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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

Sorbensyl
(FAD-2017-0064; CRL/170043)

Sorbiflore® Advance
(FAD-2017-0066; CRL/170041)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2017-0064 - CRL/170043** **FAD-2017-0066 – CRL/170041**
Name of Product(s):	***Sorbensyl*** ***Sorbiflore® Advance***
Active Agent (s):	**Lactobacillus rhamnosus (CNCM I-3698)** **Lactobacillus farciminis (CNCM I-3699**
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** **18/10/2019**
Report approved by: Date:	**Christoph von Holst** **18/10/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) under the category / functional group 1(k) 'technological additives' / 'silage additives' (*Sorbensyl*)[1] and under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers' (*Sorbiflore® Advance*)[2], according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* in *silage* for all animal species (*Sorbensyl*) and in *feedingstuffs* for chickens for fattening (*Sorbiflore® Advance*).

According to the Applicant, both *feed additives* i.e. *Sorbensyl and Sorbiflore® Advance* are of identical composition and contain as active substances viable but non-cultivable cells of the non-genetically modified strains *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699). These *products* are to be marketed as a powder containing equal amounts of both *Lactobacillus* spp. strains (CNCM I-3698 and CNCM I-3699) with a minimum total content of $5x10^8$ so-called Forming Unit (FU)/g.

Sorbensyl is intended to be added to *silage* at a minimum dose of $2.5x10^7$ or of $8x10^7$ FU/kg of fresh *silage*, depending on the raw material ensiled. *Sorbiflore® Advance* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $5x10^7$ FU/kg of complete *feedingstuffs*.

For the quantification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) in the *feed additives*, *premixtures* and *feedingstuffs*, the Applicant submitted a single-laboratory validated and further verified (for *feedingstuffs*) method based on real-time quantitative Polymerase Chain Reaction (qPCR). Based on the available performance characteristics, the EURL recommends this method for official control for the quantification of the overall *Lactobacillus* spp. (CNCM I-3698 and CNCM I-3699) in the *feed additives*, *premixtures* and *feedingstuffs*.

The Applicant did not provide any experimental method or data for the quantification of the *Lactobacillus* spp. (CNCM I-3698 and CNCM I-3699) in *silage*. Since the unambiguous quantification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) added to *silage* is not achievable by analysis, the EURL cannot evaluate nor recommend any method for official control to quantify the active substances in *silage*.

The Applicant did not provide any method suitable for the identification at strain level of non-cultivable cells of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) present in the different feed matrices, thus the EURL cannot evaluate nor

[1]FAD 2017-0064;[2] FAD 2017-0066

recommend any method for official control to identify at strain level the target active substances in the *feed additive, silage, premixtures* 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

Lactobacillus rhamnosus (CNCM I-3698), *Lactobacillus farciminis* (CNCM I-3699) technological additives, silage additives, zootechnical additives, other zootechnical additives

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) for *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) under the category / functional group 1(k) 'technological additives' / 'silage additives' (*Sorbensyl*)[1] and under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers' (*Sorbiflore® Advance*)[2], according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for the use of the *feed additive* in *silage* for all animal species (*Sorbensyl*) and in *feedingstuffs* for chickens for fattening (*Sorbiflore® Advance*) [2].

According to the Applicant, both *feed additives*, i.e. *Sorbensyl* and *Sorbiflore® Advance*, are of identical composition and contain as active substances viable but non-cultivable cells of the non-genetically modified strains *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) [3]. Both strains are deposited at the Collection Nationale de Cultures de Micro-organismes (CNCM, Institut Pasteur, Paris) [3].

The *feed additives* are to be marketed as a powder containing equal amounts of both *Lactobacillus* spp. strains (CNCM I-3698 and CNCM I-3699) with a minimum total content of $5x10^8$ so-called Forming Unit (FU)/g [3].

Sorbensyl is intended to be added to *silage* at a minimum dose of $2.5x10^7$ or of $8x10^7$ FU/kg of fresh *silage*, depending on the raw material ensiled [4]. *Sorbiflore® Advance* is intended to be used directly in *feedingstuffs* or through *premixtures* at a minimum dose of $5x10^7$ FU/kg of complete *feedingstuffs* [5].

[1]FAD 2017-0064;[2] FAD 2017-0066

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 *Sorbensyl* and *Sorbiflore® Advance* 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 quantification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) in the *feed additives*, *premixtures* and *feedingstuffs* the Applicant submitted a method based on real-time quantitative Polymerase Chain Reaction (qPCR) [6].

This method has been single-laboratory validated and further verified by an external laboratory for *feedingstuffs*. Additionally, the repeatability of the method has also been verified for the *feed additives* and *premixtures* [6,7]. Furthermore, the Applicant demonstrated the suitability of the proposed method for the *feed additives* in the frame of stability studies [8].

