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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

Streptococcus salivarius K12

(FAD-2021-0060; CRL/210000)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0060 - CRL/210000**
Name of Product / Feed Additive:	***Streptococcus salivarius K12***
Active Agent (s):	**Streptococcus salivarius K12**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**María José González de la Huebra** **22/11/2022**
Report approved by: Date:	**Christoph von Holst** **25/11/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *Streptococcus salivarius K12* under the category / functional group 1(j) 'technological additives' / 'acidity regulators', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for cats, dogs and other non-food producing animals.

The active substance of the *feed additive* is *Streptococcus salivarius K12* strain with a minimum content of $1.0x10^{11}$ Colony Forming Unit (CFU) / g product.

The *feed additive* is intended to be used directly in liquid *feedingstuffs* with a minimum proposed dose of the active substance of $1.0x10^{8}$ CFU / ml *feedingstuffs*. The *feed additive* is not intended for use in dry vitamin/mineral premixtures.

For the enumeration of *Streptococcus salivarius K12* in the *feed additive* and *feedingstuffs* the Applicant submitted single-laboratory validated and further verified spread plate (or spiral plate) methods on Columbia Blood Agar containing yeast extract, glucose and calcium carbonate.

Based on the performance characteristics and all available information, the EURL recommends for official control the single-laboratory validated and further verified spread plate (or spiral plate) methods on Columbia Blood Agar containing yeast extract, glucose and calcium carbonate for the enumeration of *Streptococcus salivarius K12* 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

Streptococcus salivarius K12, technological additives, acidity regulators, dogs and other non-food producing animals.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *Streptococcus salivarius K12* under the category / functional group 1(j) 'technological additives' / 'acidity regulators', 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, dogs and other non-food producing animals [2].

The *feed additive* is intended to be marketed as a freeze-dried powder preparation [3]. The active substance of the *feed additive* is *Streptococcus salivarius K12* strain with a minimum content of $1.0x10^{11}$ Colony Forming Unit (CFU) / g product [4,5].

The *Streptococcus salivarius K12* is non-genetically modified strain, which is deposited in the American Type Culture Collection (ATCC) as ATCC BAA and in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkuturen GmbH) under the deposition number DSM 13084 [6].

The *feed additive* is intended to be used directly in liquid *feedingstuffs* with a minimum proposed dose of the active substance of $1.0x10^{8}$ CFU / ml *feedingstuffs* [7,8]. The *feed additive* is not intended for use in dry vitamin/mineral premixtures [8].

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 *Streptococcus salivarius K12* 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 *Streptococcus salivarius K12* in the *feed additive* and *feedingstuffs* the Applicant initially proposed [9] the ring-trial validated EN 15787 method, which is dedicated for the enumeration of lactobacilli spp. in animal feed [10], without providing the corresponding applicability data. However, in the frame of the request for a supplementary information [11], the Applicant proposed [12] and submitted a single-laboratory validated and further verified method for the enumeration of *Streptococcus salivarius K12* in the *feed additive* and *feedingstuffs* [13-15].

According to the specific protocols of the method, the samples of the *feed additive* and of *feedingstuffs* are suspended in appropriate volume of phosphate buffered saline (PBS) and the suspensions are serially diluted using the PBS. The aliquots from appropriate dilutions are then deposited on Petri plates using spread plate (or spiral plate) methods on Columbia Blood

Agar containing yeast extract, glucose and calcium carbonate. The agar plates are incubated at 37 °C in the air containing 5 % of carbon dioxide for 24 h [13-15].

The performance characteristics, reported in the frame of the single-laboratory validation [16,17,20,21] and verification [18-21] studies (as re-calculated by the EURL [22] using logarithmically transformed CFU values) are presented in Table 1.

Furthemore, the Applicant reported a limit of quantification (LOQ) of 3.7 $\log_{10}$ CFU/g of logarithmically transformed CFU values for the method to enumerate of *Streptococcus salivarius K12* in *feedingstuffs* [17].

In addition, the Applicant presented the stability studies of the *feed additive* [16,20,23] and the homogeneity studies of the active substance in *feedingstuffs* [24]. The performance characteristics demonstrated from these studies were similar to the ones obtained during the validation and verification studies of the method for the enumeration of *Streptococcus salivarius K12* in the *feed additive* and *feedingstuffs*.

Based on the performance characteristics and all available information, the EURL recommends for official control the single-laboratory validated and further verified spread plate (or spiral plate) methods on Columbia Blood Agar containing yeast extract, glucose and calcium carbonate for the enumeration of *Streptococcus salivarius K12* 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.

