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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/AS/Ares

Second amendment to the EURL report FAD-2006-0014 Sorbiflore®

(D08/FSQ/CVH/RL/D(2007) 11384)

In the *Sorbiflore*® report (FAD 2006-0014) [1] the EURL evaluated analytical methods based on the original proposal of register entry that described the conditions of use for piglets with concentrations expressed in colony forming units (c.f.u.) per kilogram (kg) of complete *feedingstuffs*.

On March 2008 new information was submitted to EFSA where the proposed Annex entry (Annex III) was changed regarding the measurement units of the active agents defined as viable fluorescent units (VFU). Consequently the EURL amended the mentioned evaluation report and recommended for quantification the direct epifluorescent filtration technique (DEFT) using an appropriate dye to stain viable metabolically active cells expressed as viable fluorescent units (VFU) [2].

In the frame of a new Application (FAD 2017-0066) [3] of the same *feed additive (Sorbiflore®),* the analytical method proposed for the quantification of the two lactobacilli strains namely *Lactobacillus rhamnosus* (CNCM-I-3698) and *Lactobacillus farciminis* (CNCM-I-3699) in the *feed additive* was based on real-time quantitative Polymerase Chain Reaction (qPCR) and thus different than the one described in the previous dossier (FAD 2006-0014). The EURL concluded in the evaluation report [3] that this new method i.e. qPCR is suitable for the official control of the two target lactobacilli strains in *feed additives*, *premixtures* and *feedingstuffs*.

In order to check, whether the method originally recommended in the above mentioned EURL amendment [2] is still valid, the EURL contacted the Applicant for clarification. The Applicant confirmed that the original method proposed for quantification of *Lactobacillus rhamnosus* (CNCM-I-3698) and *Lactobacillus farciminis* (CNCM-I-3699) in the *feed additive* i.e. direct epifluorescence filtration technique (DEFT) was no longer valid [4] and that the qPCR method applies. Based on this new information the old method needs to be replaced by the qPCR based method, thus keeping consistency with the one recently evaluated.

Recommended text for the registry entry (analytical method)

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

References

[1] CRL report - D08/FSQ/CVH/RL/D(2007) 11384

[2] ***Amendment*** *to CRL report* (D08/FSQ/CVH/RL/D(2007) 11384) - D08/FSQ/CvH/RL/D(2008)17156

[3] EURL Evaluation Report – JRC.F.5/CvH/MGH/ACS/ Ares(2019)6461358

[4] FAD-2017-0066 Supplementary information_change of analytical method, Ares(2019)6759321

Second amendment
- Prepared by María José González de la Huebra
- Reviewed and approved by Christoph von Holst (EURL-FA) Geel, 31/10/2019

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

CRL Feed Additives

Geel, 07/07/2008
D08/FSQ/CvH/RL/D(2008)17156

European Food Safety Authority
Animal Feed Unit
Mrs. C. Roncancio Peña
Largo N. Palli 5/A
I-43100 Parma, Italy

Subject: ***Amendment*** *to CRL report* (D08/FSQ/CVH/RL/D(2007) 11384) *on the dossier related to EFSA-Q-2006-062 (FAD-2006-0014) (Sorbiflore®)*

Dear Mrs. Roncancio Peña,

In relation to the dossier evaluation of Sorbiflore® (EFSA-Q-2006-062), EFSA asked the CRL-FA (e-mail of 29 April 2008) to reconsider its May 2007 Evaluation Report due to fact that EFSA received new information from the applicant including a change of the proposed annex entry. Following this request from EFSA, the CRL-FA contacted the applicant for additional information regarding the method of analysis.

The attached amendment to the CRL-FA report (D08/FSQ/CVH/RL/D(2007)11384) contains the evaluation by the CRL-FA and an Amended Executive Summary. In addition, the CRL recommends that *this* summary instead of the summary of the former CRL report is included in EFSA's opinion on this dossier.

Best regards,

Christoph von Holst

Christoph von Holst

Cc. Franz Ulberth (Food Safety and Quality, HoU), Willem Penning (DG SANCO, HoU).

