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Safety and efficacy of *Lactobacillus parafarraginis* DSM 32962 as a silage additive for all animal species

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Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed was asked to deliver a scientific opinion on the safety and efficacy of *Lactobacillus parafarraginis* DSM 32962 when used as a technological additive intended to improve the production of silage at a proposed application rate of 1×10^8 colony forming units (CFU)/kg fresh material. The bacterial species *L. parafarraginis* 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 clearly established and no acquired antimicrobial resistance determinants of concern were detected, the use of the strain as a silage additive is considered safe for livestock species, for consumers of products from animals fed the treated silage and for the environment. The additive is not an eye or dermal irritant but should be considered a potential respiratory sensitiser. No conclusions can be drawn on the skin sensitisation potential of the additive. Three studies with laboratory-scale silos were made using samples of easy and moderately difficult to ensile forage. In each case, replicate silos containing untreated forage were compared with identical silos containing the same forage to which *Lactobacillus parafarraginis* DSM 32962 was added to reach an intended concentration of 1×10^8 CFU/kg fresh matter. The results showed that the addition of the additive improves significantly the aerobic stability of the silage tested.

Keywords: technological additive, silage additive, *Lactobacillus parafarraginis*, safety, efficacy, QPS

Requestor: European Commission

Question number: EFSA-Q-2019-00626

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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 Lactosan GmbH; Co. KG[2] for authorisation of the product *Lactobacillus parafarraginis* (DSM 32962), when used as a feed additive for all animal species (category: technological additives; functional group: silage additives).

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 12 November 2019.

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 the product *Lactobacillus parafarraginis* DSM 32962, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

The additive is a preparation containing viable cells of *Lactobacillus parafarraginis* (current name *Lentilactobacillus parafarraginis,* Zheng et al., 2020) DSM 32962. It has not been previously authorised as a feed additive in the European Union.

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[3] in support of the authorisation request for the use of *Lactobacillus parafarraginis* DSM 32962 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 agent in animal feed. The Executive Summary of the EURL report can be found in Annex A.[4]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of *Lactobacillus parafarraginis* DSM 32962 is in line with the principles laid down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012) and Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b).

[1] 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.
[2] Lactosan GmbH & Co.KG, Industriestraße West 5, 8605, Kapfenberg, Austria.
[3] FEED dossier reference: FAD-2019-0062.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports?title=FAD-2019-0062&combine=&field_eurl_date_of_report_value%5Bvalue%5D%5Byear%5D=&field_eurl_date_of_report_value_1%5Bvalue%5D%5Byear%5D=
[5] 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.

3. Assessment

The additive is a preparation of viable cells of *Lactobacillus parafarraginis* DSM 32962 (current name *Lentilactobacillus parafarraginis*) intended for use as a technological additive (functional group: silage additives) for use in forages for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the active agent

The active agent was isolated from silage. It was deposited in the Deustche Sammlung von Mikroorganismen und Zellkuturen with the accession number DSM 32962.[6] It has not been genetically modified.

The full genome of the strain [redacted]) was obtained and used for characterisation purposes.[7] Taxonomical identification was achieved [redacted]

The bacterial strain was tested for antibiotic susceptibility using broth microdilution techniques.[8] The battery of antibiotics used included those recommended by EFSA (EFSA FEEDAP Panel, 2018a). All the minimum inhibitory concentration values were below the corresponding EFSA cut-off values for obligate heterofermentative lactobacilli, except for tetracylicne (MIC: 128 mg/L *vs* cut-off value: 8 mg/L). However, *L. parafarraginis* is phylogenetically related to the heterofermentative *Lactobacillus buchneri* (Endo and Okada, 2007; Felis and Dellaglio, 2007), therefore the cut-off value for this species (128 mg/L) is to be applied. Therefore, the strain is considered to be susceptible to all the relevant antibiotics.

The whole genome sequence of the strain [redacted] was searched for acquired antibiotic resistance genes [redacted][9] No significant matches were detected [redacted] Therefore, the strain does not harbour known acquired antimicrobial resistance genes.

