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Safety and efficacy of *Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699 as a feed additive for all animal species

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Abstract

Following a request from the European Commission, the EFSA 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 *Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699 when used as a silage additive in forage for all animal species. The additive is the result from the fermentation of milk-based broth with *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699. It is intended to be used in easy and moderately difficult to ensile materials at a minimum proposed application rate of 2.5×10^7 and maximum 8×10^7 VFU/kg complete feed for all animal species. 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 shelf-life of the additive. Both strains fulfil the requirements of the Qualified Presumption of Safety (QPS) approach to the assessment of safety and consequently, are presumed safe for the target animals, consumers of products from animals receiving the additive and the environment. The additive should be considered a respiratory sensitiser. In the absence of data, no conclusions can be drawn on the irritancy of the additive to skin and eyes or on its dermal sensitisation potential. No conclusions can be drawn on the efficacy of the additive to improve the ensiling process of easy and moderately difficult to ensile materials.

Keywords: Technological additives, silage additive, *Lactobacillus rhamnosus* CNCM I-3698, *Lactobacillus farciminis* CNCM I-3699, safety, efficacy

Requestor: European Commission

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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 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from STI Biotechnologie[2] for authorisation of *Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699, when used as a feed additive for all animal species (category: Technological additives; functional group: Silage additive).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 22 May 2018.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of *Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699, when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

The additive is a preparation containing *Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699. EFSA has issued three opinions on the safety and efficacy of a microbial product containing *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699 when used as a zootechnical additive in feed for piglets (EFSA, 2008; EFSA FEEDAP Panel, 2020a) and for chickens for fattening (EFSA FEEDAP Panel, 2020b).

The additive under assessment has not been previously authorised as a technological additive in the European Union, but the active agents *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699 were authorised as a zootechnical additive for use with piglets.[3,4,5] 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 a technical dossier[6] in support of the authorisation request for the use of *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699 as a feed additive.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active agents in animal feed. The Executive Summary of the EURL report can be found in Annex A.[7]

[1] Regulation (EC) No s1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] STI Biotechnologie, Zone Artisanale du Coglais, 35460 St. Etienne en Coglès, France.
[3] 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.
[4] Commission Regulation (EC) No 899/2009 of 25 September 2009 amending Regulation (EC) No 1290/2008 as regards the name of the holder of the authorisation of a preparation of *Lactobacillus rhamnosus* (CNCM-I-3698) and *Lactobacillus farciminis* (CNCM-I-3699) (Sorbiflore). OJ L 256, 29.9.2009, p. 11.
[5] Commission Regulation (EC) No 1334/2013 of 13 December 2013 amending Regulation (EC) No 1290/2008 as regards the name of the holder of the authorisation and as regards the recommended dose of a preparation of *Lactobacillus rhamnosus* (CNCM-I-3698) and *Lactobacillus farciminis* (CNCM-I-3699) (Sorbiflore). OJ L 256, 29.9.2009, p. 11.
[6] FEED dossier reference: FAD-2017-0064.
[7] The full report is available on the EURL website: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports/fad-2006-0014?search&form-returnplus amendments

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699 is in line with the principles laid down in Regulation (EC) No 429/2008[8] and the relevant guidance documents: Guidance on technological additives (EFSA FEEDAP Panel, 2012a), Technical guidance: Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Guidance on studies concerning the safety of use of the additive for users/ workers (EFSA FEEDAP Panel, 2012b) and Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).

3. Assessment

The additive containing *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699 is intended to be used as a technological additive (silage additive) in forages for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the additive

The additive under assessment is the result from the fermentation of a milk-based broth with *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699. The manufacturing process foresees a two-stage process in which cultures are first grown separately in skimmed milk-based medium and then used to inoculate the production broth, in which the skimmed milk content is increased and molasses are added. Subsequently, the co-culture is mixed with the carrier materials to reach the final concentration of 10% biomass, 60.4% extruded corn meal, 11.34% soybean meal, 10% micronised wheat hulls, 7.2% algae meal, 1% silicon oxide[9] and 0.06% of an antifungal preservative based on calcium propionate, sodium diacetate and calcium formate with a mineral carrier.