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Amendment to CRL report (D08/FSQ/CVH/RL/D(2007) 11384) on the dossier EFSA-Q-2006-062 (FAD-2006-0014) (Sorbiflore®)

This Amendment was prepared by Renata Leuschner, revised by Giuseppe Simone and Piotr Robouch on 7 July and approved by Christoph von Holst 7 July 2008.

1. INTRODUCTION

In its report (D08/FSQ/CVH/RL/D(2007) 11384) the CRL-FA evaluated analytical methods based on the original proposal of register entry (Annex III) that described the conditions of use for piglets with concentrations expressed in colony forming units (c.f.u.) per kilogram (kg) of complete feedingstuffs.

Based on new information submitted by the applicant to EFSA [1], the proposed Annex entry (Annex III) was changed regarding the measurement units of the active agents defined as viable fluorescent units (VFU). The product Sorbiflore® contains a mixture of two lactobacilli strains (1×10^{11} viable fluorescent unit (VFU)/kg) at a ratio of 1:1 mixed onto a vegetable carrier. The conditions of use are proposed at concentrations of 1×10^{8} to 2×10^{9} VFU/kg complete feedingstuffs. The applicant mentions [1] that in the supplemented compound feed used for the efficacy trials, only viable metabolically active cells were detected and that cultivable (colony forming) cells were not detected.
The two lactobacilli strains, *Lactobacillus rhamnosus* (MA27/6B) CNCM-I-3698 and *Lactobacillus farciminis* (MA27/6R) CNCM-I-3699 are deposited at the Collection Nationale de Cultures Microorganismes (CNCM), at the Pasteur Institute, Paris, France.

3. EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5 Control methods), in the following referred to as 'the Guidelines'.

Description of some of the methods listed under item 2.5.1. of the Guidelines

Qualitative and quantitative composition of the additive

The applicant analyses the viable but non cultivable active agents, *Lactobacillus rhamnosus* CNCM-I-3698 and *Lactobacillus farciminis* CNCM-I-3699, of the product Sorbiflore® by direct epifluorescence filtration technique (DEFT) [1]. DEFT is described in the European Pharmacopoeia as an alternative method for control of microbiological quality [2]. The applicant based the method protocol on an European Standard (EN 13783) [3, 5]. VFUs are determined by the applicant using DEFT and a Chemchrom B® dye (Chemunex) and an excitation wavelength between 450 – 490 nm [5]. The method was validated in the Laboratory of Food Microbiology at the University of Caen in France [4]. The coefficients of variation of the DEFT method were demonstrated to be low, with a maximum of 2 % [4], well below values of 10 – 15 % which is considered acceptable in the European Pharmacopoeia [6]. A differentiation of the two lactobacilli strains is possible by DEFT based on the appearance of *L. farciminis* cells in pairs and of *L. rhamnosus* cells as short chains [5]. This allows determination of the ratio between the two strains. DEFT is considered suitable for the intended purpose by the CRL-FA.

The applicant confirmed that the use of pulsed-field gel electrophoresis (PFGE) as a tool to identify the two lactobacilli strains is applicable at all stages during the production process where viable cell that are able to form colony forming units on agar plates are present [6].

Description of qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (cf. requirements of Guidelines section 2.5.2)

For the enumeration of the metabolically active cells of the two strains, *Lactobacillus rhamnosus* CNCM-I-3698 and *Lactobacillus farciminis* CNCM-I-3699, in *premixtures* and *feedingstuffs*, the applicant proposes the same method as mentioned above [1].

The application of DEFT was validated in the literature for quantitative analysis of the active agents of Sorbiflore® by Bernadeau et al. (2001) [4][1] using premixture and feed samples. The coefficients of variation were 2 % derived from logarithmic measurement values to the basis of 10 [4].

The applicant submitted on the request of the CRL-FA additional information [5] including a detailed method protocol and additional validation data for the accuracy and precision of DEFT in feed supplemented with Sorbiflore®. Coefficients of variation of around 5 % were established using feed samples supplemented with the product Sorbiflore®, which is considered acceptable in the European Pharmacopoeia [6]. The limit of quantification (LOQ) was determined by the applicant to be 10^4 VFU/g which is well below the anticipated target concentrations in feed [5].

