SCIENTIFIC OPINION

efsa JOURNAL

ADOPTED: 31 January 2023

doi: 10.2903/j.efsa.2023.7858

Safety and efficacy of a feed additive consisting of *Lacticaseibacillus rhamnosus* CNCM I-3698 and *Companilactobacillus* sp. CNCM I-3699 for weaned piglets (STI Biotechnologie)

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of Sorbiflore® ADVANCE, a feed additive consisting of *Lacticaseibacillus rhamnosus* CNCM I-3698 and *Companilactobacillus* sp. CNCM I-3699 intended to be used as a zootechnical additive (functional group: other zootechnical additives) in feed for weaned piglets to improve their performance. In a previous opinion, the additive was described as containing viable but not cultivable cells of the two strains in a 1:1 ratio, with a minimum of total lactic acid bacteria counts of 5×10^8 viable-forming units (VFU)/g additive. However, in that opinion, the Panel could not fully characterise the additive or conclude on its dermal/ocular irritancy or sensitisation potential. In the current assessment, the applicant provided supplementary information to address the missing information for the characterisation of the additive. The proposed methodology to discriminate and individually quantify the two strains composing the additive still presented limitations. Therefore, the Panel concluded that the data available do not allow to fully characterise the additive. The Panel was not in the position to conclude on the taxonomical identification of the strain CNCM I-3699, and consequently, on its eligibility for the application of the qualified presumption of safety (QPS) approach. Therefore, the previous conclusions on the safety of the additive based on the QPS approach could not be confirmed. The Panel was not in the position to conclude on the safety of the additive for the target species, consumer and the environment. Sorbiflore® ADVANCE is not irritant to skin. The Panel could not conclude on the eye irritancy or skin sensitisation potential of the additive.

Keywords: zootechnical additives, other zootechnical additives, *Lacticaseibacillus rhamnosus* CNCM I-3698 and *Companilactobacillus* sp. CNCM I-3699, piglets, safety

Requestor: European Commission

Question number: EFSA-Q-2021-00535

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Declarations of interest: If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

Acknowledgements: The Panel wishes to thank the following for the support provided to this scientific output (in alphabetical order of the last name): Natalia Alija Novo and the Microbiology WG.

Suggested citation: EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Bampidis V, Azimonti G, Bastos ML, Christensen H, Dusemund B, Durjava M, Kouba M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Cocconcelli PS, Pettenati E, Anguita M and Brozzi R, 2023. Scientific Opinion on the safety and efficacy of a feed additive consisting of *Lacticaseibacillus rhamnosus* CNCM I-3698 and *Companilactobacillus* sp. CNCM I-3699 for weaned piglets (STI Biotechnologie). EFSA Journal 2023;21 (4):7858, 8 pp. https://doi.org/10.2903/j.efsa.2023.7858

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

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1. Introduction

1.1. Background and terms of reference as provided by the requestor

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 9 defines the terms of authorisation by the Commission.

The applicant, STI Biotechnologie, is seeking a Community authorisation of *Lactobacillus rhamnosus* and *Lactobacillus farciminis* as an other zootechnical additive for piglets (Table 1).

Table 1: Description of the substances

Category of additive	**Zootechnical additives**
Functional group of additive	Other zootechnical additives
Description	*Lactobacillus rhamnosus* & *Lactobacillus farciminis*
Target animal category	Piglets (weaned)
Applicant	STI Biotechnologie
Type of request	New opinion

On 19 March 2020, the Panel on Additives and Products or Substances used in Animal Feed of the European Food Safety Authority ('Authority'), in its opinion on the safety and efficacy of the product, could not conclude because the data provided do not allow a full characterisation of the additive, and therefore, uncertainty remains on the nature of the product in terms of viability, on the ratio between the active agents and on the stability of the additive.

During the discussions with the Member States at a meeting of the Standing Committee on Plants, Animals, Food and Feed (Animal Nutrition section), it was suggested to check for the possibility to demonstrate viability and stability of the additive.

The Commission gave the possibility to the applicant to submit supplementary information and data in order to complete the assessment and to allow a revision of the EFSA's opinion. The new data have been received on 19 February 2021 and the applicant has been requested to transmit them to EFSA as well.