The applicant describes the product as containing viable but not cultivable cells of the two strains in a 1:1 ratio, with a minimum total lactobacilli (LAB) number of 5×10^8 Viable Forming Units (VFU)/g additive.[10]

To characterise the additive and confirm the inclusion level in the feed used in the efficacy studies, the applicant has developed a method [redacted][11]

Compliance with the specifications, in terms of total lactobacilli counts, was confirmed by analysis of 10 batches (mean value of 5.7×10^8 VFU/g, range= 5.15–6.5 $\times 10^8$ VFU/g, coefficient of variation CV = 8.5%), using the PMA-qPCR method.[12] Individual counts of the two strains were tested in the same 10 batches to support the declared qualitative and quantitative (1:1 ratio) composition.[13]

The Panel notes that, following the EURL report on the methodology and the data provided by the applicant, the unambiguous discrimination between the two lactobacilli strains is not possible, which does not allow to conclude on the ratio between the two strains in the product. Therefore, the method does not allow a full characterisation of the additive in terms of viability, on the ratio between the active agents, and uncertainty remains on the nature of the product and on its shelf-life.

Based on the above, the method used by the applicant does not allow a full characterisation of the additive, and therefore, uncertainty remains on the nature of the product and on its stability.

Three batches of the additive were analysed for microbial contaminants.[14] Results confirm compliance with limit levels (coliforms < 500 CFU/g, *Salmonella* absence in 25 g, *Listeria monocytogenes* absence in 25 g, yeasts and filamentous fungi < 1.5×10^4 CFU/g. Chemical contaminants were analysed in one batch of the additive (lead < 1.0 mg/kg, arsenic < 1,0 mg/kg, cadmium < 0.25 mg/kg, mercury < 0.01 mg/kg, aflatoxins B1, B2, G1 and G2 < 0.01 mg/kg, deoxynivalenol: 1.11 mg/kg, ochratoxin A (< 0.01 mg/kg).[15,16]

[8] 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.
[9] Feed additive currently under re-evaluation.
[10] Technical dossier/Supplementary information April 19/FAD-2017-0066.
[11] Technical dossier/Section II/Annexes II-8, [redacted]
[12] Technical dossier/Section II/Annex II-8.
[13] Technical dossier/Section II/Annex II-18.
[14] Technical dossier/Section II/Annex II-9.
[15] Technical dossier/Supplementary information April 19/Annex 3.
[16] LOD or LOQ not provided.

Five batches of the additive were analysed for dusting potential with the Stauber-Heubach dustmeter. Results showed values of 36–83 g/m^3 (average: 58 g/m^3).[17]

3.1.2. Characterisation of the active agents

Both strains of the additive were isolated from the rumen of healthy goats and deposited in the National Micro-organism Collection of Pasteur Institute (CNCM, Paris) with the accession numbers CNCM I-3698 and CNCM I-3699.[18]

The full genome sequence of both strains was obtained and used for characterisation purposes. [redacted] Although the analysis was not conducted in full compliance with the relevant FEEDAP Guidance, [redacted] the data are deemed sufficient to identify the strains as *L. rhamnosus* and *L. farciminis*.

The susceptibility of both strains to the antibiotics recommended by the relevant FEEDAP Guidance (EFSA FEEDAP Panel, 2018) was tested by broth microdilution.[20] All the minimum inhibitory concentration (MIC) values for *L. rhamnosus* CNCM I-3698 were lower than the EFSA cut-off values, consequently, the strain is considered to be susceptible to these relevant antibiotics. The only exception was the MIC for chloramphenicol which was exceeded by two dilutions (MIC: 16 mg/L vs. cut-off value: 4 mg/L).