The use of pulsed-field gel electrophoresis (PFGE) as a tool to identify the two lactobacilli strains is not applicable for feed as the applicant confirms that no colony forming units are expected to be present [5].

4. CONCLUSIONS AND RECOMMENDATIONS

Having evaluated the method performance criteria of the method of analysis that was suggested by the applicant, the CRL-FA comes to the conclusion that DEFT is an appropriate technique for official controls in the context of the authorisation sought.
On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Quantification: Direct epifluorescent filtration technique (DEFT) using an appropriate dye to stain viable metabolically active cells expressed as viable fluorescent units (VFU)

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[1] The applicant confirmed that *Lactobacillus acidophilus* MA27/6R was reclassified as *Lactobacillus farciminis* MA27/6R [4]

References

[1] Additional information of the applicant sent to EFSA dated 25 March 2008
[2] European Pharmacopoeia 5.5, 07/2006:50106, 5.1.6. Alternative methods for control of microbiological quality, section 2.3.3. Direct epifluorescent filtration technique (DEFT)
[3] European Standard EN 13783, Foodstuffs – Detection of irradiated food using Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC) – Screening method
[4] Bernadeau M., Vernoux J.P., Gueguen M. 2001. Usefulness of epifluorescence for quantitative analysis of lactobacilli in probiotic feed. J. Appl. Microbiol. 91, 1103-1109
[5] Additional information and method protocol provided by the applicant on request of the CRL-FA dated 6 July 2008
[6] European Pharmacopoeia 5.5, 07/2006:50106, 5.1.6. Alternative methods for control of microbiological quality, section 3.3 Validation of alternative quantitative tests for enumeration of micro-organisms, section 3.3.2. Precision

Amended Executive Summary to CRL report (D08/FSQ/CVH/RL/D(2007) 11384) on the dossier EFSA-Q-2006-062 (FAD-2006-0014) (Sorbiflore®)

In the current application authorisation is sought for the microbial feed additive Sorbiflore® under the category ‘zootechnical additives’, functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive are viable but non colony forming cells of two microorganism strains, *Lactobacillus rhamnosus* CNCM-I-3698 and *Lactobacillus farciminis* CNCM-I-3699. The additive is a light brown free-flowing powder containing both strains at a ratio 1:1 mixed onto a vegetable carrier. Specifically, authorisation is sought to use Sorbiflore® for piglets from weaning up to two months. The conditions of use are proposed with a recommended dosage of 1 x 10^8 to 2 x 10^9 viable fluorescent units (VFU)/kg complete feedingstuffs.

For the quantification of the active agents (*Lactobacillus rhamnosus* CNCM-I-3698 and *Lactobacillus farciminis* CNCM-I-3699) of Sorbiflore® in the *feed additive*, *premixtures* and *feedingstuffs* the applicant proposes direct epifluorescence filtration technique (DEFT) which is based on a European Standard method[2]. DEFT is described in the

[2] European Standard EN 13783

European Pharmacopoeia[3] as an alternative method for control of microbiological quality. The application of DEFT was described and validated in the literature[4] for quantitative analysis of the active agents of Sorbiflore® using feed samples. The applicant submitted on the request of the CRL-FA additional information including a detailed method protocol and additional validation data for the accuracy and precision of DEFT in feed supplemented with Sorbiflore®. Coefficients of variation were established using the product Sorbiflore®, premixtures and feed samples. The values reported in the literature[4] were maximally 2 % and those reported by the applicant around 5 %. These coefficients of variations were below 10 – 15 % which is considered acceptable in the European Pharmacopoeia[5]. The limit of quantification (LOQ) was determined by the applicant to be 10^4 VFU/g which is well below the anticipated target concentrations in feed.

