European Food Safety Authority

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SCIENTIFIC OPINION

Scientific Opinion on the safety and efficacy of *Lactobacillus kefiri* (DSM 19455) as a silage additive for all animal species[1]

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)[2,3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Lactobacillus kefiri is intended to improve the ensiling process at a dose of 5×10^7 CFU/kg fresh material. This species is considered by EFSA to be suitable for the qualified presumption of safety approach to safety assessment. As the identity of the strain has been established and no resistance to antibiotics of human and veterinary clinical significance was detected, the use of the strain in the production of silage is presumed safe for livestock species, consumers of products from animals fed the treated silage and for the environment. Given the proteinaceous nature of the active agent and the high dusting potential of the preparation tested, the FEEDAP Panel considers it prudent to treat this additive as a skin and respiratory sensitiser. It is also considered an irritant. The results of three efficacy studies indicated that *L. kefiri* has the potential to improve the aerobic stability of silage from forages with dry matter content above 40 % at the inclusion level of 5×10^7 CFU/kg forage.

KEY WORDS

Technological additive, silage additive, *Lactobacillus kefiri*, QPS, safety, efficacy

[1] On request from the European Commission, Question No EFSA-Q-2012-00645, adopted on 13 March 2013

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[3] Acknowledgement: The Panel wishes to thank the members of the Working Group on Silage, including Andrew Chesson, Pier Sandro Cocconcelli and Miklós Mézes, for the preparatory work on this scientific opinion.

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SUMMARY

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 for the target animals, consumer, user and the environment and on the efficacy of a product based on a specific strain of *Lactobacillus kefiri*, when used individually as a technological additive intended to improve the ensiling process at a proposed dose of 5×10^7 CFU/kg fresh material.

The species *L. kefiri* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment. Therefore, it does not require any specific demonstration of safety other than confirming the susceptibility to antibiotics of human and veterinary clinical significance. As the identity of the strain has been clearly established and no antibiotic resistance of concern was detected, the use of the strain in the production of silage is presumed safe for livestock species, consumers of products from animals fed the treated silage and for the environment.

Although users at the farm level are exposed to the additive for only a short period of time when preparing the aqueous suspension, in the absence of data, its potential to be irritant and/or to act as skin/respiratory sensitiser cannot be excluded. The dustiness of the preparation tested indicated a potential for users to be exposed via inhalation. Given the proteinaceous nature of the active agent, the additive should be considered to have the potential to be a skin/respiratory sensitiser and treated accordingly.

Three studies carried out in laboratory-scale silos are described. Each lasted at least 90 days and used samples of grass and whole-crop maize of differing water-soluble carbohydrate content and representing materials easy and moderately difficult to ensile. In each case, replicate silos containing treated forage at 5×10^7 CFU/kg of silage were compared with identical silos containing the same but untreated forage. *L. kefiri* showed the potential to improve the aerobic stability of silage from forages with dry matter content above 40 % at the inclusion level of 5×10^7 CFU/kg forage..

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BACKGROUND

Regulation (EC) No 1831/2003[4] 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 Biomin GmbH[5] for authorisation of the product *Lactobacillus kefiri* (DSM 19455), when used as a feed additive for all animal species (category: technological additive; functional group: silage additive) under the conditions mentioned in Table 1.

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). EFSA received directly from the applicant the technical dossier in support of this application.[6] According to Article 8 of that Regulation, 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. The particulars and documents in support of the application were considered valid by EFSA as of 4 September 2012.

The product *Lactobacillus kefiri* (DSM 19455) has not been previously authorised in the European Union (EU).

TERMS OF REFERENCE

According to Article 8 of Regulation (EC) No 1831/2003, EFSA shall 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 animal(s), consumer, user and the environment and the efficacy of the product *Lactobacillus kefiri* (DSM 19455), when used under the conditions described in Table 1.

[4] Regulation (EC) No 1831/2003 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.
[5] Biomin Holding GmbH, Industriestraße 21, 3130 Herzogenburg, Austria.
[6] EFSA Dossier reference: FAD-2012-0018.