The applicant provided two sets of data on WGS interrogation for the presence of antimicrobial resistance (AMR) genes. [redacted]

Regarding *L. farciminis* CNCM I-3699, the MIC values for ampicillin, gentamicin and streptomycin were below or equal to the corresponding EFSA cut-off values; consequently, the strain is considered to be susceptible to these relevant antibiotics. The MIC values for the remaining antibiotics were excceded by one or more dilutions (i.e. vancomycin by several dilutions (MIC: > 128 mg/L vs. cut-off value: 2 mg/L), erythromycin by more than three dilutions (> 8 mg/L vs. 1 mg/L), kanamycin and tetracycline by two dilutions (64 vs. 16 μg/mL and 16 mg/L vs. 4 mg/L) and clindamycin and chloramphenicol and by one dilution (8 mg/L vs. 4 mg/L). Exceedance of the cut-off value by one dilution is considered to be within the normal range of variation and thus, not a matter of concern. In order to analyse the elevated MICs observed for erythromycin, kanamycin and tetracycline, the genome interrogation was performed as described above. [redacted] Therefore, these resistances are not related to the presence of acquired resistance genes and are considered of no concern.

3.1.3. Shelf-life

The shelf-life of the additive during storage was studied using three batches stored for 12 months at 25°C, 60% relative humidity (RH) and at 40°C and 75 RH% (packaging during storage was not described).[23] Losses in total LAB counts (measured using qPCR PMA-coupled method) were negligible (< 0.5 log) in the first case after 12 months whilst reached 0.8 log after 6 months in the second case.

[17] Technical dossier/Supplementary information April 19/Annex 5.
[18] Technical dossier/Section II/AnnexII-11.
[redacted]
[20] Technical dossier/Section II.
[redacted]
[23] Technical dossier/Section II/Annexes II-19 and 20.

3.1.4. Conditions of use

The additive is intended to be used in easy and moderately difficult to ensile materials and for all animal species at a minimum proposed application rate of 2.5×10^7 VFU/kg forage and at a maximum proposed application rate of 8×10^7 VFU/kg forage.

3.2. Safety

3.2.1. Safety for the target species, consumers and environment

The bacterial species *Lactobacillus rhamnosus* and *Lactobacillus farciminis* are considered by EFSA to be potentially suitable for the qualified presumption of safety approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strains to be conclusively established and evidence that the strains do not harbour acquired antimicrobial genes to clinically relevant antibiotics. In the view of the FEEDAP Panel, the antibiotic resistance qualification has been met and the identity of the strains established as *L. farciminis* and *L. rhamnosus*. Therefore, both strains are considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment and, consequently, are presumed safe for the target species, consumers of products from animals fed treated silage and the environment.

3.2.2. Safety for user

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.

Once an active agent has been authorised as a silage additive, different formulations can be placed on the market with reference to that authorisation. The applicant listed several cryoprotectants and carriers which would allow multiple formulations of the additive to be produced and consequently, not all forms can be directly tested for user safety. However, for assessing the safety for the user of the additive, the active agent is the principal concern provided that other components do not introduce safety issues. For this specific product, the excipients used in the preparation of the final formulation do not introduce additional risks.[24]

3.3. Efficacy

Three studies were reported with different forage materials varying in dry matter (DM) and water soluble carbohydrates (WSC) content. In study 1,[25] maize (30% DM, 3.7% WSC in fresh matter (FM)) representing material easy to ensile was used. In the other studies, forage representing moderately difficult to ensile materials as specified by Regulation (EC) No 429/2008, were used, namely ryegrass (31% DM, 2.8–2.9% WSC in FM, study 2[26]) and lucerne (40% DM, 2.1–2.3% WSC in FM, study 3[27]). Even though some of the experimental procedures in the three studies can be considered acceptable, flaws have been detected, in particular in the statistical analysis of the data. First, with only three replicates per experimental group, data should have been analysed with non-parametric tests. In addition, in study 1, the statistical analysis was not properly reported. Furthermore, two different batches of the additive were tested in this study, what can be regarded as an additional source of replication, but not as two independent studies. As for studies 2 and 3, the statistical analysis provided is not acceptable as time effects (from samples taken at different times after ensiling) were not properly included in the statistical model, so that the actual replication from the real experimental units (each mini-silo) was not appropriately considered. Consequently, the FEEDAP Panel deems that the results provided from the three studies cannot be used to conclude on the efficacy of the additive to improve the ensiling process in easy and moderately difficult to ensile materials.