A differentiation of the two lactobacilli strains by DEFT based on the appearance of *L. farciminis* CNCM-I-3698 cells in pairs and of *L. rhamnosus* CNCM-I-3699 cells as short chains was demonstrated by the applicant. This allows determination of the ratio between the two strains. Pulsed-field gel electrophoresis (PFGE) as a tool to identify the two lactobacilli strains is applicable at all stages during the production process where viable cell that are able to form colony forming units on agar plates are present. It is not applicable e.g. when the product has been added to feed as no colony forming units are expected to be present.
DEFT is considered by the CRL-FA appropriate for official controls in the frame of the authorisation concerning the quantitative determination of the viable fluorescent units of the active agents in the *feed additive*, *premixtures* and *feedingstuffs*.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

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[3] European Pharmacopoeia, 5.5, 07/2006:50106, 5.1.6., 2.3.3.

[4] J. Appl. Microbiol. 2001, 91, 1103-1109

[5] European Pharmacopoeia 5.5, 07/2006:50106, 5.1.6., 3.3.

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D08/FSQ/CVH/RL/D(2007)11384

CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2006-062 FAD-2006-0014
Name of Additive:	Sorbiflore® for piglets
Active Agent(s):	*Lactobacillus rhamnosus* MA27/6B, *Lactobacillus farciminis* MA27/6R
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
Report prepared by:	Renata Leuschner (CRL-FA)
Report checked by:	Giuseppe Simone and Renata Leuschner (CRL-FA)
Date:	10/05/2007
Report approved by:	Christoph von Holst (CRL-FA)
Date:	10/05/2007

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Sorbiflore® under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive are viable cells of two microorganism strains, *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R. The additive is a light brown free-flowing powder containing equivalent numbers of both strains at concentrations of 0.5 x 10^8 colony forming units (c.f.u.) per gram additive. Specifically, authorisation is sought to use Sorbiflore® for piglets from weaning up to two months. The conditions of use are proposed with a recommended dosage of 1 to 5 x 10^8 c.f.u./kg complete feedingstuffs.

For the quantification of the active agents (*Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R) of Sorbiflore® in the *feed additive*, *premixtures* and *feedingstuffs* the applicant uses a microbiological plate count enumeration method and epifuorescence microscopy. The methods are appropriate for the purpose.

For official controls in the frame of the authorisation concerning the quantitative determination of the colony forming units of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been fully ring-trial validated (Food Microbiol. 2003, 20, 57-66). The method performance characteristics include a relative standard deviation for repeatability (RSD_r) of around 1 to 3 % and a relative standard deviation for between-laboratory reproducibility (RSD_R) of around 2 to 5 %. The limit of quantification (LOQ) for the method is around 2 to 3 x 10^6 c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

The identity of the bacterial strains, *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R, was analysed by pulsed-field gel electrophoresis (PFGE) which showed a sufficient degree of differentiation. PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

KEYWORDS

Sorbiflore®, feed additive, *Lactobacillus rhamnosus, L. farciminis*, zootechnical additive, piglets

1. BACKGROUND

Sorbiflore® is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Sorbiflore® is provided in form of a powder supplemented with two strains in equal numbers, *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R. According to the applicant Sorbiflore® contains a mixture of 'Colony Forming Units (CFU)' determined according to classical microbiological methods and 'viable but non cultivable cells' (FU for Fluorescent Units) determined according to the method Bernardeau et al. (2001) [1, 5]. The content of each strain is 0.5 x 10^8 colony forming units (c.f.u.) and 1 x 10^8 florescent units (FU) per gram feed additive and in total for both strains 1 x 10^8 c.f.u./g and 2 x 10^8 FU/g [1]. The two strains are deposited at the Institut Pasteur, Paris, France. The intended use of the current application (EFSA-Q-2006-062) is for piglets from weaning up to two months. The proposed conditions of use are a recommended dosage of 1 to 5 x 10^8 c.f.u./kg complete feedingstuffs [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application. For this particular dossier, the methods of analysis submitted in connection with the Sorbiflore® dossier (EFSA-Q-2006-062) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5 Control methods), in the following referred to as 'the Guidelines'.