Table 1: Description and conditions of use of the additive as proposed by the applicant

Additive	*Lactobacillus kefiri* BIO 94 IFA 94, DSM 19455
Registration number/EC No/No	-
Category(-ies) of additive	Technological Additives
Functional group(s) of additive	1 k Silage additives

Description			
Composition, description	Chemical formula	Purity criteria	Method of analysis
Preparation of *Lactobacillus kefiri* BIO 94 IFA 94, DSM 19455	Not applicable	compliant with EU law on microbial quality, heavy metals, toxins and undesirable substances	EN 15787:2009

Trade name	-
Name of the holder of authorisation	-

Conditions of use				
Species or category of animal	Maximum Age	Minimum content	Maximum content	Withdrawal period
		CFU/kg of complete feedingstuffs		
All animal species and categories				

Other provisions and additional requirements for the labeling	
Specific conditions or restrictions for use	store in cool, dry place (room temperature or lower)
Specific conditions or restrictions for handling	Face mask, goggles and gloves recommended. Use original container. When using do not eat, drink or smoke. After use wash hands and face. Avoid contac with eyes. Change and clean spoiled work clothing
Post-market monitoring	not applicable
Specific conditions for use in complementary feedingstuffs	not applicable

Maximum Residue Limit (MRL)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues

ASSESSMENT

1. Introduction

Six genera of lactic acid-producing bacteria, including *Lactobacillus*, are commonly associated with forage species and collectively contribute to the natural ensiling process. The present application concerns a strain of *Lactobacillus kefiri* intended to be added to forages to promote ensiling (technological additive, functional group: silage additive) for eventual use in the silage for all animal species. This species of *Lactobacillus* is considered by EFSA to be suitable for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007, 2012a). This approach requires the identity of the strain to be conclusively established and evidence that that it does not show resistance to antibiotics of human and veterinary importance to be demonstrated.

2. Characterisation

2.1. Characterisation of the active substance

The *L. kefiri* strain was isolated from sauerkraut and has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen with the accession number DSM 19455.[7] It has not been genetically modified. Taxonomic identification of the strain was achieved by phenotypic tests and sequence analysis of the 16S rRNA gene.[8] Pulsed-field gel electrophoresis (PFGE) with *Asc*I and *Sfi*I is used as a strain-specific method of detection.[9] The same technique was used to assess genetic stability after several generations and the pattern has remained unchanged since the DSM 19455 deposition in 2007.[10]

The strain was tested for antibiotic susceptibility using the broth microdilution method. The battery of antibiotics tested included those recommended by EFSA (2012b).[11] In addition, susceptibility to neomycin, ciprofloxacin, linezolid and rifampicin was examined. As all minimum inhibitory concentration (MIC) values were equal to or below the cut-off values defined by the FEEDAP Panel, no further investigation is required. Only in the case of chloramphenicol was the cut-off value exceeded by a single dilution. This is within the normal variation around a mean and is not considered to be cause for concern by the FEEDAP Panel.

2.2. Production and characterisation of the product

The manufacturing process has been detailed and material safety datasheets for cryoprotectants and carrier materials were provided in the dossier. All excipients are of food grade and do not introduce safety concerns. Data on five production batches showed that the minimum specification (1×10^{10} CFU/g additive) was exceeded in all cases (mean 1×10^{11} CFU/g additive).[12]

The additive is routinely monitored for microbial contamination at various points in the manufacturing process and in the final product. Limits are set for coliforms (< 10 CFU/g additive), yeasts and filamentous fungi (<10 CFU/g additive), *Escherichia coli* (< 10 CFU/g additive) and *Salmonella* (absence in 25 g additive). Data from five batches confirmed compliance with the set microbiological values.[13]

Given the nature of the fermentation medium and the food-grade excipients, the probability of contamination with heavy metals or mycotoxins is considered to be low and consequently not included in routine monitoring. Three batches of the additive were, however, sent for analysis to confirm that

[7] Technical dossier/Section II/Annex II-26.
[8] Technical dossier/Section II/Annexes II-27.
[9] Technical dossier/Section II/Annexes II-28.
[10] Technical dossier/Section II/Annex II-29.
[11] Technical dossier/Section II/Annexes II-30.
[12] Technical dossier/Section II/Annexes II-3 to 7.
[13] Technical dossier/Section II/Annexes II-9 to 13.

this was indeed the case.[14] Aflatoxins B_1, B_2, G_1 and G_2, the metals lead, mercury and cadmium and arsenic could not be detected.

Three batches of the additive (formulation declared to be as intended to be marketed) were examined for particle size distribution by sieving. The measurements showed that 9.8 %, by volume, of the additive consisted of particles with diameters below 50 µm; no information on the percentage of particles with diameter < 10 µm was given.[15] The same batches were used to measure the dusting potential with a Heubach dustometer. The mean value was 5.6 g/m^3, which is considered high.[16]

2.3. Stability and homogeneity

The stability of three batches of the additive was studied in sealed aluminium foil bags or sealed plastic containers at 4 and 22 °C for up to 18 months.[17] Essentially no losses were observed under the conditions tested.