In view of the above, the Commission asks the Authority to deliver a new opinion on *Lactobacillus rhamnosus* & *Lactobacillus farciminis* as a feed additive for weaned piglets based on the additional data submitted by the applicant, in accordance with Article 29(1)(a) of Regulation (EC) No 178/2002.

1.2. Additional information

EFSA issued two opinions on the safety and efficacy of the product consisting of *Lactobacillus rhamnosus*, CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699 (Sorbiflore® ADVANCE) when used as a zootechnical additive for weaned piglets (EFSA, 2008; EFSA FEEDAP Panel, 2020a), one for chickens for fattening (EFSA FEEDAP Panel, 2020b) and one when used as a silage additive for all animal species (EFSA FEEDAP Panel, 2020c). Since the last opinions, the taxonomic designation of the species under assessment has been updated from *Lactobacillus rhamnosus* to *Lacticaseibacillus rhamnosus* and *L. farciminis* to *Companilactobacillus farciminis* (EFSA BIOHAZ Panel, 2020).

Sorbiflore was authorised as a zootechnical additive for use in piglets.[2] This authorisation expired on 8 January 2019.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of supplementary information[3] to a previous application on the same product.[4]

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Commission Regulation (EC) No 1290/2008 of 18 December 2008 concerning the authorisation of a preparation of *Lactobacillus rhamnosus* (CNCM-I-3698) and *Lactobacillus farciminis* (CNCM-I-3699) (Sorbiflore) as a feed additive. OJ L 340, 19.12.2008, p. 20, plus amendments.
[3] FEED dossier reference: EFSA-Q-2021-00535.
[4] Dossier reference: FAD-2018-0027.

In accordance with Article 38 of the Regulation (EC) No 178/2002 and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation, and of the Decision of EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[5] a non-confidential version of the supplementary information has been published on Open.EFSA.[6]

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA, peer-reviewed scientific papers, to deliver the present output.

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Lactobacillus rhamnosus*, CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699 (Sorbiflore® ADVANCE) is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEEDAP Panel, 2017), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).

3. Assessment

The additive under assessment with the tradename Sorbiflore® ADVANCE is the result of the fermentation of a milk-based broth with two lactic acid bacteria (*L. rhamnosus* CNCM I-3698 and *C. farciminis* CNCM I-3699). Sorbiflore® ADVANCE is intended to be used as a zootechnical additive (functional group: other zootechnical additives) at the minimum level of 1.25×10^8 viable-forming units (VFU)/kg and the maximum level of 5×10^8 VFU/kg complete feed for weaned piglets, in order to improve their performance.

In a previous opinion (EFSA FEEDAP Panel, 2020a), the applicant described the product as containing viable but not cultivable cells of the two strains in a 1:1 ratio, with a minimum total lactic acid bacteria (LAB) count of 5×10^8 VFU/g additive. However, in that opinion, the data available did not allow the Panel to fully characterise the additive or to conclude on its dermal/ocular irritancy potential and on its dermal sensitisation potential.

The applicant has produced new data to address the limitations identified by the Panel, that are described below.

3.1. Characterisation of the additive

In the previous assessments (EFSA, 2008; EFSA FEEDAP Panel, 2020a), the strain CNCM I-3698 was taxonomically identified as *Lactobacillus rhamnosus*, and the strain CNCM I-3699 as *Lactobacillus farciminis*. The taxonomic designation of these species has been updated to *Lacticaseibacillus rhamnosus* and *Companilactobacillus farciminis*, respectively. The current names are used hereafter in the opinion.

Additionally, in the new data set, the applicant indicated that the strain CNCM I-3699 has been reassigned to the species *Companilactobacillus formosensis* on the basis of the whole genome sequence (WGS) analysis.[7] However, no information was provided on the WGS-based analyses that assigned the strain CNCM I-3699 to the new species *C. formosensis*. In particular, no details were provided on the bioinformatics tools used, or whether representative type strains of species of the genus *Companilactobacillus* were included in the analysis. Consequently, the FEEDAP Panel is not in the position to conclude on the taxonomical identification of the strain CNCM I-3699.