3.1.2. Manufacturing process and characterisation of the additive

[redacted] The product consists of approximately [redacted] biomass and [redacted] cryoprotectants/carriers and has a minimum declared content of 5×10^{11} CFU/g additive.

Analysis of five batches showed a mean value of 6.4×10^{11} CFU/g (range $6.1–6.8 \times 10^{11}$ CFU/g).[11]

Microbial contamination is routinely monitored at various points in the manufacturing process and in the final product.[12] Limits are set for enterobacteria (10^2 CFU/g), yeasts and filamentous fungi (10^2 CFU/g) and *Salmonella* spp. (absent in 25 g). Analysis of three batches of the additive showed compliance with these limits.[13] Three batches of the additive were tested for aflatoxins (B1, B2, G1 and G2), deoxynivalenol, zearalenone, lead, mercury, cadmium and arsenic; results showed levels below the respective limits of detection, except for two batches that showed levels of 0.16 and 0.17 mg Pb/kg and 0.02 mg Hg/kg.[14,15]

[6] Technical dossier/Section II/Annex II 2-1.
[7] Technical dossier/Section II/Annexes II 2-2 and II_2-2a.
[8] Technical dossier/Section II/Annex II 2-5.
[9] Technical dossier/Section II/Annex II 2-7 and Supplementary information January 2020/Annex I.
[10] Technical dossier/Section II/Annex II 3-1.
[11] Technical dossier/Section II/Annex II 1-2.
[12] Technical dossier/Section II/Annex II 3-1.
[13] Technical dossier/Section II/Annex II 1-3.
[14] Technical dossier/Section II/Annexes II 1-4 and II 1-5.
[15] Limit of detection: aflatoxins (B1, B2, G1, and G2): 0.03 μg/kg, deoxynivalenol 10 μg/kg, zearalenone (5 μg/kg), Pb (0.141 mg/kg), Hg (0.001 mg/kg), Cd (0.064 mg/kg) and arsenic (0.156 mg/kg).

The additive is a powder with hygroscopic properties. The dusting potential of the additive was measured in three batches (by the Stauber–Heubach method) and showed a mean value of 1.36 mg/m^3 air (range: 1.32–1.39 mg/m^3 air). The same three batches were tested for particle size distribution by laser diffraction[16]; results showed that approximately 52% of the additive consists of particles with diameters below 100 μm, 31% below 50 μm and 5% below 10 μm.

3.1.3. Stability and homogeneity

Three batches of the additive were maintained in sealed aluminium foil bags at three storage conditions: at 4°C for 24 months, at 25°C for 12 months and at 40°C for 2 months. Negligible losses of *L. parafarraginis* viable cells were observed over any of the periods and conditions tested (plate counts decreased by less than 0.5 log).[17]

Three samples from three different batches of the additive (each of 1 g) were suspended in 19 mL water giving a count of 2.6 × 10^{10} CFU/ml and stored for 2 days at room temperature and for 7 days at 4°C.[18] No loss of viability was detected after 3 days and even after seven days at 4°C losses were ≤ 0.3 log of the initial value.

3.1.4. Conditions of use

The additive is intended for use in forages with dry matter (DM) content ranging from 30% to 70% and at a proposed minimum concentration of 1 × 10^8 CFU/kg forage, for all animal species. It is to be applied as such or as an aqueous suspension.

3.2. Safety

3.2.1. Safety for the target species, consumer and the environment

The species *L. parafarraginis* is considered by EFSA to be suitable for the Qualified Presumption of Safety (QPS) approach to safety assessment (EFSA, 2007; EFSA BIOHAZ Panel, 2020). This approach requires the identity of the strain to be conclusively established and evidence that the strain lacks acquired determinants of resistance to antibiotics of human and veterinary importance. In the view of the FEEDAP Panel, the identity of the strain was established as *L. parafarraginis* and the antibiotic resistance qualification has been met. Consequently, *Lactobacillus parafarraginis* DSM 32962 is considered to be suitable for the QPS approach to safety assessment and therefore is presumed safe for the target species, consumers of products from animals fed treated silage and the environment.

3.2.2. Safety for user

No specific studies investigating the effects of the additive on the respiratory system were submitted. The dusting potential reported (1.3 g/m^3) suggests that exposure by inhalation is possible. Owing to the proteinaceous nature of the active agent, the additive should be considered a respiratory sensitiser.