Description of some of the methods listed under item 2.5.1. of the Guidelines

Qualitative and quantitative composition of the additive

The applicant quantifies the strains *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R in the additive by classical microbiological methods and by an epifluorescence microscopy technique [3, 4, 5]. Colony forming units are determined by plate count enumeration technique. Fluorescent units (FU) are determined by staining cells with appropriate fluorochromes and by counting them using an epifluorescence microscope. The proposed methods are suitable for the intended purpose. However, for official controls in the frame of the authorisation epifluorecence microscopy is not appropriate as colony forming units can only be determined by a plate count enumeration technique. A fully ring-trial validated plate count method is recommended for official controls in the frame of the authorisation [6].

The genetic identity of the strains is examined by pulsed-field gel electrophoresis (PFGE) however all method details were not provided [7]. PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

Qualitative and quantitative composition of any impurities in the additive

The applicant analyses the feed additive for microbial contaminants such as faecal coliforms, *Salmonella* species, yeasts and fungi. Heavy metals including chromium, lead, arsenic, mercury and other impurities are further monitored [8]. Method description or protocols are not provided. Therefore, internationally recognised standardised methods such as from the International Organization for Standardization (ISO) and the European Committee for Standardisation (CEN) where available are recommended for official controls in line with current EU legislation

Description of qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (cf. requirements of Guidelines section 2.5.2)

For the enumeration of the two strains of the active agents, *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R, in *premixtures* and *feedingstuffs*, the applicant proposes similar methods as mentioned above [3, 4, 5]. The methods are suitable for the intended purpose.

For official controls the fully ring-trial validated method as cited above is recommended [6]. The ring-trial validated method includes a preparation of an initial sample suspension using 20 g for premixtures and 50 g for feed samples in phosphate buffered saline.

Decimal dilutions in peptone salt diluent are prepared. Subsequently, appropriate dilutions are spread on de Man, Rogosa, Sharp (MRS) agar and plates were incubated at 37 °C for 24 – 48 h. This method revealed relative standard deviations for repeatability (RSD_r) between 1.2 – 3.4 % and for inter-laboratory reproducibility (RSD_R) between 2.2 – 5.2 %.

The applicant used a range of techniques to identify the *Lactobacillus rhamnosus* MA27/6R and *Lactobacillus farciminis* MA27/6B strains used as active agent as described above [7]. Pulsed-field gel electrophoresis (PFGE) is considered as a suitable method for official controls.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant uses appropriate conventional methods to enumerate the active agent. A ring-trial validated method using a MRS agar is recommended for official controls in the frame of the authorisation [6]. Pulsed-field gel electrophoresis (PFGE) is widely used by reference laboratories to identify bacterial isolates and it is therefore recommended as a suitable methodology in this context for official controls.
On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Enumeration: Spread plate method using MRS agar and 37 °C as incubation temperature
Identification: Pulsed-field gel electrophoresis (PFGE)

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive Sorbiflore® for piglets for fattening have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Technical dossier, section II, 2.4 Conditions of use of the additive
[2] Proposal of Register entry
[3] Technical dossier, section II, 2.1.3 Qualitative and quantitative composition
[4] Technical dossier, section II, .5. Control methods
[5] Bernardeau M., Vernoux J.P., Gueguen M. 2001. Usefulness of epifluorescence for quantitative analysis of lactobacilli in probiotic feed. J. Appl. Microbiol. 91, 1103-1109

[6] Leuschner R.G.K., Bew J.,Coeuret V., Vernoux J.P., Gueguen, M. 2003. A collaborative study of a method for the enumeration of probiotic lactobacilli in animal feed. Food Microbiol. 20, 57-66
[7] Technical dossier, section II, 2.2. Biological origin
[8] Technical dossier, section II, 2.1.4. Qualitative and quantitative composition of any impurities

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- VITO, Mol, Belgium
- National Reference Laboratory RO Praha, Praha, Czech Republic
- Laboratoire de Rennes, Rennes, France
- Laboratori Agroalimentari, Cabrils, Spain