Table 1: Performance characteristics of the analytical method for the determination of the sum of *Lactobacillus* spp. strains (CNCM I-3698 and CNCM I-3699) in *feed additives* (FA), *premixtures* (PM) and *feedingstuffs* (FS)

Matrix	Bacterial Counts (log_{10} FU/g)		S_r (log_{10} FU/g)		S_{ip} (log_{10} FU/g)		LOQ (log_{10} FU/g)	
	Val	Ver	Val	Ver	Val	Ver	Val	Ver
FA	-	6.32	-	0.15	-	-	-	5.00
PM	-	5.89	-	0.12	-	-	-	5.00
FS	5.11 -5.91	5.20 - 6.08	0.09 -0.23	0.12 -0.35	0.09	0.34	4.00	5.00

S_r: standard deviation for repeatability; S_{ip} standard deviation for intermediate precision; LOQ: Limit of quantification; Val: Validation; Ver: verification

A *feed additive*, *premixture* or *feedingstuffs* sample is dispersed in a buffer containing polyvinyl pyrrolidone (PVPP), stirred while incubated and further homogenised. An aliquot of the obtained suspension is filtered, sonicated and briefly centrifuged to sediment the debris. The supernatant is collected, intensively centrifuged and discarded. The pellet (bacterial cells) is then re-suspended in the buffer without PVPP and treated with propidium mono azide (PMA) to avoid further amplification of exposed DNA from dead cells. From this cell suspension, DNA is extracted on FTA® Elute cards, purified with ultra-pure water and amplified by qPCR with primers enabling amplification of DNA targets specific for each bacterial strain. For quantifying the target cells expressed in terms of FU, a conversion equation (from Ct to FU) is established for each strain with a "calibration sample" provided by the Applicant upon request. This "calibration sample" consists of the targeted strains that have been previously characterized for cell content by plate counting of the respective cultivable cells on MRS agar [9]. Before DNA extraction for qPCR analysis, serial dilutions of the cell suspension collected from the "calibration sample", and treated with PMA, are made to obtain enough data to establish the conversion equation. *Lactobacillus farciminis* (CNCM I-3699) and *Lactobacillus rhamnosus* (CNCM I-3698) estimated counts (FU) of unknown samples are calculated from their Ct values with their respective conversion equation and summed up to obtain the total content of viable cells in the *feed additives*, *premixtures* and *feedingstuffs* [6].

The main performance characteristics reported by the Applicant in the frame of the validation and verification [7] studies are summarised in Table 1.

Based on the available performance characteristics, the EURL recommends for official control the qPCR method described above for the quantification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) in the *feed additives*, *premixtures* and *feedingstuffs*.

The Applicant did not provide any experimental method or data for the quantification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) in *silage*. Furthermore, the unambiguous quantification of the *Lactobacillus* spp. (CNCM I-3698 and CNCM I-3699) added to *silage* is not achievable by analysis. Therefore, the EURL cannot evaluate nor recommend any method for official control to quantify the *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) strains in *silage*.

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)

The Applicant applied Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains, for the identification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) isolated in pure cultures [3]. 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 [10]. However the use of PFGE requires the presence of cultivable cells in order to be able to isolate them in a pure culture [11]. Therefore it cannot be considered as suitable in the frame of this dossier.

The Applicant did not provide any method suitable for the identification at strain level of the non-cultivable cells of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) present in the different *feed matrices*, thus the EURL cannot evaluate nor recommend any method for official control to identify at strain level the target active substances in the *feed additive, silage, premixtures* 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 the single validated and further verified real-time quantitative Polymerase Chain Reaction (qPCR) method for the quantification of *Lactobacillus rhamnosus* (CNCM I-3698) and *Lactobacillus farciminis* (CNCM I-3699) in the *feed additives (Sorbensyl* and *Sorbiflore® Advance), premixtures* and *feedingstuffs*.

As the unambiguous quantification of the *Lactobacillus* spp. (CNCM I-3698 and CNCM I-3699) added to *silage* is not achievable by analysis the EURL cannot evaluate nor recommend any method for official control for their quantification in *silage.*

Recommended text for the register entry (analytical method)

- Quantification in the *feed additive, premixtures* and *feedingstuffs*: real-time quantitative Polymerase Chain Reaction (qPCR)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Sorbensyl* and *Sorbiflore® Advance* 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] [1, 2]Application, References SANTE E5: F.A. 1831/0053-2017 and 1831/0051-2017

[2] [1, 2]Application, Proposal for Register Entry, Annex A

[3] [1, 2]Technical dossier, Section II: 2.2 Characterisation of the Active Substance

[4] [1]Additional information, Scientific Summary: 1.2.5 Conditions of use of the additive

[5] [2]Technical dossier, Section II: 2.5 Conditions of use of the additive

[6] [1]Supplemetary information: 2.6 Methods of analysis and reference samples

[7] [1]Supplemetaryl information: Annex_II_29 & Annex_II_30

[8] [1, 2]Technical dossier, Section II: 2.4 Physical-chemical and technological properties of the additive)

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

[10] 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)

[11] Lahti C.J. (1996). Pulsed field gel electrophoresis in the clinical microbiology laboratory. Journal of Clinical Laboratory Analysis 10: 326-330

[1]Refers to Dossier no: FAD- FAD-2017-0064; [2]Refers to dossier no: FAD-2017-0066

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)