The additive is described as containing viable but not cultivable cells of the two bacterial strains in a 1:1 ratio, with a minimum total LAB number of 5×10^8 VFU/g additive. In the former opinion, the applicant proposed a method based on the use of propidium monoazide (PMA) coupled with real-time quantitative polymerase chain reaction (qPCR) to characterise the additive and confirm its inclusion level

[5] Decision available at: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements
[6] Available at: https://open.efsa.europa.eu/questions/EFSA-Q-2021-00535
[7] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.
[8] 4. ANALYTICAL METHOD, 2. Report *L. rhamnosus* FULL VERSION and 3. Report *L. farciminis* FULL VERSION.

in the feed. The methodology foresees that the PMA is cell membrane-impermeable and selectively penetrates the dead cells with damaged membranes and, after photoactivation, cross-links with DNA. The resulting DNA monoadducts prevent the DNA amplification. Consequently, only the DNA from viable cells with intact membrane can be subject to qPCR following a proper lysis step. This method was evaluated in the previous opinion and the FEEDAP Panel concluded that it did not allow an unambiguous discrimination between the two lactobacilli strains. Consequently, a full characterisation of the additive in terms of nature, compliance with the specifications and stability could not be established.

The applicant developed a new methodology using PMA coupled with qPCR and TaqMan probes to individually enumerate the two strains in feed. The methodology was based on a set of primers and an internal probe designed to target strain-specific genes present in single copy in the genome of *L. rhamnosus* CNCM I-3698 or *Companilactobacillus* sp. CNCM I-3699.[7] However, details on the target genes were not provided.

The specificity of the primers and probe used in the qPCR analysis aimed at identifying and quantifying *L. rhamnosus* CNCM I-3698 was tested on 30 DNA samples from eight species of *Lactobacillaceae* and 16 other bacterial species from different genera. No amplification signal was observed in any of the samples tested. Similarly, the same pool of strains was used to test the specificity of the primers and probe selected for *Companilactobacillus* sp. CNCM I-3699. No amplification signal was observed in any of the samples tested. However, the Panel notes that the set of primers/probe for *L. rhamnosus* CNCM I-3698 was not tested on *Companilactobacillus* sp. CNCM I-3699, and vice versa. Consequently, data showing that the proposed qPCR methods are strain-specific and adequate to discriminate and quantify *L. rhamnosus* CNCM I-3698 and *Companilactobacillus* sp. CNCM I-3699 in the additive is still lacking.

As concerns the controls and sensitivity tests of the methodologies proposed, the applicant submitted studies conducted on the DNA extracted from the two strains of the additive with or without the PMA treatment which are described below.

The quantification efficiency of the qPCR methods was tested using the DNA extracted from dilutions of a broth culture from which the cell counts (CFU/ml) were determined by plate counting on MRS medium. Five logarithmic dilutions (from 7.8×10^5 to 78 genome-equivalent units (GU) per PCR tube of 2 μL) for *L. rhamnosus* CNCM I-3698 and four logarithmic dilutions (from 1×10^6 to 1×10^3 GU per PCR tube of 2 μL) in the case of *Companilactobacillus* sp. CNCM I-3699 were used. The experiments were made in triplicate. The Ct values of the calibration curve experiments were provided, while slope values were not.

The limit of detection of the qPCR methods was determined by using 10 replicates per strain and per dilution at DNA concentrations of 78, 39, 19, 9 and 5 GU for *L. rhamnosus* CNCM I-3698 and 500, 250, 125, 62 and 31 GU for *Companilactobacillus* sp. CNCM I-3699 calculated per PCR tubes of 2 μL. In the absence of PMA, DNA of *L. rhamnosus* CNCM I-3698 was detected in all the 10 replicates only at the highest concentration, while only in seven to eight replicates out of 10, the amplification occurred in the experiments with *Companilactobacillus* sp. CNCM I-3699. The PMA treatment was efficient in eliminating the free *Companilactobacillus* sp. CNCM I-3699 DNA in samples with a concentration $\leq$ 125 GU per PCR tube. Differently, the PMA treatment was unable to eliminate the free DNA in all the samples of *L. rhamnosus* CNCM I-3698 tested at lower concentrations ($\leq$ 39 GU per PCR tube). No data were provided on the efficacy of the PMA treatment and the qPCR methods to differentiate between viable, dead or inactivated cells.

