *Pediococcus acidilactici* NRRL-B-50959, *Pediococcus acidilactici* NRRL-B-50964, NS AH007, technological additives, preservatives, acidity regulators, chickens for fattening, chickens reared for laying

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *NS AH007* under the category / functional groups 1 (a & j) 'Technological additives' / 'preservatives' & 'acidity regulators', according to Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the *feed additive* for chickens for fattening and chickens reared for laying [1, 2].

According to the Applicant, the *feed additive* contains as active substances viable spores of the non-genetically modified strain *Bacillus velezensis* NRRL-B-50910, and viable cells of the non-genetically modified strains *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 [3]. The strains are deposited at the Agricultural Research Service Culture Collection of the United States Department of Agriculture (NRRL, Peoria, IL, USA) under the deposit numbers NRRL-B-50910, NRRL-B-50959 and NRRL-B-50964, respectively [4].

The *feed additive* is to be marketed as a powder containing a minimum content of *Bacillus velezensis* NRRL-B-50910 of $3x10^{10}$ Colony Forming Unit (CFU) / g and of total *Pediococcus acidilactici* (sum of the two strains) of $1x10^{10}$ CFU /g [5].

The *feed additive* is intended to be used in *feedingstuffs* at a minimum dose of total active substances (sum of the three strains) of 4×10^8 CFU / g complete *feedingstuffs* [2, 6].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus* spp and *Pediococcus* 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 *NS AH007* 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 velezensis* NRRL-B-50910 in the *feed additive*, 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].

For the enumeration of total *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 in the *feed additive*, the Applicant submitted the ISO 15214 method [9] which is not ring trial validated.

The ISO 15214 method is based on plating dilutions of the sample and counting the colonies on MRS (de Man, Rogosa and Sharpe) agar at pH 5.7 ($MRS_{5.7}$) after anaerobic incubation at 30°C for 72 hours. No performance characteristics for the method ISO 15214 are available.

However, the EURL identified the ring-trial validated spread plate method EN15786 which was specifically developed by CEN for the determination of *Pediococcus* spp. [10].

Additionally, upon EURL request, the Applicant confirmed the equivalence between the EN15786 method using acidified MRS agar (AMRSA), and the proposed ISO 15214 method. Both methods have been already evaluated and recommended by the EURL in the frame of previous *Pediococcus* spp. dossiers [7].

The Applicant did not provide any method or experimental data for the quantification of the total active substances in *feedingstuffs*; bacterial and physical parameters determined in the frame of stability studies [11] are not suitable for the purpose. However, both CEN spread plate methods, the EN 15784 and the EN 15786 using AMRSA were ring trial validated for the enumeration of respectively *Bacillus* spp. and *Pedioccocus* spp. in *feedingstuffs*.

According to the EN 15784 method, the sample is suspended in an initial diluent: phosphate buffered saline for *feed additive* or 0.2 % sodium hydroxide for *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 under aerobic conditions for 16 to 24 hours. The following

performance characteristics were reported from the validation study after logarithmic transformation of the CFU values [8]:

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

In addition, the EURL calculated a limit of quantification (LOQ) of $3x10^4$ CFU / g following the recommendations of the ISO 7218 standard [12].

According to the EN 15786 method, the sample is suspended and diluted in a phosphate buffered saline solution; the appropriate dilutions are then spread on AMRSA (de Man, Rogosa, Sharp) agar plates. The agar plates are incubated at 37 °C under anaerobic conditions for 36 to 48 hours before enumeration of the colonies. The following performance characteristics were reported from the validation study after logarithmic transformation of the CFU values [10]:

- a standard deviation for *repeatability* (S_r) ranging from 0.07 to 0.16 log_{10} CFU / g, and
- a standard deviation for *reproducibility* (S_R) ranging from 0.12 to 1.72 log_{10} CFU / g.

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

Based on the performance characteristics and the experimental data available, the EURL recommends for the official control the EN 15784 spread plate method for the enumeration of *Bacillus velezensis* NRRL-B-50910 in the *feed additive* and *feedingstuffs* and the EN 15786 spread plate method using AMRSA or the ISO 15214 plate count method on $MRS_{5.7}$ incubated anaerobically for the enumeration of total *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 in the *feed additive* and *feedingstuffs*.

Note: For the estimation of the total active substances in *feedingstuffs*, the EURL recommends to apply an arithmetic addition of the results obtained by applying both enumeration methods i.e. EN 15784 and EN 15786 or ISO 15214 to *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 *Bacillus velezensis* NRRL-B-50910, *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 at the strain level, the Applicant applied Pulsed Field Gel Electrophoresis (PFGE) [3]. PFGE, is a generally recognised methodology for the genetic identification of bacterial strains.

The EURL recommends for official control the 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 [14].

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 velezensis* NRRL-B-50910, *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964; (ii) the EN 15784 spread plate method for the enumeration of *Bacillus velezensis* NRRL-B-50910 in the *feed additive* and *feedingstuffs*; and (iii) the EN 15786 spread plate method using AMRSA or the ISO 15214 plate count method on $MRS_{5.7}$ incubated anaerobically for the enumeration of total *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 in the *feed additive* and *feedingstuffs*.

Note: For the estimation of the total active substances in *feedingstuffs*, the EURL recommends to apply an arithmetic addition of the results obtained by applying both enumeration methods i.e. EN 15784 and EN 15786 or ISO 15214 to *feedingstuffs*.

Recommended text for the register entry (analytical method)

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration of *Bacillus velezensis* NRRL-B-50910 in the *feed additive* and *feedingstuffs*: Spread plate method on triptone soya agar (EN 15784)
- Enumeration of *Pediococcus acidilactici* NRRL-B-50959 and *Pediococcus acidilactici* NRRL-B-50964 in the *feed additive* and *feedingstuffs*: Spread plate method on acidified MRS agar (EN 15786) or plate count method on MRS incubated anaerobically (ISO 15214)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *NS AH007* 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. 1831-0006-2021

[2] *Application, Proposal for Register Entry, Annex A

[3] *Technical dossier, Section II: II.2 Characterisation of the active agents

[4] *Technical dossier, Section II: Annex_II_2_8, Annex_II_11 & Annex_II_14

[5] *Technical dossier, Section II: II.1.3 Qualitative and quantitative composition

[6] *Technical dossier, Section II: II.5 Conditions of use of the additive

[7] #EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/default/files/finrep_fad-2018-0002_minalac.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep_fad-2020-0076_0077ped_pentosaceus.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2017-0025-pediococcus-pent.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2020-0049-bacillus-amyloliquefaciens.docx_.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep_fad-2020-0058_bio-three.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2019-0090-mixbalac.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2019-0086_correlinkabs1781.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2019-0074_correlink.pdf

[8] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.

[9] ISO 15214:1998 – Microbiology of food and animal feedingstuffs – Horizontal method for the enumeration of mesophilic lactic acid bacteria- Colony-count technique at 30 °C

[10] EN 15786:2009- Animal feeding stuffs - Isolation and enumeration of presumptive *Pediococcus* spp.

[11] Technical dossier, Section IV: Annex IV.1, Annex IV.2 & Annex IV.3

[12] EN ISO 7218:2007 & and EN ISO 7218/A1:2013- Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations

[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) and Report 20873/3 EN (2002) ISBN 92-894-6252-3 (Vol. III)

[14] prEN 17697 - Animal feeding stuffs: Methods of analysis - PFGE typing of Lactobacilli, Pediococci, Enterococci and Bacilli in animal feeds

*Refers to Dossier no: FAD-2021-0001

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

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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)