The skin[19] and eye[20] irritation potential of the additive was tested in valid studies performed according to OECD guidelines 439 and 405, respectively, showing that the product is not a skin or an eye irritant. No data on skin sensitisation potential were provided.

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.

3.2.2.1. Conclusions on safety for the user

The additive is not a dermal or an ocular irritant but should be considered a respiratory sensitiser. No conclusions can be drawn on the skin sensitisation potential of the additive.

[16] Technical dossier/Section III/Annex III 3-1.
[17] Technical dossier/Section II/Annex II 4-1.
[18] Technical dossier/Supplementary information January 2020/Annex II.
[19] Technical dossier/Section III/Annexes III 3-3.
[20] Technical dossier/Section III/Annexes III 3-4.

3.3. Efficacy

Four studies using laboratory scale silos were submitted. The forages used were of different origin and showed DM and a water soluble carbohydrates contents representing material easy to ensile (studies 1,[21] 3[22] and 4[23]) and moderately difficult to ensile (study 2[24]), as specified by Regulation (EC) No 429/2008 (Table 1). All the studies included two treatment groups. In the treated group, *Lactobacillus parafarraginis* DSM 32962 was applied to the forage at a concentration of 1×10^8 CFU/kg of fresh forage. No analytical confirmation of the compliance of the *L. parafarraginis* counts present in the specific batch of the additive used for the trials or in the silage after its application was provided for any of the studies to ensure compliance with specifications of the additive. An aqueous suspension of the mixture was prepared and then sprayed on the forage prior to ensiling. In the control silos, the same volume of water was added but without the additive. In all studies, three replicated silos of 1.5- and 5-litre capacity for each treatment were used (the 1.5-L silos were opened at intermediate times and the 5 L at the end of the ensiling period). Silos were opened after 90 days in studies 1 and 2, 91 in study 4 and 92 in study 3. The ambient temperature during ensiling was 20 ± 2°C.

Table 1: Characteristics of the forage samples used in the four ensiling experiments

Study	Test material	Dry matter content (%)	Water-soluble carbohydrate content (% fresh matter)
1	Whole crop maize	32	4.6
2	Grass (2nd cut)[1]	35	2.9
3	Grass (2nd cut)[1]	51	6.1
4	Maize cob mix	66	3.8

[1]: 100% *Festuca arundinacea*.

Silos were opened at the end of the experiment and the contents were analysed to determine silage DM content, pH, lactic, acetic, butyric and propionic acids and ethanol concentrations. The method of Honig (1990) was used to determine aerobic stability of the silage. At the end of each experiment, samples were taken from each silo and exposed to air with continuous monitoring of temperature. A rise of 3°C above room temperature was considered as an indicator of silage deterioration, and the time at which that rise was observed was taken as a measure of the aerobic stability of treated and control silages. A minimum increase of stability of the treated silage of 2 days compared to that shown by the untreated control is considered as evidence of aerobic stability.

Non-parametric tests were used for the statistical analyses to compare treated vs control silos. Significance was declared at $p < 0.05$. Results are shown in Table 2.

Table 2: Summary of the analysis of ensiled material recovered at the end of the ensiling period with *Lactobacillus parafarraginis* DSM 32962

Study	Application rate (CFU/kg forage)	Dry matter loss (%)	pH	Lactic acid (% dry matter)	Acetic acid (% dry matter)	Ammonia-N (% total N)	Aerobic stability (days)
1	0	1.8	3.8	1.8	0.3	6.1	2.2
	1.5×10^8	2.6*	3.8	1.7	1.6*	5.8	> 12*
2	0	3.1	4.6	1.6	0.5	9.9	3.1
	1.5×10^8	3.7	4.4*	1.2*	1.4*	8.7	> 11*
3	0	3.1	4.7	1.1	0.5	3.3	9.0
	1.5×10^8	3.9	4.2*	1.3	2.4*	2.8	> 13*
4	0	1.2	4.0	1.3	0.2	4.7	1.3
	1.5×10^8	1.2	4.2*	0.4	1.4*	4.8	> 9*

*: Means in a column within a given trial are significantly different to the control $p < 0.05$.