Stability in water was tested using three batches of the additive diluted in water to an average level of 7.8×10^5 CFU/mL and stored at 22 °C.[18] The average count after 48 hours was 3.2×10^5 CFU/mL, with a survival rate of 41 %. The applicant recommends the use of the solution within hours after dilution in water.

2.4. Conditions of use

The additive is intended for use with forages for all animal species at a proposed minimum dose of 5×10^7 CFU/kg fresh matter and to be applied to silage directly (granular application) or by spraying as an aqueous suspension (liquid application).

2.5. Evaluation of the analytical methods by the European Union Reference Laboratory (EURL)

EFSA has verified the EURL report as it relates to the methods used for the control of the active agent in animal feed. The Executive Summary of the EURL report can be found in the Appendix.

3. Safety

In the view of the FEEDAP Panel, the antibiotic susceptibility qualification has been met and the identity of the strain established. Consequently, *L. kefiri* is suitable for QPS approach to safety assessment and no further assessment of safety for the target species, consumers of products from animals fed treated silage or the environment is required.

No data were provided on skin/eye irritation caused by the additive. Although users at the farm level are exposed to the additive for only a short period of time when preparing the aqueous suspension, in the absence of data the potential of the additive to be irritant and/or to act as skin sensitiser cannot be excluded. The dustiness of the preparation tested indicated a potential for users to be exposed via inhalation. Given the proteinaceous nature of the active agent, the additive should be considered to have the potential to be a respiratory sensitiser and treated accordingly.

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 as carrier materials and cryoprotectants inulin and soy peptone, which can be added in a concentration range that 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

[14] Technical dossier/Section II/Annexes II-14 to 17.
[15] Technical dossier/Section II/Annexes II-18 to 20.
[16] Technical dossier/Section II/ Annexes II-21 to 23.
[17] Technical dossier/Section II/ Annex II-44.
[18] Technical dossier/Supplementary information November 2012.

the principal focus provided that other components do not introduce concerns. For this specific product, the excipients used would not introduce any additional risk as the active agent is already regarded as hazardous by any route of exposure.

4. Efficacy

A total of three laboratory studies are described, each using different forage materials and lasting at least 90 days (91, 93 and 90 days for studies 1, 2 and 3, respectively). All of the studies used 5.8-L mini-silos. In each case, the contents of three replicate treated silos were sprayed with the additive at 5×10^7 CFU/kg forage. Forage for the control silos was sprayed with an equal volume of water but without the additive. Forage was packed in the silos at 6 bars pressure. Ambient temperature was controlled at 22 (±2) °C. The forages used in the three studies are shown in Table 2, and represented easy (study 1) and moderately difficult to ensile materials (studies 2 and 3), as defined in Regulation (EC) No 429/2008.[19]

Table 2: Forages used in the efficacy studies of *Lactobacillus kefiri* DSM 19455 as silage additive

Study	Forage	Dry matter content (%)	Water soluble carbohydrate content (% fresh matter)
1[20]	Grass (second cut of a permanent grassland)	65.0	4.3
2[21]	Whole crop maize (dough ripe stage)	43.5	2.9
3[22]	Whole crop maize (full ripe stage)	42.7	2.0

Replicate silos were opened at the end of the experiments and the contents were analysed for dry matter content, pH, lactic acid and volatile fatty acids concentration, ethanol, ammonia (as a percentage of total nitrogen), as well as aerobic stability (using a rise of 3 °C as indicative of instability and spoilage) (Table 2). Statistical analysis was carried out using non-parametric tests (Kruskal–Wallis test) comparing differences between treated and untreated samples. [23]

Table 3: Summary of the analysis of ensiled material recovered at the end of the experiments with *Lactobacillus kefiri* DSM 19455

Study	Treatment (CFU/kg)	Dry matter loss (%)	pH	Lactic acid (% dry matter)	Acetic acid (% dry matter)	NH3-N (% total N)	Aerobic stability (days)
1	0	7.4	5.5	0.0	0.6	3.3	4.3
	5×10^7	3.7	4.8*	2.5*	1.4*	3.5	13.0*
2	0	0.6	4.0	4.1	0.9	5.0	2.1
	5×10^7	2.2*	4.1*	3.2*	3.2*	7.4	7.7*
3	0	4.1	4.1	3.3	0.7	6.4	3.6
	5×10^7	2.7	4.4*	0.6*	3.6*	8.3	16.7*

*Significantly different from control at $P < 0.05$.