[24] Silicon oxide currently under re-evaluation.
[25] Technical dossier/Section IV/Annexes IV_1 and IV_2 and Supplementary information April 19/Annexes 7-9.
[26] Technical dossier/Section IV/Annexes IV_3 and IV_4 and Supplementary information April 19/Annexes 11, 13, 15 and 16.
[27] Technical dossier/Section IV/Annexes IV_3 and IV_4 and Supplementary information April 19/Annexes 10, 12, 14 and 16.

3.3.1. Conclusions on efficacy

No conclusions can be drawn on the efficacy of the additive to improve the ensiling process of easy and moderately difficult to ensile materials.

4. Conclusions

The data produced 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 shelf-life of the additive.

L. rhamnosus CNCM I-3698 and *L. farciminis* CNCM I-3699 fulfil the requirements of the QPS approach to safety assessment and consequently, can be presumed safe for the target animals, consumers products derived from animals fed with the additive and the environment.

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 or on its dermal sensitisation potential.

No conclusions can be drawn on the efficacy of *L. rhamnosus* CNCM I-3698 and *L. farciminis* CNCM I-3699 to improve the ensiling process of easy and moderately difficult to ensile materials.

5. Documentation as provided to EFSA/Chronology

Date	Event
09/11/2017	Dossier received by EFSA. Dossier name. Submitted by STI Biotechnologie
23/11/2017	Reception mandate from the European Commission
22/05/2018	Application validated by EFSA – Start of the scientific assessment
20/06/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterization, safety*
25/07/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: method of analysis*
22/08/2018	Comments received from Member States
10/04/2019	Reception of supplementary information from the applicant
14/06/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation*
12/09/2019	Reception of supplementary information from the applicant
15/10/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
18/10/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
19/03/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012b. Guidance on studies concerning the safety of use of the additive for users/workers. EFSA Journal 2012;10(1):2539, 5 pp. https://doi.org/10.2903/j.efsa.2012.2539

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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, Kouba M, Kos Durjava 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, Glandorf B, Prieto Maradona M, Saarela M, Rychen G, Pettenati E and Brozzi R, 2020b. Safety and efficacy of Sorbiflore® ADVANCE (*Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699) as a feed additive for chickens for fattening. EFSA Journal 2020;18(4):6080, 12 pp. https://doi.org/10.2903/j.efsa.2020.6080

Abbreviations

AMR	antimicrobial resistance
BLAST	Basic Local Alignment Search Tool
CFU	colony-forming unit
DM	Dry matter
EURL	European Union Reference Laboratory
FEEDAP	Additives and products or Substances used in Animal Feed
FM	fresh matter
qPCR	quantitative Polymerase Chain Reaction
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
RH	relative humidity
VFU	Viable Forming Units
WSC	water soluble carbohydrates contents

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Lactobacillus rhamnosus* CNCM I-3698 and *Lactobacillus farciminis* CNCM I-3699

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*)[28] and under the category/functional group 4(b) 'zootechnical additives'/'gut flora stabilisers' (*Sorbiflore® Advance*),[29] 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 *L. rhamnosus* (CNCM I-3698) and *L. 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 5×10^8, so-called Forming Unit (FU)/g.

Sorbensyl is intended to be added to silage at a minimum dose of 2.5×10^7 or of 8×10^7 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 5×10^7 FU/kg of complete feedingstuffs.

For the quantification of *L. rhamnosus* (CNCM I-3698) and *L. 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 *L. rhamnosus* (CNCM I-3698) and *L. 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 *L. rhamnosus* (CNCM I-3698) and *L. 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.

[28] FAD 2017-0064.
[29] FAD 2017-0066.