Six samples (three batches of the additive, the additive in a premixture, a mash and a pelleted feed) were analysed with and without the PMA treatment for the quantification of both strains, using the qPCR method described above. No certificate of analysis was provided for any of the test items. The detected GU values were substantially identical between the PMA treated and untreated samples, suggesting that most of the cells present an intact cellular membrane. However, no data were provided to allow reaching conclusions on the individual enumeration of the two strains, and therefore, on compliance with the specifications of the additive.

The proposed methodology to individually enumerate the two strains composing the additive still presents limitations. In particular: (i) the capacity of the specific qPCR detection and quantification methods to discriminate between the two strains has not been tested (i.e. the method developed for CNCM I-3698 was not applied on CNCM I-3699 and vice versa), (ii) no demonstration of the efficacy of the PMA treatment and the qPCR methods to differentiate between viable, dead or inactivated cells was provided. Therefore, based on the available data, the Panel is not in the position to conclude on the full characterisation of the additive under assessment.

3.2. Safety

In the former opinion (EFSA FEEDAP Panel, 2020a), the Panel concluded that the active agents (*Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699) fulfilled the requirements of the QPS approach to the assessment of safety, no concerns were expected from other components of the additive; therefore, Sorbiflore® ADVANCE was presumed to be safe for the target animals, consumers and the environment.

In view of the applicant's new statement that strain CNCM I-3699 has been reassigned to the species *Companilactobacillus formosensis*, but owing to the lack of data, the FEEDAP Panel is not in the position to conclude on the taxonomical identification of this strain, and consequently, on its eligibility for the application of the qualified presumption of safety (QPS) approach to safety assessment. Therefore, the former conclusions on the safety of the additive based on the QPS approach cannot be confirmed (EFSA FEEDAP Panel, 2020a).

As regards the safety for the user, in the former opinion the Panel concluded that 'Despite the request, no information was provided on the inhalation toxicity of the additive or on its skin/eye irritation and skin sensitisation potential. The dustiness of the preparations tested indicated a potential for users to be exposed via inhalation to be likely. Given the proteinaceous nature of the active agents, the additive should be considered a respiratory sensitiser. In the absence of data, the FEEDAP Panel cannot conclude on the irritancy of the additive to skin and eyes and on its dermal sensitisation potential'.

The applicant has now submitted a skin irritation study.[9] The skin irritation potential of Sorbiflore® ADVANCE was investigated in an *in vitro* study performed according to OECD TG 439. The results indicated that the additive is non-irritant to skin and classified in accordance with UN GHS as 'no Category'.

In the absence of data, the Panel cannot conclude on the eye irritancy potential of the additive.

The FEEDAP Panel notes that the OECD test guidelines available at present are designed to assess the skin sensitisation potential of chemical substances only and that currently no validated assays for assessing the sensitisation potential of microorganisms are available. Therefore, no conclusions can be drawn on the skin sensitisation potential of the additive.

4. Conclusions

The proposed methodology to discriminate and individually quantify the two strains composing the additive still presents limitations. Therefore, the Panel concludes that the data available do not allow to fully characterise the additive under assessment.

The FEEDAP Panel is not in the position to conclude on the taxonomical identification of one of the two bacterial strains composing the additive (strain CNCM I-3699), and consequently, on its eligibility for the application of the qualified presumption of safety (QPS) approach to safety assessment in the present assessment. Therefore, the former conclusions on the safety of the additive based on the QPS approach cannot be confirmed. The Panel cannot conclude on the safety of Sorbiflore® ADVANCE for the target species, consumer and the environment.

The Panel reiterates its previous conclusions that Sorbiflore® ADVANCE should be considered a respiratory sensitiser. Based on the new data provided, the additive is not irritant to skin. The Panel cannot conclude on the eye irritancy or skin sensitisation potential of the additive.

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Abbreviations

FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
LOD	limit of detection
qPCR	quantitative polymerase chain reaction
PMA	propidium monoazide
VFU	Viable forming unit
WGS	Whole genome sequence