Table 1. The performance characteristics of the single-laboratory validated and further verified method for the enumeration of *Streptococcus salivarius K12* in the *feed additive* and *feedingstuffs*.

	Feed additive		***Feedingstuffs***	
	Validation	***Verification***	***Validation***	***Verification***
Amount, $\log_{10}$ CFU/g	11.6		9.2	
S_r, $\log_{10}$ CFU/g	0.08	0.09	0.05	0.05
S_{ip}, $\log_{10}$ CFU/g	0.08	0.1	0.05	0.07
R_{Rec}, %	100	99	100	100
Reference	[16,20,22]	[18,20,22]	[17,21,22]	[19,21,22]

S_r and S_{ip}: standard deviations for *repeatability* and *intermediate precision,* respectively;
R_{Rec} - a *recovery* rate;

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 *Streptococcus salivarius K12* at strain level, the Applicant proposed a Pulsed-Field Gel Electrophoresis (PFGE) [9]. The PFGE methodology, which is a generally recognised methodology for the genetic identification of bacterial strains has been already recommended by the EURL in former reports for similar dossiers [25]. Furthermore, this methodology has been recently ring-trial validated [26] and is supposed to become a CEN Technical Specification [27].

In addition, for the identification of the microorganisms at strain level, the Applicant used DNA sequencing methods such as whole genome sequencing [6].

The EURL considers that both methodologies (PFGE and DNA sequencing methods) are suitable for official control for the bacterial identification of *Streptococcus salivarius K12* at a strain level.

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) or ii) DNA sequencing methods for the identification of *Streptococcus salivarius K12* and (iii) the single-laboratory validated and further verified spread plate (or spiral plate) methods on Columbia Blood Agar containing yeast extract, glucose and calcium carbonate for the enumeration of *Streptococcus salivarius K12* in the *feed additive* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

- Identification: Pulsed-Field Gel Electrophoresis (PFGE) or DNA sequencing methods
- Enumeration in the *feed additive* and *feedingstuffs*: spread plate (or spiral plate) methods on Columbia Blood Agar containing yeast extract, glucose and calcium carbonate

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Streptococcus salivarius K12* 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. 1831-0050-2021

[2] *Application, Annex 1 – submission number 1616512706656-2942

[3] *Technical dossier, Section II: 2.1.1. Name of the additive

[4] *Technical dossier, Section II: 2.2. Characterisation of the active substance(s)/agent(s)

[5] *Technical dossier, Section II: 2. Introduction

[6] *Technical dossier, Section II: 2.2.1.2. Micro-organisms

[7] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition

[8] *Technical dossier, Section II: Section II: 2.4.1. Stability of the additive used in premixtures and feedingstuffs

[9] *Technical dossier, Section II: 2.6. Methods of analysis and reference samples

[10] EN 15787:2021 – Animal feeding stuffs: Methods of sampling and analysis - Detection and enumeration of Lactobacillus spp. used as feed additive

[11] *Supplementary information – 0_FAD-2021-0060_SIn_Addendum_EURL_230322

[12] *Supplementary information – 0_FAD-2021-0060_Addendum_AppReply-22

[13] *Supplementary information – Annex_II_6_1_8_BLTINGSOP2_AdditiveSpread

[14] *Supplementary information – Annex_II_6_1_7_BLTINGSOP1_AdditiveSpiral

[15] *Supplementary information – Annex_II_6_1_9_BLT-FGAF-SOP1_Feed

[16] *Supplementary information – Annex_II_6_1_3_Validation_BLIS_Additive

[17] *Supplementary information – Annex_II_6_1_6_Validation_BLIS_Feed

[18] *Supplementary information – Annex_II_6_1_2_Verification_Cawthron_Additive

[19] *Supplementary information – Annex_II_6_1_5_Verification_Cawthron_Feed

[20] *Supplementary information – Annex_II_6_1_1_Calc_Lab1_Lab2_FA

[21] *Supplementary information – Annex_II_6_1_4_Calc_Lab1_Lab2_feed

[22] *Supplementary information – EURL calculation performance characteristics

[23] *Technical dossier, Section II – Annex_II.2.4.1.1_Shelf_life

[24] *Technical dossier, Section II – Annex_II.2.4.2.1_Homogeneity

[25] EURL reports: https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en

[26] CEN project TC 327 WI00327127 (2020): DNA fingerprinting of lactobacilli, pediococci, enterococci and bacilli in animal feeds by pulsed field gel electrophoresis (PFGE) Draft Report of a validation trial

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

*Refers to Dossier no: FAD-2021-0060

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:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)