[19] 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. 1–65.
[20] Technical dossier/Section IV/Annexes IV_01 and IV_04.
[21] Technical dossier/Section IV/Annexes IV_01 and IV_03.
[22] Technical dossier/Section IV/Annexes IV_01 and IV_05.
[23] Technical dossier/Supplementary information December 2012/SIn addendum 19455.

L. kefiri DSM 19455 significantly improved aerobic stability 90 days after ensiling by increasing acetic acid production during fermentation (Table 3). *L. kefiri* DSM 19455 also significantly reduced silage pH within the first seven days in all trials (data not shown), although, after 90 days of ensiling, this decrease in pH remained only in study 1. *L. kefiri* DSM 19455 improved aerobic stability of silage from easy and moderately difficult to ensile materials at the inclusion level of 5×10^7 CFU/kg forage.

CONCLUSIONS

As the identity of the strain of *Lactobacillus kefiri* has been established and no antibiotic resistance of concern detected, following the QPS approach to safety assessment, the use of the strain in the production of silage is considered safe for target species, for consumers of products from animals fed treated silage and for the environment.

Although users at the farm level are exposed to silage additive for only a short period of time when preparing the aqueous suspension, its potential to be an irritant and/or to act as a skin sensitiser cannot be excluded. Given the proteinaceous nature of the active agent and the high dusting potential of the product tested, the FEEDAP Panel considers it prudent to treat this additive as a skin and respiratory sensitiser.

L. kefiri has the potential to improve the aerobic stability of silage from forages with dry matter content above 40 % at the inclusion level of 5×10^7 CFU/kg forage.

DOCUMENTATION PROVIDED TO EFSA

1. *Lactobacillus kefiri* BIO 94 IFA 94 DSM 19455. June 2012. Submitted by Biomin GmbH.
2. *Lactobacillus kefiri* BIO 94 IFA 94 DSM 19455. Supplementary information. November 2012. Submitted by Biomin GmbH.
3. *Lactobacillus kefiri* BIO 94 IFA 94 DSM 19455. Supplementary information. December 2012. Submitted by Biomin GmbH.
4. Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for *Lactobacillus kefiri*
5. Comments from Member States received through the ScienceNet.

REFERENCES

EFSA (European Food Safety Authority), 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. The EFSA Journal, 587, 1–16.

EFSA Panel on Biological Hazards (BIOHAZ), 2012a. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2012 update). EFSA Journal, 10(12):3020. 84 pp.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012b. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal, 10(6):2740. 10 pp.

APPENDIX

Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for *Lactobacillus kefiri*[24]

In the current application authorisation is sought under Article 4(1) for Lactobacillus kefiri BIO 94 IFA 94 – DSM 19455, under the category/functional group 1(k), "technological additives/silage additives", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the feed additive for all animal species and categories. The feed additive is to be placed on the market as a powder, containing a minimum concentration of 1×10^{10} CFU/g of *Lactobacillus kefiri* BIO 94 IFA 94 – DSM 19455. It is intended to be mixed directly into silage or suspended in water and sprayed on silage with a minimum concentration of 5×10^{7} CFU/kg *fresh forage*.

For enumeration of *Lactobacillus kefiri* BIO 94 IFA 94 – DSM 19455 in *feed additive*, the Applicant submitted the ring-trial validated spread plate CEN method (EN 15787), using MRS agar. The performance characteristics of the method reported after logarithmic transformation are:

- a standard deviation for *repeatability* (S_r) of 0.24 $\log_{10}$ CFU/g;

- a standard deviation for *reproducibility* (S_R) ranging from 0.29 to 0.38 $\log_{10}$ CFU/g;

and

- a limit of detection (LOD) of 10^{5} CFU/kg *feedingstuffs*.

Based on the performances characteristics presented, the EURL recommends for official control, the CEN method (EN 15787) for the determination of *Lactobacillus kefiri* BIO 94 IFA 94 – DSM 19455 in the feed additive per se.

The Applicant did not provide any experimental method or data for the determination of Lactobacillus kefiri BIO 94 IFA 94 – DSM 19455 in silage. Furthermore, the unambiguous determination of the content of *Lactobacillus kefiri* BIO 94 IFA 94 – DSM 19455 added to silage is not achievable by analysis. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *Lactobacillus kefiri* BIO 94 IFA 94 – DSM 19455 in silage.

Molecular methods were used by the Applicant to identify the active agent in the feed additive. The EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification. 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) is not considered necessary.

[24] The full report is available on the EURL website: http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2012-0018.pdf