[21] Technical dossier/Section IV/Annexes IV.1.
[22] Technical dossier/Section IV/Annexes IV.3.
[23] Technical dossier/Section IV/Annexes IV.4.
[24] Technical dossier/Section IV/Annexes IV.2.

The addition of *Lactobacillus parafarraginis* DSM 32962 at the minimum recommended inclusion level of 1×10^8 CFU/kg forage caused effects on silage fermentation end-products similar to those expected from a heterofermentative lactobacillus. Acetic acid production was significantly increased in the four studies, resulting in a significantly lower pH in two of them. However, this had no effect in terms of the direct preservation of nutritional value. The value of addition was seen in a more extended aerobic stability of the silage after exposure to air, which was seen in all studies. The time to detectable deterioration of silage was greater than 2 days in all the treated silages compared to control silages, and these differences reached significance in all studies.

3.3.1. Conclusions on efficacy

The use of *Lactobacillus parafarraginis* DSM 32962 in the ensiling process has the potential to increase the aerobic stability of the silage. This has been shown in forages with a DM content ranging between 30% and 70%.

4. Conclusions

The identity of the active agent has been established as *Lactobacillus parafarraginis* DSM 32962 and the strain does not show acquired antimicrobial resistance determinants for antibiotics of human and veterinary interest. Following the QPS approach to safety assessment, the use of the strain as a silage additive is considered safe for the target species, consumers of products from animals fed treated silage and the environment.

The additive is not a skin or an eye irritant but should be considered a respiratory sensitiser. No conclusions can be drawn on the skin sensitisation potential of the additive.

Lactobacillus parafarraginis DSM 32962 at a concentration of 1×10^8 CFU/kg plant material showed a potential to significantly improve the aerobic stability of silage from forage material with a dry matter content ranging from 30% to 70%.

5. Documentation as provided to EFSA/Chronology

Date	Event
23/09/2019	Dossier received by EFSA. *Lactobacillus parafarraginis* DSM 32962. Submitted by Lactosan GmbH & Co.KG
06/05/2019	Reception mandate from the European Commission
12/11/2019	Application validated by EFSA – Start of the scientific assessment
11/12/2019	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*
31/01/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
12/02/2020	Comments received from Member States
12/02/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
01/07/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

CFU	colony forming unit
DM	dry matter
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
MIC	minimum inhibitory concentration
OECD	Organisation for Economic Co-operation and Development
PFGE	pulsed field gel electrophoresis
QPS	Qualified Presumption of Safety

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 parafarraginis* DSM 32962

In the current application authorisation is sought under Article 4(1) for a preparation of *Lactobacillus parafarraginis* DSM 32962 under the category/functional group 1(k) 'technological additives'/'silage additives', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all animal species and categories.

According to the Applicant, the active substance in the feed additive consists of viable cells of the non-genetically modified strain *Lactobacillus parafarraginis* DSM 32962. The *feed additive* is to be marketed as a powder preparation containing a minimum *Lactobacillus parafarraginis* DSM 32962 content of 5×10^{11} Colony Forming Unit (CFU)/g. The feed additive is intended to be added to *silage* at a minimum dose of 1×10^{5} CFU/kg fresh *silage*.

For the enumeration of *Lactobacillus parafarraginis* DSM 32962 in the *feed additive* and *silage*, the Applicant submitted the ring-trial validated spread plate method EN 15787 (Animal feeding stuffs, Isolation and enumeration of *Lactobacillus* spp.) which was already evaluated by EURL in the frame of previous *Lactobacillus* spp. dossiers. Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated EN 15787 method for the enumeration of *Lactobacillus parafarraginis* DSM 32962 in the *feed additive*.

Since the unambiguous determination of the content of *Lactobacillus parafarraginis* DSM 32962 initially added to silage is not achievable by analysis, the EURL cannot evaluate nor recommend any method for official control to determine the *feed additive* in *silage*.

For the identification of *Lactobacillus parafarraginis* DSM 32962, the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised methodology for genetic identification of bacterial strains.

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.